

***BOTH THE AT<sub>1</sub> AND AT<sub>2</sub> RECEPTOR SUBTYPES MEDIATE  
ANGIOTENSIN II-STIMULATED GROWTH IN VASCULAR  
SMOOTH MUSCLE CELLS***

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**Both the AT<sub>1</sub> and AT<sub>2</sub> Receptor Subtypes Mediate Angiotensin II-Stimulated Growth in  
Vascular Smooth Muscle Cells**

**BY**

**Laura Seward**

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University  
of Manitoba in partial fulfillment of the requirements of the degree  
of  
Doctor of Philosophy**

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## LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ACE	Angiotensin Converting Enzyme
AngII	Angiotensin II
AT1	Angiotensin Type 1 Receptor
AT2	Angiotensin Type 2 Receptor
bFGF	Basis Fibroblast Growth Factor
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CRE	cAMP-responsive element
DMEM	Dulbecco Modified Eagles's Medium
DTT	Dithiothreitol
FBS	Fetal Bovine Serum
ECL	Enhanced Chemiluminescence
FAK	Focal Adhesion Kinase
GAPDH	Glyceraldehyde pyruvate dehydrogenase
GRK	G-protein receptor kinase
IGF	Insulin-like Growth Factor
IRE	Insulin Responsive Element
IRS1	Insulin Receptor Substrate 1
losartan	(2-butyl-4-chloro-5 hydroxymethyl-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole); also DuP753
LY294002	2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MAPK	Mitogen-activated Protein Kinase
MAPKK	Mitogen-activated Protein Kinase Kinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide
PA	Plasminogen activator
PBS	Phosphate-buffered Saline
PCA	Porcine Coronary Artery
PCNA	Proliferating Cell Nuclear Antigen
PD123319	(S)-1-[[4-(di-methylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)4,5,6,7-tetrahydro-1H-imidazol[4,5-c]pyridine-6-carboxylic acids
PDGF	Platelet-derived Growth Factor
PDK1	3-phosphoinositide-dependent kinase 1
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphoinositidol phosphate
PIP2	Phosphoinositidol-4,5-biphosphate, phosphoinositidyl-3,4-biphosphate
PIP3	Phosphoinositidol (3,4,5) triphosphate
PMSF	Phenylmethylsulfonyl fluoride

ABBREVIATIONS - cont.

SH2	Src-homology 2
SMC	Smooth Muscle Cell
SSC	Standard Saline Citrate
STAT	Signal Transducers and Activators of Transcription
TBS-T	Tris-buffered Saline -Tween 20
TCA	Trichloroacetic Acid
TGF $\beta$	Transforming Growth Factor $\beta$
TLC	Thin Layer Chromotography
VSMC	Vascular Smooth Muscle Cells
VWBr	VonWillebrand Factor VIII

## **ABSTRACT**

Angiotensin II (AngII), a key growth factor for smooth muscle cells (SMCs), contributes to the pathophysiology of cardiovascular diseases. Although at least two distinct receptor subtypes have been identified, the AT<sub>1</sub> receptor is regarded as the principal mediator of AngII's actions and the biological role of the AT<sub>2</sub> receptor remains unclear. The hypothesis that activation of both AT<sub>1</sub> and AT<sub>2</sub> receptors contribute to SMC proliferation through distinct signalling pathways was investigated. A comprehensive study of the relative contribution of i) the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes to AngII-mediated SMC growth and ii) receptor-specific intracellular signalling pathways was undertaken in primary cultures of porcine coronary artery (PCA) SMCs. Both AT<sub>1</sub> and AT<sub>2</sub> receptors were coupled to the mitogenic response based on the inhibition of AngII-stimulated RNA and DNA synthesis, PCNA expression and hyperplasia by the non-peptide receptor antagonists losartan (AT<sub>1</sub>) or PD123319 (AT<sub>2</sub>). A novel role for phosphatidylinositol 3-kinase (PI3K) in AT<sub>1</sub>-mediated signalling was established with assays for PI3K activity and the formation of a p85 signalling complex with IRS-1, paxillin, FAK and the AT<sub>1</sub> receptor. AngII regulated PI3K through transient tyrosine phosphorylation of p85 and translocation of the p85 and p110 subunits to different intracellular compartments. A role for PI3K in AngII-stimulated growth was indicated by the inhibitor LY294002. A key signalling pathway for the AT<sub>2</sub> receptor was identified as prostaglandin synthesis, based on the ability of indomethacin to inhibit AngII-stimulated growth and PD123319 to block AngII-stimulated arachidonic acid release. In support of these data, AngII-stimulated growth of fetal rat A10 SMCs was mediated by AT<sub>2</sub> receptors, via a prostaglandin synthesis pathway, that involved a novel requirement for insulin pretreatment. A cellular mechanism for crosstalk between the RAS and insulin system was demonstrated by insulin regulation of AT<sub>2</sub> receptor mRNA levels. In conclusion, these data support the hypothesis and challenge the accepted view of the AT<sub>1</sub> receptor as the sole mediator of the mitogenic actions of AngII in SMCs.

## 1. REVIEW OF THE LITERATURE

### 1.1 RENIN-ANGIOTENSIN SYSTEM

#### 1.1.1 Systemic RAS

1.1.1.1 *Components of Systemic RAS*: An intrinsic role for the renin-angiotensin system (RAS) in the development of hypertension was established in the 1960s. At this time, the RAS was viewed as an endocrine system with the primary role of synthesis of angiotensin II (AngII) with various associated substrates and enzymes produced in distinct organ systems and several effector organs. For a review of the RAS, refer to Peach (1981). The basic substrate of the RAS enzymatic cascade is angiotensinogen which is continuously synthesized and released from the liver into the circulation. While several hormones may regulate the synthesis of angiotensinogen, including AngII, the concentration of angiotensinogen itself is not rate-limiting. Renin cleaves angiotensinogen with a high degree of specificity to produce the biologically inactive precursor angiotensin I (AngI). The major source of circulating active renin is the renal juxtaglomerular cell which produces prorenin. Prorenin is converted to active renin through the proteolytic removal of the prosegment by several candidate enzymes including cathepsins B (Neves et al., 1996), D (Morris, 1978) and G (Dzau et al., 1987b), and tissue kallikrein (Derkx et al., 1979). It is well established that renin is the rate-limiting step in regulating plasma AngII levels (Lutterotti et al., 1994). Subsequent cleavage of AngI by angiotensin converting enzyme (ACE) produces the bioactive octapeptide AngII. ACE is primarily found on the luminal surface of the pulmonary vasculature. Unlike renin, ACE is a more promiscuous enzyme and also cleaves several small peptides such as bradykinin. AngII is released into the systemic circulation and acts as a regulatory hormone in several organ systems. The biological response to AngII is mediated by cell-surface receptors of two main subtypes, AT<sub>1</sub> and AT<sub>2</sub>, which will be discussed later in more detail.

**1.1.1.2 Role of Systemic RAS:** The RAS was initially studied as an important physiological system for both the acute regulation and long-term maintenance of blood pressure (Peach, 1981). Activation of the RAS occurs in situations with low sodium, low extracellular fluid or low blood volume. These physiological signals stimulate the production of renin from the kidneys which results in increased levels of AngII. The circulating AngII facilitates the body's attempt to increase the blood volume through its actions on several different effector organs such as sodium and water reabsorption directly through proximal tubule epithelial cells, as well as indirectly through aldosterone secretion from the adrenal gland (Peach, 1981). In the brain, AngII has been shown to activate the thirst response as well as stimulate the sympathetic CNS. Finally, the vasoconstrictive effect of circulating AngII on vascular smooth muscle cells results in an increase in total peripheral resistance. The cardiovascular actions of AngII include a direct effect on cardiac contractility, rhythm, metabolism and structure. Thus, the RAS represents a multi-level system for the regulation of blood pressure and cardiovascular function.

**1.1.1.3 Expanded View of RAS:** For an extended period, it was believed that our understanding of the multi-step cascade outlined for the systemic RAS was complete. In recent years, however, our view of the RAS has expanded to include other bioactive fragments of angiotensinogen, alternative enzymes pathways independent of renin and ACE, and heterogeneity in the receptors. This new view of the RAS requires a careful interpretation of the data in past and present studies to appreciate the divergence and selectivity among tissues in terms of processing pathways and cellular functions of the angiotensins. With intervention in this increasing complex cascade a clinically relevant goal for the treatment of a number of cardiovascular diseases, it is important to examine the details and relative importance of these alternative pathways.

One of the most significant modifications to our view of the RAS has been the

discovery of other bioactive peptides in this cascade (Ardailou and Chansel, 1998). These processing pathways may be especially important when interpreting findings obtained with pharmacological doses of AngII. Ferrario et al. (1991) have proposed a novel view of the RAS which is based on the concept of biotransformation to define the biologically important components. Within this context, both AngII and Ang(1-7) fit the criteria of end-point products of the RAS with non-linear multiple pathways for their formation. Numerous studies have shown that AngII is not an obligatory end point for AngI metabolism. Ang(1-7) can be generated directly by the action of prolyl-endopeptidase enzyme on the C-terminus (Chappell et al., 1990) of either AngI or AngII (Chappell *et al.*, 1989; Chappell *et al.*, 1990). In addition, recent studies suggest that Ang(1-7) is an endogenous substrate for ACE (Chappell et al., 1998) that may inhibit its activity (Deddish et al., 1998). This RAS product may play an important role in the regulation of blood pressure and heart function that is distinct from the actions of AngII through its ability to mediate neuronal excitation, coronary vasoconstriction, natriuresis, and the release of vasopressin and prostanoids (Ferrario et al., 1998a; Ferrario et al., 1991). In essential hypertension, Ang(1-7) levels were correlated with elevated blood pressure (Ferrario et al., 1998b). Although the actions of Ang(1-7) differ from those of AngII, they may involve activation of the AT<sub>1</sub> receptor and/or a novel receptor subtype that remains to be characterized (Benter *et al.*, 1993). Even more intriguing is the ability of this peptide to antagonize the growth response to AngII through AT<sub>2</sub> receptor activation (Freeman *et al.*, 1996) and to inhibit neointimal proliferation following vascular injury (Strawn et al., 1999). The ability of Ang(1-7) to stimulate the release of different prostaglandins in comparison to AngII through AT<sub>2</sub> receptor activation suggests a distinct signalling pathway is activated by this peptide (Jaiswal *et al.*, 1993a). The biological activities of Ang(1-7) may be particularly significant in clinical situations where ACE inhibition results in a significant increase in AngI and, subsequently, Ang(1-7) (Lawrence

et al., 1990).

N-terminal cleavage of AngII results in the production of other peptide fragments that also exhibit some biological activity. For example, AngIII (Ang(2-8)) exhibits many of the properties of AngII through activation of both AT<sub>1</sub> and AT<sub>2</sub> receptors (Wright and Harding, 1995; Reaux et al., 1999). Dipeptidase cleavage of the N-terminal of AngII results in the formation of the hexapeptide Ang IV (Ang(3-8)) which has been shown to mediate effects distinct from AngII (Braszko *et al.*, 1988; Dostal *et al.*, 1990) through a pharmacologically unique receptor (tentatively designated AT<sub>4</sub>) (Louframi et al., 1999; Swanson *et al.*, 1992). Several recent studies have demonstrated that AngIV possesses unique biological attributes that are potentially important to cardiovascular function. Although it has been difficult to identify the function of this receptor (Briand et al., 1998), it can antagonize AngII-mediated growth (Baker and Aceto, 1990) and regulation of ventricular function (Slinker et al., 1999), as well as stimulate endothelium-dependent vasodilation (Haberl et al., 1991) through nitric oxide synthesis and release (Hill-Kapturczak et al., 1999). Further insight into the biological contributions of the AT<sub>4</sub> receptor should be possible with the specific antagonist WSU1291 (Kerins et al., 1995). In addition, another peptidic fragment, Ang(3-7), has been reported to exhibit pressor activity (Benter *et al.*, 1993) and affect memory (Braszko *et al.*, 1991), probably through the AT<sub>4</sub> receptor (Jarvis *et al.*, 1992). These results demonstrate that the RAS is a finely-tuned multi-factorial pathway that is able to produce a number of downstream effects in response to its environment.

### 1.1.2 Tissue-specific RAS

In the past decade, a major shift in our view of the RAS has occurred with the realization that, in addition to the blood-borne RAS, there are multiple independent RAS localized within individual tissues (Danser et al., 1992). The discovery that angiotensinogen is the principal protein in cerebrospinal fluid and AngII receptors exist in the brain offered

the first evidence of a distinct RAS, since this organ is isolated from the blood-borne RAS (Suzuki *et al.*, 1986). This new perspective is especially valid with respect to functions of the RAS in various organ systems, including the cardiovascular system. Thus, cardiovascular tissues not only respond to mechanical and humoral influences, but are also biologically-active organs capable of synthesizing a variety of autocrine/paracrine factors.

**1.1.2.1 Evidence for Tissue-specific RAS:** Components of the RAS have been shown to be coexpressed in several tissues including the heart, blood vessel, adrenal, kidney, brain, pancreas and gonads (Paul *et al.*, 1993; Gomez *et al.*, 1990; Dzau *et al.*, 1987a; Dzau *et al.*, 1988; Campbell and Habener, 1986). Specifically, the heart and blood vessels exhibit significant expression of angiotensinogen mRNA (Naftilan *et al.*, 1991) and ACE mRNA (Yamada *et al.*, 1991). The most convincing evidence of a functional tissue-specific RAS has been demonstrated in isolated systems through the local generation of AngII in stretched neonatal cardiomyocytes (Sadoshima *et al.*, 1993), rat aortic SMC (Kubo *et al.*, 1999), isolated perfused rat hearts (Lindpaintner *et al.*, 1988) and isolated rat limb arteries (Hilgers *et al.*, 1993; Admiraal *et al.*, 1991). The source of renin in tissue-specific RAS, however, remains controversial. Although renin mRNA has been detected in some tissues (Paul *et al.*, 1993; Dzau *et al.*, 1987a), the sequestration of plasma renin by certain tissues has limited the acceptance of data which shows tissue-specific AngII production (Loudon *et al.*, 1983). An immediate decrease in tissue-specific renin activity has been clearly demonstrated following nephrectomy (Nussberger *et al.*, 1991). In contrast, renin activity persists in the tissues such as the heart, aorta and adrenal gland which continue to produce AngII even after nephrectomy (Campbell *et al.*, 1993). The synthesis of renin in cultures of VSMCs also strongly suggests that extrarenal tissues participate in the production of renin under certain conditions (Re *et al.*, 1982). In a recent study, the ability of hyperthyroidism to induce cardiac hypertrophy directly correlated with the induction of cardiac renin mRNA expression

(Kobori et al., 1999). In addition, the expression of a novel renin isoform has been characterized recently in brain tissue (Lee-Kirsch et al., 1999). Thus, the body of evidence from the published literature supports local renin gene expression; however, both mechanisms may be important in the formation of interstitial AngII.

**1.1.2.2 Regulation of Tissue-specific RAS:** The factors involved in the regulation of tissue-specific RAS remain undefined. While cell culture studies have provided much of the information to-date, some insight into the consequences of tissue-specific RAS overexpression has been obtained using *in vivo* gene transfer. Tissue-specific RAS differs from the systemic RAS where the rate-limiting step is plasma renin activity and ACE is abundant. Transfection of ACE and/or renin cDNA into culture vascular smooth muscle cells (VSMC) resulted in increased production of AngII (Morishita *et al.*, 1993). A synergistic effect between ACE and renin indicated that both act as rate-limiting factors. In humans, the circulating level of AngI is often higher than AngII and arterially delivered AngI is physiologically important (MaassenVanDenBrink et al., 1999).

**1.1.2.3 Role of Tissue-specific RAS:** The rationale for the existence of a separate RAS within individual tissues has been extensively debated. Although the systemic RAS represents a system for acute hemodynamic stability, tissue-specific RAS may be involved in more long-term maintenance of hemodynamics and remodelling events (Admiraal et al., 1993). In addition, tissue-specific RAS provides an important autocrine/paracrine system capable of controlling the cellular response at the local tissue level. In this way, the local environment can control the expression of the various components of the RAS. Locally produced AngII can also regulate the local matrix environment through direct effects on the synthesis and degradation of various extracellular matrix components (refer to Section 1.2.2). A recent study concluded the local RAS is functionally significant in the vasoconstrictive actions of AngII in human coronary arteries (MaassenVanDenBrink et al., 1999). Although

the role of tissue-specific RAS in normal physiology has been difficult to delineate, the activation of tissue-specific RAS has been extensively studied in the adaptive processes associated with several cardiovascular disorders (refer to Section 1.1.3).

**1.1.2.4 Alternative RAS Pathways:** Concurrent with the discovery of a tissue-specific RAS, our view of the traditional RAS expanded with the identification of other non-renin, non-ACE enzymes capable of participating in the production of AngII. Since intervention into this increasingly complex cascade has clinical applications, it is important to define the relative importance of these alternative pathways. Several non-renin enzymes capable of generating AngI and/or AngII from angiotensinogen have been described (Dzau *et al.*, 1993; Okunishi *et al.*, 1987; Boucher *et al.*, 1974). It is also possible to bypass ACE, since chymase (Urata *et al.*, 1990b) and cathepsin G (Klickstein *et al.*, 1982) can cleave AngI to AngII. The increased production of AngII in cultures of VSMC from SHR was due to increased levels of cathepsin D as well as ACE (Fukuda *et al.*, 1999). The quantitative roles of ACE and chymase in the conversion of AngI to AngII appear to differ between rodent and human heart (Urata *et al.*, 1990a; Urata *et al.*, 1990b). However, both pathways for AngII generation may be significant in some species, since both ACE- and chymase-dependent mechanisms were functional in vasoconstriction of human coronary arteries (MaassenVanDenBrink *et al.*, 1999; Padmanabhan *et al.*, 1999) and balloon-catheter injury of canine carotid arteries (Wei *et al.*, 1999). The potential pathophysiological role of chymase has been reviewed in Fukami *et al.* (1998). Thus, in contrast to the original view of a sequential linear cascade to produce AngII, the RAS can be viewed as a non-linear biochemical cascade of multiple pathways with an end-point being the production of biologically active products, such as AngII and Ang(1-7).

In conclusion, a large body of evidence supports the concept that the local RAS is an important modulator of tissue function and structure. Although some controversy still exists

as to whether renin is produced locally or derived from the systemic RAS, the fact remains that AngII is produced independently of the systemic RAS. While regulation of the tissue-specific RAS remains poorly defined, the existence of multiple pathways for the formation of AngII and interplay with the systemic RAS further complicates the study of how these systems are modulated. Most studies are now focussed on defining the effect of local RAS under pathophysiological conditions.

### **1.1.3 Role of RAS in the Pathophysiology of the Cardiovascular System**

"The long range aim of antihypertensive treatment should be not only to attenuate arterial pressure rises but also, if possible, to induce a true structural regression so that geometric increase in resistance and vascular reactivity would be eliminated. In the end, these are tough problems we have to deal with these days" (Schalekamp,1991). This quote reflects the theory behind intervention in the RAS. Unlike other systems that solely influence hemodynamic control, the RAS offers a two-tiered approach to intervention in cardiovascular pathophysiology due to the participation of the systemic RAS in acute hemodynamic control as well as the tissue-specific RAS in the remodelling of cardiovascular tissues. Currently, ACE inhibitors are preferred for the treatment of human hypertension and heart failure; however, the relative contribution of the additional non-RAS effects of ACE inhibition on bradykinin metabolism and prostaglandin actions remains to be elucidated. The accumulated body of evidence does suggest that activation of the RAS can be considered a new risk factor for cardiovascular disease (Oparil and Oberman, 1999). Genetic linkage studies have confirmed the relative importance of the RAS components in cardiovascular pathophysiology, since ACE and AT<sub>1</sub> receptor polymorphisms correlate with essential hypertension (Bengtsson et al., 1999; Kainulainen et al., 1999), microvascular wall thickening (Teranishi et al., 1999) and the risk of coronary artery disease (Alvarez et al.,

1998).

Cardiovascular disease covers a broad spectrum of conditions, ranging from dysfunction of the vasculature in atherosclerosis, hypertension and restenosis to ventricular dysfunction that leads to heart failure. Excessive VSMC growth has been established as a critical turning point in the development of these cardiovascular diseases. Originally, the RAS was identified as an important neuroendocrine system in the control of vascular tone and cardiac function (Francis et al., 1990; Morgan and Baker, 1991). Recently, the RAS has been implicated in the development and progression of chronic conditions such as congestive heart failure, in part, through its role in vascular and cardiac remodelling, independent of blood pressure changes (Jacobi et al., 1999). The benefits of ACE inhibitors in the treatment of cardiovascular disease have been established in a number of clinical trials and animal studies. In both the SOLVD and SAVE studies (SOLVD, 1991; Lamas et al., 1995), the beneficial effects of ACE inhibitors on left ventricular dysfunction and survival was studied in patients following myocardial infarction. ACE inhibitor treatment significantly reduced mortality as well as the incidence of heart failure and recurrent myocardial infarction (Pfeffer et al., 1992). These benefits have been attributed to inhibition of the local RAS, since the circulatory RAS is only involved in the acute reaction to myocardial infarction (Pfeffer et al., 1992). In addition, ACE inhibitors have been shown to reverse endothelial dysfunction in coronary artery disease (Mancini et al., 1998). Although the vascular mechanisms of chronic administration of ACE inhibitors are not defined, their beneficial anti-hypertensive effects have been predominantly attributed to the inhibition of AngII formation by tissue-based RAS in rats (Tokita et al., 1995). Sustained inhibition of tissue RAS, in particular renal ACE, correlated with the beneficial effects of long-term ACE treatment and survival after myocardial infarction in the rat (Wollert et al., 1994). In fact, the differences in efficacy of various classes of ACE inhibitors have been directly correlated with the differential effects

of these inhibitors on the gene expression of tissue-specific RAS and, consequently, their effect on cardiac hypertrophy (Keuneke et al., 1995).

The efficacy of ACE inhibitors in humans can be expected to differ significantly from rodent models due to the prevalence of human chymase that has been shown to be the predominant pathway for the production of AngII in the human vasculature (Liao et al., 1995; Okunishi et al., 1993) and heart (Balcells et al., 1997). Also, prolonged ACE inhibition results in increased levels of renin and angiotensin I (Nishida et al., 1997) which may overcome the blockade of this enzyme or provide excess substrate for alternative AngII formation pathways. In fact, physiologically active levels of AngII levels do persist during long-term ACE inhibitor therapy (Baruch et al., 1999) and, as a result, the inclusion of AT<sub>1</sub> receptor blockade may be beneficial in the treatment of patients with heart failure and post-myocardial infarction (Brunner-La Rocca et al., 1999; Di Pasquale et al., 1999; Azizi et al., 1997). Currently, clinical trials such as ELITE II and CHARM are underway to assess the efficacy of AT<sub>1</sub> receptor antagonism in the treatment of cardiovascular disease (Baan 1999). However, AT<sub>1</sub> receptor blockade, as well as ACE inhibitor treatment, may result in significantly increased levels of renin activity and AngII levels (Gavras and Gavras 1999). Preliminary results suggest that the efficacy of AT<sub>1</sub> antagonists may differ significantly from ACE inhibitors in some cardiovascular diseases, possibly due to increased activation of other angiotensin receptors (van der Meulen et al., 1999; Weber, 1999; Iyer et al., 1998; Lui et al., 1997; Pitt et al., 1997).

The prominent role of the tissue RAS in cardiovascular disease has been demonstrated in transgenic models that provide key evidence of the functional consequence of local expression of RAS components. Transgenic rats that overexpress the mouse Ren-2 renin gene in several tissues but exhibit low levels of circulating renin, develop severe hypertension and secondary complications such as heart failure, stroke and renal sclerosis

that results in premature death (Mullins *et al.*, 1990). Similarly, overexpression of vascular ACE resulted in angiotensin-mediated vascular hypertrophy *in vivo*, independent of blood pressure effects, which demonstrates the importance of autocrine/paracrine AngII formation in the vessel wall (Morishita *et al.*, 1994). AT<sub>1</sub> receptor overexpression in cardiac myocytes resulted in myocyte hyperplasia with bradycardia and heart block that led to premature death (Hein *et al.*, 1997b). In addition, a number of animal studies have correlated increased expression of components of the tissue-specific RAS with various cardiovascular disease states. Recently, increased production of AngII by the local RAS in VSMC was implicated in the development of hypertension in SHR (Fukuda *et al.*, 1999). Myocardial infarction and left ventricular hypertrophy are associated with upregulation of ACE and angiotensinogen mRNA in the rat (Hirsch *et al.*, 1991; Finckh *et al.*, 1991; Lindpainter *et al.*, 1993). Specifically, increased ACE activity was identified throughout the hypertrophied left ventricle which correlated with increased AngII production (Schunkert *et al.*, 1990), infarct size following myocardial infarction (Dzau *et al.*, 1989) and the development of heart failure (de Mello and Crespo, 1999). Balloon injury of the rat carotid or abdominal artery resulted in induction of ACE (Rakugi *et al.*, 1994) and angiotensinogen (Rakugi *et al.*, 1993) expression and paracrine or autocrine AngII-mediated VSMC growth (Daemen *et al.*, 1991; Osterrieder *et al.*, 1991). These studies have demonstrated the isolated activation of the RAS in the injured/remodelled tissue which supports the concept that activation of only the local RAS, independent of the circulatory RAS, is necessary for the initiation and progression of cardiovascular disease.

## **1.2 ANGIOTENSIN II AND SMC**

### **1.2.1 Growth Factor Effects of Angiotensin II**

Until recently, the best understood physiological function of AngII in smooth muscle was vasoconstriction of the arteries to increase systemic blood pressure. The concept that

AngII acts as a growth factor in addition to a vasoconstrictor was initially met with controversy. The traditional view of the physiological role of AngII was drastically modified when the ability of this hormone to directly stimulate growth was clearly demonstrated *in vitro* in a number of cell culture systems. In SMCs, AngII acts as a growth factor that results in a hypertrophic or hyperplastic growth response (Gibbons et al., 1992; Chiu et al., 1991b; Geisterfer et al., 1988) with a rapid induction of proto-oncogene expression (Naftilan et al., 1990; Taubman et al., 1989). However, an accompanying increase in DNA synthesis or mitogenesis is often delayed (Weber et al., 1994) and may depend on an autocrine mechanism of enhanced expression and secretion of endogenous growth factors such as epiregulin, TGF $\beta$ 1 and PDGF-A (Thomas et al., 1999; Hanada et al., 1999; Linseman et al., 1995; Hahn et al., 1991). The cellular mechanisms involved in AngII-mediated mitogenesis are undefined and may be distinct from other mitogenic agents. Within the context of an autocrine growth mechanism, AngII may act as "competence factor" that initiates the growth response and stimulates other growth factors that act as "progression factors" to complete the cell cycle. Thus, stimulation of cellular hypertrophy or hyperplasia by AngII may involve secondary factors such as PDGF, IGF-I, bFGF and TGF $\beta$  (Itoh et al., 1993). Recent studies have identified the EGF receptor as a key secondary growth factor pathway intrinsic to AngII-stimulated mitogenesis (Eguchi et al., 1998; Murasawa et al., 1998) and TGF $\beta$  synthesis (Moriguchi et al., 1999).

### **1.2.2 Additional Vascular Effects of Angiotensin II**

In addition to AngII's direct vasoactive and mitogenic effects, this peptide has been shown to modulate VSMC function through alterations in its environment. The matrix surrounding a SMC is thought to modulate the cellular response to growth factors, chemotactic factors and injury through its interaction with cell surface integrins. Thus, alterations in extracellular environment can lead to inappropriate proliferation and synthetic

behaviour that, in turn, facilitate vascular disease. Specifically, AngII has been shown to stimulate the expression of collagen (Kato et al., 1991), laminin (Regenass et al., 1994), osteopontin (deBlois et al., 1996), thrombospondin, fibronectin and tenascin (Hahn et al., 1993). The ability of AngII to stimulate tenascin in VSMC is significant due to the association of tenascin with remodelling events associated with disease and developmental processes (Mackie et al., 1992). In addition, AngII stimulates expression of osteopontin which may correlate with neointimal SMC proliferation (deBlois et al., 1996), as well as the development of heart failure in SHR (Singh et al., 1999). Another mechanism by which AngII can modify the local cellular environment is through induction of matrix-degrading enzymes such as collagenases (Brilla et al., 1994).

AngII-dependent regulation of other key cellular processes potentially contributes to the pathophysiology of vascular disease. AngII has been shown to induce migration of VSMC, an important step in vascular remodelling and disease (Liu G et al., 1997; Dubey et al., 1995; Bell et al., 1990). AngII may directly stimulate the formation of atherosclerotic foam cells through stimulation of cellular cholesterol biosynthesis (Keidar et al., 1999) and upregulation of cellular receptors for oxidized low-density lipoproteins (LOX-1) (Morawietz et al., 1999). In addition, AngII may contribute to the atherosclerotic process by inducing the expression of inflammatory genes in VSMCs, such as the SMC receptor for monocytes, VCAM-1 (Tummala et al., 1999), as well as plasminogen activator (PA) (Bell et al., 1990) and PA inhibitor-1 and -2 (Feener et al., 1995). AngII has also been shown to stimulate tissue factor expression in VSMC which acts as a regulator of coagulation and thrombosis (Taubman et al., 1993).

### **1.3 ANGIOTENSIN RECEPTORS**

#### **1.3.1 Receptor Subtypes**

The effectors responsible for the wide-range of biological effects produced by the

RAS are the cell-associated receptors. The initial discovery of cell-surface angiotensin receptors in 1970 and acknowledgement of their intrinsic role as modulators of AngII's diverse physiological effects (Lin and Goodfriend, 1970) was closely followed by the recognition of multiple receptor subtypes (Papdimitriou and Worcel, 1974). Although the possibility of receptor diversity was proposed based on differences in membrane binding properties (Gunther, 1984) and differential sensitivity of AngII binding to sulfhydryl reagents (Birabeau et al., 1984), it was the development of non-peptide receptor antagonists in 1989 that made it possible to establish the heterogeneous nature of AngII receptors (Chiu et al., 1989a). This discovery enabled the identification of at least two AngII receptor subtypes, AT<sub>1</sub> and AT<sub>2</sub>. The pharmacological characterization of the receptor subtypes is based on their relative affinity for angiotensin peptides and non-peptide antagonists, as well as their sensitivity to sulfhydryl-reducing agents such as dithiothreitol (DTT)(Chiu et al., 1989b). A major breakthrough in our understanding of the heterogeneity in AngII receptors occurred with the cloning of the AT<sub>1</sub> receptor in 1991 (Murphy et al., 1991; Sasaki et al., 1991) and the AT<sub>2</sub> receptor later in 1993 (Kambayashi et al., 1993b; Mukoyama et al., 1993). To date, multiple receptor subtypes and isoforms have been identified in different species and tissues, revealing a much more complex picture of the RAS receptor system.

**1.3.1.1 AT<sub>1</sub> Receptor:** The AT<sub>1</sub> receptor has been designated a member of the seven-transmembrane domains G-protein-coupled receptor superfamily. This receptor is comprised of 359 amino acid residues with a molecular weight of approximately 35-41 kDa. The observed variance in size has been attributed to varying degrees of glycosylation; however, no role for the glycosylation in ligand binding has been identified (Yamano et al., 1992). This receptor displays a high degree of sequence homology at the amino acid level (over 94% identical between all mammalian species), and the high degree of conservation within the transmembrane domain suggests these residues play an important role in signal

transmission. In fact, site-directed mutagenesis within the transmembrane domains markedly alters the ligand binding profile and allows the receptor to relax into an active conformation without stimulation (Balmforth et al., 1997). The overall structure of the AT<sub>1</sub> receptor consists of four extracellular domains, each with four cysteine residues ideally situated for disulphide bridge formation. Not only are these cysteines absolutely required for ligand binding, they likely account for the sensitivity to sulfhydryl agents such as DTT. The coupling of the AT<sub>1</sub> receptor to a G-protein signal transduction mechanism via the second cytoplasmic loop and the carboxyl-terminal tail has been well-documented (Ohyama et al., 1992).

Two isoforms of the AT<sub>1</sub> receptor subtype have been identified in rat and mouse cells and designated AT<sub>1A</sub> and AT<sub>1B</sub> (Iwai et al., 1992a; Sasamura et al., 1992). The coding region for both AT<sub>1A</sub> and AT<sub>1B</sub> receptors are contained within a single exon that are 94-96% homologous at the amino acid level, with the differences isolated to 17-22 amino acids located primarily in the intracellular domains (Iwai et al., 1992a). Despite these similarities, the two AT<sub>1</sub> receptor subtypes are the products of different genes localized on separate chromosomes. In the rat, the AT<sub>1A</sub> receptor gene is found on chromosome 17 and the AT<sub>1B</sub> gene is localized on chromosome 2 (Szpirer et al., 1993). Interestingly, these two AT<sub>1</sub> subclasses differ in tissue distribution and regulation; however no pharmacological or functional difference has been detected. In the rat, the AT<sub>1A</sub> receptor is the dominant form expressed in the liver, kidney, vasculature, lung, ovary, testis and heart, whereas the AT<sub>1B</sub> receptor is expressed in greater quantities in the adrenal gland, pituitary and uterus (Iwai et al., 1992b; Sandberg et al., 1992). The structural differences in the intracellular domains may be involved in receptor desensitization, although both AT<sub>1A</sub> and AT<sub>1B</sub> receptors appear to be equally sensitive to AngII-induced receptor internalization (Sasamura et al., 1992). Due to differences involving serine residues in the carboxy terminal region, these two isoforms may

undergo differential phosphorylation which could contribute to functional and regulational differences (Dzau et al., 1993). Although the functional significance of the AT<sub>1A</sub> receptor in comparison to the AT<sub>1B</sub> receptor remains to be defined, a large degree of redundancy in function is indicated by the ability of AT<sub>1B</sub> receptors to mediate all of the vasoactive effects of AngII in AT<sub>1A</sub> receptor-deficient mice (Oliverio et al., 1997). At the present time, only one AT<sub>1</sub> receptor subtype has been identified in human tissues and it more closely resembles the AT<sub>1B</sub> receptor subclass (Szpirer et al., 1993). Thus, the variation in AT<sub>1</sub> receptor isoforms may be significant only in specific tissues or cells.

Activation of the AT<sub>1</sub> receptor initiates a wide range of signal transduction mechanisms that vary in their temporal pattern from a rapid response (seconds) to a long-term response (minutes). Although the mechanisms for this differential response are not fully understood, they are consistent with the myriad of biological effects mediated by AngII. It has been proposed that isomerization of multiple conformations or active and inactive receptors may facilitate the selective coupling to downstream signalling molecules (Thomas, 1999). The hierarchy of various mechanisms of regulation such as receptor dimerization, phosphorylation of the carboxy-terminus, internalization and intracellular targeting requires further research and clarification.

**1.3.1.2 AT<sub>2</sub> Receptor:** Although the AT<sub>2</sub> receptor subtype was identified as a component of the RAS more than ten years ago, its remains enigmatic. Our understanding of the function of the AT<sub>2</sub> receptor was hampered due to the lack of information on its structure, DNA sequence and signalling pathways until the receptor was cloned in 1993 from PC12W cells (Kambayashi et al., 1993b) and rat fetus (Mukoyama et al., 1993). Based on the cloned sequence, it was determined that this receptor subtype also has a putative seven-transmembrane domain structure and conservation of residues that are known to be crucial for G-protein-coupling. Although they exhibit a similar binding affinity for AngII, the AT<sub>2</sub>

receptor is only 32-34% identical in sequence (53% homologous based on conservative substitutions) to the AT<sub>1</sub> receptor, mostly in the transmembrane domains, with the lowest homology in the intracellular loop that couples to G-proteins and signalling proteins. The sequence for this receptor is highly conserved with 92% homology at the amino acid level between rodent and human AT<sub>2</sub>, and the greatest variability is localized in the amino terminal region. The gene for the AT<sub>2</sub> receptor has been mapped as a single gene on the X chromosome in both the mouse and human (Martin and Elton, 1995; Ichiki et al., 1994; Koike et al., 1994). This gene encodes a 363 amino acid protein with an apparent molecular weight of 41 kDa. Due to the absence of introns in the coding region, variation with alternatively spliced transcripts does not occur. Large variations in the reported size of the AT<sub>2</sub> receptor (68-113 kDa) have been attributed to extensive glycosylation in the amino terminal region of this protein (Lazard et al., 1994; Servant et al., 1994); however, this modification does not appear to influence the binding characteristics of the receptor (Servant et al., 1996).

The two main defining characteristics originally used to identify AT<sub>2</sub> receptors are the susceptibility to sulfhydryl reducing agents such as DTT and the apparent absence of coupling to known G-proteins. However, this criteria is no longer relevant since several studies have demonstrated G-protein coupling, although the involvement of "classical" G-proteins is inconsistent or controversial (refer to Section 1.4.3.2). In contrast, several studies have found a direct G-protein coupling, especially to Gi proteins. The pharmacological definition of the AT<sub>2</sub> receptor includes an enhanced (or unchanged) affinity for AngII following treatment with a sulfhydryl agent such as DTT (Chiu et al., 1989b).

The current view considers the existence of only a single gene for the AT<sub>2</sub> receptor, but heterogeneity within the AT<sub>2</sub> receptor subtype has been reported. Tsutsumi et al. (1992) first reported heterogeneity of AT<sub>2</sub> receptors in the rat brain based on marked differences in

their sensitivity to GTP $\gamma$ S and pertussis toxin. The most convincing evidence of AT<sub>2</sub> receptor heterogeneity was demonstrated with the identification of at least two immunologically distinct AT<sub>2</sub> receptor populations in N1E-115 neuroblastoma cells that exhibited differences in their sensitivity to DTT and PD122319 (Reagan et al., 1993; Siemens et al., 1994). In agreement with this conclusion, heterogeneity in the AT<sub>2</sub> subtypes has been clearly demonstrated by structure-activity studies with CGP42112A analogues, a highly selective peptide AT<sub>2</sub> competitor, in fetal and newborn rat kidney tissue (Ciuffo et al., 1996). *In situ* hybridization with riboprobes exhibited different patterns of AT<sub>2</sub> receptor distribution that supported the variations observed in binding assays. RT-PCR analysis of the promoter and coding regions exhibited marked differences in their affinity to the primer sequences (Ciuffo et al., 1996). Possibly, the AT<sub>2</sub> receptor subtype may include two pharmacologically distinct subclasses: AT<sub>2A</sub> (coupled to G-protein) and AT<sub>2B</sub> (not coupled to G-proteins). Interestingly, AngII stabilized a complex between two differentially regulated AT<sub>2</sub> receptors (66 kDa and 100 kDa) which may represent a multimeric protein complex comprising both of these proteins (Yee et al., 1994). Recently, homology-based PCR was employed to clone these two distinct AT<sub>2</sub>-like receptors from N1E-115 cells, however, this approach cloned only one receptor which indicates these genes are distinctly different (Yee et al., 1997).

**1.3.1.3 Other Receptor Subtypes:** In previous studies, the product of the *mas* oncogene was identified as an AngII receptor subtype (designated AT<sub>3</sub>) (Jackson et al., 1988) based on the ability of the cDNA expressed in frog oocytes to confer AngII-mediated activation of a chloride channel. However, little conclusive evidence exists that this gene product serves as an AngII receptor *in vivo*. A recent study has concluded that *mas* actually functions as part of the signalling pathway for AT<sub>1</sub> receptors (Halbach et al., 2000). A new class of angiotensin receptors, AT<sub>4</sub>, has recently been identified that exhibits both high specificity and affinity for AngIV (Ang(3-8)) and a distinct binding profile from the other angiotensin

receptors including a lack of sensitivity to AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists or guanine nucleotides (Hall et al., 1993). The presence of high concentrations of this novel angiotensin binding site has been identified in rat tissues (Swanson et al., 1992), bovine aortic SMCs and endothelial cells (Hall et al, 1993), and bovine coronary endothelial cells (Hall et al., 1995). In many other species, there are receptors that differ from these defined receptor subtype classes. Several reports have characterized high affinity angiotensin binding sites that are insensitive to both AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists and may couple to intracellular signalling pathways. In Neuro-2A murine neuroblastoma cells, a unique non-AT<sub>1</sub>/AT<sub>2</sub> AngII site has been identified that is associated with differentiation and coupled to cyclic GMP formation (Inagami et al., 1992; Chaki and Inagami, 1992). Similarly, an atypical high affinity AngII site has been characterized on IEC-6 rat intestinal epithelial cell line that is insensitive to losartan and coupled to cyclic GMP formation (Smith, 1996). At present, little information is known about the molecular significance of multiple AngII receptors in cells.

### **1.3.2 Receptor Antagonists**

The success of ACE inhibitors in the treatment of several cardiovascular diseases has initiated a search for additional modes of intervention in the RAS. Blockade of the effects of AngII at the receptor level offers the most direct and selective method for intervention. Peptidic receptor antagonists have been available since the 1970's and provided the first effective blockers of the RAS; however, it was not until the development of the peptide antagonist saralasin (Sar<sup>1</sup>Ala<sup>8</sup>AngII) that an important tool for delineating the physiological effects of AngII was available (Moore and Fulton, 1984). Although later studies demonstrated only a limited clinical usefulness due to the agonistic properties of this compound and its short half-life, it provided a critical first step in defining the contribution of cell-surface AngII receptors to the downstream actions of this hormone. As a result, the search for effective non-peptide AngII antagonists followed to avoid the disadvantages

associated with peptide antagonists. The search for nonpeptide compounds that competed directly with AngII for binding proved to be very challenging due to the specific characteristics of AngII as a small highly flexible peptide which is capable of many different conformations in solution. The discovery of potent non-peptide receptor antagonists to selectively block two distinct receptor subtypes for AngII, AT<sub>1</sub> and AT<sub>2</sub> (Chiu et al., 1989a; Bumpus et al., 1991), represented an important advance in our understanding of the effector system for AngII.

**1.3.2.1 AT<sub>1</sub> Receptor Antagonists:** Losartan, previously known as DuP753 (2-butyl-4-chloro-5 hydroxymethyl-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole), is the prototypical, best-characterized AT<sub>1</sub> receptor antagonist (Wong et al., 1991; Chiu et al., 1991a); however, there is an ever-growing list of AT<sub>1</sub>-selective antagonists currently being investigated in experimental and clinical models that are based on modifications of losartan (Timmermanns, 1999). The pharmacology of these antagonists vary with surmountable and insurmountable antagonism that reflects the stability of their interaction with the receptor, interaction with the matrix or sequestration in tissue compartments (McConnaughey et al., 1999; Vanderheyden et al., 1999). In general, losartan has been shown to act as a competitive antagonist with a very high degree of selectivity for the AT<sub>1</sub> receptor with a 10,000-fold greater affinity than for the AT<sub>2</sub> receptor and a K<sub>D</sub> of 6 nM (Chiu et al., 1990). The primary metabolite of losartan, EXP3174, has also been studied clinically as a potent, insurmountable AT<sub>1</sub> antagonist (McConnaughey et al., 1999; Wong et al., 1991).

It is important to note that recent studies have expanded our understanding of the actions of losartan and complicated the interpretation of its AT<sub>1</sub> receptor effects. More than 50% of the [<sup>3</sup>H]-DuP753 (losartan) binding characterized in rat livers was attributed to novel, non-angiotensin sites (Widdowson et al., 1993; Grove and Speth, 1993). In addition, several studies have described alternative binding sites for losartan. Losartan has been shown to be

a competitive antagonist of thromboxane A<sub>2</sub>/prostaglandin H<sub>2</sub> receptors in human platelets (Liu et al., 1992a) which raises the possibility that some of the protective effects attributed to AT<sub>1</sub> receptor antagonism could result from losartan blockade of thromboxane A<sub>2</sub>'s actions in platelet activation (Guerra-Cuesta et al., 1999) and cardiovascular disease (Hall, 1991). Agonistic effects of losartan has also been reported since losartan could activate the AT<sub>1</sub> receptor directly and stimulate prostacyclin synthesis (Jaiswal et al., 1991). The AT<sub>1</sub> receptor antagonists losartan, EXP3174 and L-158,809 have also been shown to bind to tachykinin NK<sub>3</sub> receptors in rat and guinea pig brain (Chretien et al., 1994), and losartan completely abolished the cardiovascular and behavioural effects of a tachykinin NK<sub>3</sub> receptor agonist *in vivo* (Picard et al., 1993). These observations may explain the reports of non-competitive binding of this receptor antagonist (Zhang et al., 1994; Lui, 1993). Although it has been previously suggested that the antihypertensive effects of losartan could not be attributed solely to the antagonism of the actions of AngII (Ohlstein et al., 1992), these newly characterized actions of losartan will require clarification of the portion of losartan's antihypertensive and physiological effects that are attributable to AT<sub>1</sub> receptor antagonism.

### 1.3.2.2 AT<sub>2</sub> Receptor Antagonists

The development of peptidic and non-peptidic antagonists for the AT<sub>2</sub> receptor has played a major role in delineating the biological function and downstream signalling pathways of this receptor. Initially, a highly potent AT<sub>2</sub> selective ligand, CGP42112 (nicotinyl-Tyr-(N-benzyloxycarbonyl-Arg)Lys-His-Pro-Ile-OH), was used to block AngII activation of the AT<sub>2</sub> receptor (Whitebread et al., 1991). This compound is a modified peptide derived from the 4-8 core of AngII, with a high affinity and selectivity for the AT<sub>2</sub> receptor (K<sub>D</sub> of 0.02 nM) and little, if any, binding to the AT<sub>1</sub> receptors (>100,000-fold difference). Although early studies utilized CGP42112 as an AT<sub>2</sub> receptor antagonist, it has been established that CGP42112 can act as an agonist at higher concentrations (Brechler

et al., 1993). The next major advance in the tools to study the function of the AT<sub>2</sub> receptor was the development of non-peptide AT<sub>2</sub> receptor antagonists. Two of the best characterized AT<sub>2</sub> receptor antagonists, PD123177 and PD123319 ((S)-1-[[4-(di-methylamino)-3-methylphenyl]methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazol[4,5-c]pyridine-6-carboxylic acids), have been employed in a number of studies due to a selectivity for the AT<sub>2</sub> receptor that is 3,500-fold greater than for the AT<sub>1</sub> receptor (AT<sub>2</sub> IC<sub>50</sub> 8.3 nM vs AT<sub>1</sub> IC<sub>50</sub> >10,000 nM) (Dudley et al., 1990). In many experiments, PD123177 or PD123319 showed absolutely no affinity for DuP753-labelled sites, even at concentrations as high as 10<sup>-4</sup> M (Chiu et al., 1990). In systems where CGP 42112 can act as a full agonist, such as the PC12W cell line that express only the AT<sub>2</sub> receptor, PD123319 acted as an antagonist only (Brechler et al., 1993). No conclusive evidence for an agonistic effect by PD123319 have been reported, although PD123319 was reported to have a stimulatory effect on cerebral blood flow autoregulation in rats (Naveri et al., 1994) and improved survival in a gerbil model of stroke (Fernandez et al., 1994). Nevertheless, it is difficult to interpret what these observations mean due to the poorly defined role of the AT<sub>2</sub> receptor in this system. Thus, it is largely accepted that PD123319 is a selective antagonist of the AT<sub>2</sub> receptor.

### 1.3.3 Regulation of Receptor Function

**1.3.3.1 AT<sub>1</sub> Receptor:** A critical control point for the cellular response to AngII is the availability of cell-surface receptors. Reflective of the complex array of actions mediated by the AT<sub>1</sub> receptor, regulation of AT<sub>1</sub> activity involves a complex synergy between multiple modes of regulation. Thomas (1999) recently proposed a hierarchical model that incorporates four stages of receptor activation and regulation: (1) ligand binding and G-protein activation, (2) receptor phosphorylation, (3) internalization and desensitization of cell-surface receptor proteins, and (4) recruitment/ coupling to signalling molecules.

The binding of AngII to the AT<sub>1</sub> receptor results in rapid internalization of the

receptor-ligand complex by endocytosis through a mechanism that involves clathrin-coated pits (Conchon et al., 1994) and possibly caveolae (Ishizaka et al., 1998). This process occurs independently of G-protein coupling (Hunyday et al., 1994a) and can be initiated by the binding of both receptor agonists and antagonists but not the non-peptide ligands (Conchon et al., 1994). Within the carboxyl terminal tail of the AT<sub>1</sub> receptor, key regions and phosphorylation sites involved in AngII-mediated endocytosis have been identified (Hunyady, 1999; Conchon et al., 1998; Thomas et al., 1998; 1995). Unlike other G-protein-coupled receptors, internalization of the cell-surface receptors does not necessarily terminate signal transduction. Although the concept that endocytosed receptors are no longer active is widely accepted, reports that internalized receptor complexes retain the capacity to couple to signalling pathways (Yang et al., 1997b; Kapas et al., 1994) add a new dimension to this form of receptor regulation. A dynamic recycling process has been identified for the AT<sub>1</sub> receptor that rapidly replenishes the number of agonist-receptor complexes present on the cell surface (Richard et al., 1997), and this dynamic equilibrium with receptor cycling to the plasma membrane persists even after desensitization of some of AT<sub>1</sub>-mediated signalling (Hein et al., 1997a). In addition, a prolonged cellular response to AngII may involve targeting of active AT<sub>1</sub> receptors to specific intracellular locations to facilitate a unique mode of signal transduction. The presence of AngII receptors on hepatocyte nuclei has been previously reported (Booz et al., 1992; Tang et al., 1992), and AngII has been shown to rapidly localize in the nuclei of SMCs (Robertson and Khairallah, 1971). Recently, AngII-induced nuclear targeting of the AT<sub>1</sub> receptor in neuronal cultures demonstrated phosphorylation of a putative nuclear localization signal in the cytoplasmic tail of AT<sub>1</sub> receptor (amino acids 307-331, KKFKK), a dose- and time-dependent translocation to the nucleus and subsequent phosphorylation of the key signalling protein in the nuclear pore complex, p62 (Lu et al., 1998b). The biological contribution of this novel form of signalling

remains to be elucidated in future studies; however, this mechanism may provide a mode for regulating transcription directly via AT<sub>1</sub> receptor.

Desensitization represents another key regulator of AT<sub>1</sub> receptor function. This form of regulation results in the loss of responsiveness of the receptor to subsequent agonist exposure. Activation of the AT<sub>1</sub> receptor by AngII has been shown to result in rapid desensitization of the receptor both *in vivo* and *in vitro* resulting in the termination of some of the downstream signals (Sasamura et al., 1994). The process of desensitization likely involves a synergy between uncoupling with the intracellular G-protein and loss of interaction with key cytoplasmic signalling molecules. Phosphorylation of the AT<sub>1</sub> receptor on key serine and tyrosine residues can lead to desensitization (Kai et al., 1994; Paxton et al., 1994); however, the kinases involved require further characterization. Different studies have established the AT<sub>1</sub> carboxyl-terminal domains implicated in desensitization are phosphorylated by PKC (Sasamura et al., 1994), PKA (Kai et al., 1994), and specific G-protein receptor kinases (GRKs) (Oppermann et al., 1996), and it serves as an *in vitro* substrate for Src family kinases (Paxton et al., 1994). Interestingly, a concentration-dependent phosphorylation of the AT<sub>1</sub> receptor by different kinases has been noted that may result in phosphorylation at distinct sites and alter the downstream response. At low AngII concentrations (1 nM), phosphorylation occurs via PKC, while at higher AngII concentrations (100 nM) phosphorylation is mediated by another kinase, likely a GRK (Balmforth et al., 1997b; Tang et al., 1998). However, conflicting results were reported by Thomas et al. (1995) with a truncated AT<sub>1</sub> receptor that lack all carboxyl-terminal phosphorylation sites but continued to exhibit agonist-induced desensitization. In addition, a phosphorylation-independent mechanism for desensitization of the AT<sub>1</sub> receptor has been proposed. Specifically, a switch between low and high affinity states is capable of explaining the ability of losartan to reverse AngII-mediated receptor desensitization (Boulay et al.,

1994). It should be noted that not all of the AT<sub>1</sub> receptor-associated signal transduction mechanisms are terminated by receptor desensitization. For example, activation of the STAT pathway requires prolonged receptor occupancy (Thomas et al., 1996).

Regulation at the level of AT<sub>1</sub> gene transcription is an important modulator of the actions of AngII. Although the cell-surface AT<sub>1</sub> receptor levels are determined by a balance between internalization/recycling and expression, the primary mechanism for upregulation of receptor number involves transcription of this gene, while downregulation of receptor number occurs through mRNA decay (Adams et al., 1999). Feedback regulation by different components of the RAS, especially AngII, has been shown to directly regulate AT<sub>1</sub> receptor levels in a variety of tissues. Several *in vitro* studies have demonstrated AngII downregulates AT<sub>1</sub> receptor expression (Oulai et al., 1997; Lassègue et al., 1995; Nickenig and Murphy, 1994; Makita et al., 1992). Also, AngII infusion downregulated AT<sub>1</sub> mRNA expression *in vivo* in rat aortic tissue through a mechanism that is independent of pressure changes (Wang et al., 1996). In transgenic renin rats, the high levels of tissue renin result in a downregulation of the AT<sub>1</sub> receptor (Nickenig et al., 1997a). Activation of the AT<sub>2</sub> receptor participates in the regulation of AT<sub>1</sub> receptor levels, since blockade of the AT<sub>2</sub> receptor (Makino et al., 1997) or ablation of AT<sub>2</sub> receptor in knockout mice (Tanaka et al., 1999) resulted in increased levels of AT<sub>1</sub> mRNA expression.

Cloning of the AT<sub>1</sub> receptor revealed heterogeneity within this receptor subtype and provided a basis for the definition of the AT<sub>1A</sub> and AT<sub>1B</sub> subclasses in rodents (Iwai et al., 1992a). Although the amino acid sequence in the coding region of these two receptors is 96% identical (94% amino acid homology), there is significant variation in their 3' and 5' flanking regions (only 34% homology) (Guo et al., 1994). These promoter differences may account for the tissue-specific expression of these two AT<sub>1</sub> receptor subclasses (Iwai et al., 1992a; Sandberg et al., 1992; Kakar et al., 1992b) and their differential regulation (Llorens-Cortes

et al., 1994; Iwai et al., 1992b). Within the rodent AT<sub>1A</sub> promoter region, several cis-regulatory regions have been tentatively identified for the POU family of transcription factors: PEA-3 (which mediates transcriptional activation by serum components, phorbol esters and proto-oncogenes), a glucocorticoid-responsive element, a cyclic AMP-responsive element (CRE) and an AP-1 recognition element (Guo et al., 1995). One binding site for a shear-stress inducible transcription factor has also been isolated which suggests that the AT<sub>1</sub> receptor gene may respond to shear stress *in vivo* (Resnick et al., 1993). A functional role for the 3' UTR of the AT<sub>1A</sub> receptor gene was conclusively demonstrated in stable transfectants that exhibited distinct differences in the receptor-mediated signalling pathways, possibly due to differences in RNA-binding proteins (Thekkumkara et al., 1998). The differential regulation of AT<sub>1A</sub> and AT<sub>1B</sub> receptor expression may have a significant role in the pathogenesis of the RAS. For instance, selective upregulation of AT<sub>1B</sub> mRNA has been observed in the hearts of SHR (Iwai et al., 1991). Although only a single human AT<sub>1</sub> receptor subtype has been identified, the heterogeneity observed in the mRNA transcripts has been attributed to alternative splicing of the 5' UTR (Su et al., 1996). The promoter region of the human AT<sub>1</sub> gene contains three exons which may offer some insight into the regulation of mRNA expression.

**1.3.3.2 AT<sub>2</sub> Receptor:** The mechanisms regulating AT<sub>2</sub> receptor levels are not clearly defined. In all cell-types studied to date, activation of AT<sub>2</sub> receptors does not result in internalization of the receptor-ligand complex (Hein et al., 1997a; Dudely et al., 1991; Pucell et al., 1991). Furthermore, regulation of this receptor by desensitization has not been described, in part due to the poorly characterized signalling pathways associated with activation of the AT<sub>2</sub> receptor. Thus, most studies in the literature have reported on the regulation of AT<sub>2</sub> receptor expression at the gene transcription level. However, a unique mode of regulation at the protein level may also be involved, since Csikos et al. (1998)

reported that an unknown endogenous factor can act as a receptor ligand and prevent AT<sub>2</sub> receptor degradation. In addition, cAMP can downregulate the AT<sub>2</sub> receptor through a unique mechanism involving destabilization of AT<sub>2</sub> mRNA (Murasawa et al., 1996).

The AT<sub>2</sub> receptor exhibits a unique tissue-specific and ontogeny-dependent expression. This receptor is the predominant AngII receptor in a number of fetal tissue, including the vasculature, but it is rapidly downregulated after birth in most tissues (Grady et al., 1991 ; Tsutsumi et al., 1991; Viswanathan et al., 1991). In adult tissues, high levels of AT<sub>2</sub> receptor expression have been observed in kidney, heart, myometrium, pancreas, adrenal medulla, ovarian granulosa as well as brain tissues (Nuyt et al., 1999; Ozono et al., 1997; Lenkei et al., 1996; Gehlert et al., 1991; Grady et al., 1991). AT<sub>2</sub> receptor levels are tightly regulated, both positively and negatively, by numerous growth factors (Li JY et al., 1998; Ichiki et al., 1995b; Dudley et al., 1993). Growth factor regulation of AT<sub>2</sub> receptor levels occurred at multiple levels including transcription rates, but not mRNA stability, as well as translation rates through increasing attachment of AT<sub>2</sub> mRNA to polysomal fractions (Li JY et al., 1999). AT<sub>2</sub> receptor levels appear to be very sensitive to culture conditions (Huang et al., 1997; Kambayashi et al., 1996), cell confluency (Heemskerk et al., 1999; Dudley et al., 1993) and cell passage number (Li JY et al., 1998). However, the numerous reports of contradictory effects indicates AT<sub>2</sub> receptor regulation is likely cell-type specific and emphasizes the importance of cautious interpretation of the data within the context of each specific study.

Analysis of the AT<sub>2</sub> receptor gene promoter has revealed several potential *cis*-acting DNA regulatory elements, including an AP-1 binding site, glucocorticoid response element, insulin response element, cAMP response element and a C/EBP site for interleukins (Ichiki et al., 1996; Murasawa et al., 1996; Ichiki et al., 1995a). A study by Dzau et al. (1995) conclusively identified *cis* regulatory elements for the trans-acting interferon regulatory

factor-1 and -2 (IRF-1 and -2) which act as negative and positive regulators of AT<sub>2</sub> receptor expression and, thus, provide a flexible control mechanism (Horiuchi et al., 1995). In addition, elements within the intronic sequence that may be necessary for efficient transcription of the gene have been identified (Warnecke et al., 1999). An *in vitro* myocyte stretch experiment demonstrated that stretch induces both AT<sub>1</sub> and AT<sub>2</sub> receptor mRNA (Kijima et al., 1996); however, the secondary factors or responsive element have not been defined.

### 1.3.4 Biological Role

The biological significance of AngII receptor multiplicity remains unclear. However, the availability of selective receptor antagonists has resulted in a rapidly expanding body of data regarding their role in specific pathophysiologies. This section will focus on the data relating to the role of the AngII receptor subtypes in the cardiovascular system.

**1.3.4.1 AT<sub>1</sub> Receptor:** A detailed overview of the role of the AT<sub>1</sub> receptor in cardiovascular physiology has been recently presented (Helin et al., 1997). Now that the physiology of the systemic RAS and its main receptor, AT<sub>1</sub>, has been extensively studied, recent research has focussed on deciphering the function of this receptor in the local RAS. The role of AT<sub>1</sub> receptor activation in the myocardium was definitively established in a study of the effects of AT<sub>1</sub> receptor overexpression in cardiomyocytes. Transgenic mice that overexpress the AT<sub>1A</sub> receptor selectively in cardiac myocytes exhibited significant myocyte hyperplasia in the atria, significant bradycardia with heart block and premature death (Hein et al., 1997b). Similarly, direct activation of the AT<sub>1</sub> receptor in cardiac myocytes *in vivo* was sufficient to induce cardiomyocyte growth and alter electrical conduction (Hein et al., 1997b). This conclusion is supported by evidence that blockade of the AT<sub>1</sub> receptor with losartan resulted in a significant decrease in heart/body weight ratios in fetal rats (Everett et al., 1997). However, mice with a targeted mutation in the AT<sub>1A</sub> gene were shown to grow and develop

normally except for a lower basal blood pressure and a poor vascular response to AngII (Coffman, 1997). Since the AT<sub>1A</sub> receptor is the predominant receptor subtype in mice, and the absence of any AT<sub>1</sub> receptor subtype in the kidneys was confirmed, this study suggests that functional redundancy or overlap must exist within the RAS to facilitate this compensatory reaction. In agreement with this conclusion, AT<sub>1B</sub> receptor knockout mice were healthy, without an abnormal phenotype (Oliverio et al., 1997); however, mice lacking both the AT<sub>1A</sub> and AT<sub>1B</sub> receptors demonstrated reduced growth and abnormal kidney structure including vascular thickening and atrophy, a phenotype that closely resembled angiotensinogen-knockout mice (Oliverio et al., 1998; Tsuchida et al., 1998).

A role for AT<sub>1</sub> receptor expression and activation has been identified in several cardiovascular pathophysiologies. AT<sub>1</sub> receptors are selectively downregulated in failing human ventricles with idiopathic dilated cardiomyopathy but not ischemic cardiomyopathy (Tsutsumi et al., 1998; Asano et al., 1997; Haywood et al., 1997). In rat models, AT<sub>1</sub> receptor expression was increased during neointimal formation following balloon angioplasty (Hutchinson et al., 1999; Viswanathan et al., 1992), vascular hypertrophy (Wang et al., 1996) and myocardial infarction (Nio et al., 1995). In addition, low-density lipoprotein dramatically upregulated AT<sub>1</sub> receptor expression in VSMC which contributed to enhanced AngII-mediated DNA synthesis (Nickenig et al., 1997b). To date, clinical studies have revealed that AT<sub>1</sub> receptor antagonism significantly improved heart function and survival in the treatment of hypertension, heart failure and post-myocardial infarction (Brunner-La Rocca et al., 1999; Di Pasquale et al., 1999). In animal models, blockade of the AT<sub>1</sub> receptor was shown to inhibit aortic atherosclerosis in a genetically hypercholesterolemic rabbit model (Hope et al., 1999), improve cardiac function and survival in a rat model of CHF (Richer et al., 1999) and improve myocardial contractility and collagen content post-myocardial infarction (Thai et al., 1999). A role for AT<sub>1</sub>-mediated SMC growth has been indicated in many *in vitro* studies

and the upregulation of the AT<sub>1</sub> receptor in the balloon-injured rat carotid artery resulted in neointimal formation (Tazawa et al., 1999); however, a recent study of AT<sub>1A</sub> knock-out mice provides conflicting data since no role for this receptor was found in neointimal formation (Harada et al., 1999).

Recent studies have established that AngII is capable of acting as both a growth factor and an apoptotic factor under different circumstances. A connection between AT<sub>1</sub> receptor activation and apoptosis has been reported in both adult and neonatal rat ventricular myocytes (Kajstura et al., 1997; Cigola et al., 1997) and in rat blood vessels (Diep et al., 1999). In human coronary artery endothelial cells, activation of the AT<sub>1</sub> receptor was shown to increase Fas, decrease bcl-2 and stimulate apoptosis (Li D et al., 1999a).

**1.3.4.2 AT<sub>2</sub> Receptor:** Unlike the AT<sub>1</sub> receptor, no consensus has been reached on the biological role of AT<sub>2</sub> receptor activation (de Gasparo and Siragy, 1999). A large body of accumulated literature details AT<sub>2</sub> receptor localization and changes in the pattern of receptor expression, however, the numerous contradictory reports of AT<sub>2</sub> receptor function have prevented any final conclusions. Although both the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes are expressed in the myocardium of the rabbit (Rogg et al., 1990), rat (Sechi et al., 1992) and humans (Nozawa et al., 1996), as well as vascular SMCS (Otsuka et al., 1998; Levy et al., 1996), until recently few studies have included both AT<sub>2</sub> and AT<sub>1</sub> receptor antagonists. This oversight can be attributed to the general assumption that only the AT<sub>1</sub> receptor mediates the cardiovascular effects of AngII. In fact, a key role for the AT<sub>2</sub> receptor in cardiovascular pathophysiology is indicated by numerous studies that correlate enhanced AT<sub>2</sub> receptor expression or activation with cardiovascular disease states such as heart failure (Tsutsumi et al., 1998; Haywood et al., 1997; Rogg et al., 1996), post-myocardial infarction (Kuizinga et al., 1998; Nio et al., 1995), ischemia (Wiemer et al., 1993), hypertension (Touyz et al., 1999; Wu et al. 1994), diabetes (Sechi et al., 1994), neointimal formation (Harada et al.,

1999) and cardiac hypertrophy due to pressure-overload (Lopez et al., 1994; Suzuki et al., 1993) or AV-shunt (Iwai et al., 1995; Pieruzzi et al., 1995). In hypertensive rats, chronic AT<sub>2</sub> receptor blockade prevented arterial hypertrophy and fibrosis, whereas chronic AT<sub>1</sub> receptor blockade normalized arterial pressure with no effect on the pathological changes in arterial structure (Levy et al., 1996). In support of these data, AT<sub>2</sub> receptor antagonism was shown to be cardioprotective (Ford et al., 1996). In contrast, AT<sub>2</sub> receptor blockade amplified new cardiac protein synthesis in response to AngII in hypertrophied rat hearts (Bartunek et al., 1999). Due to the fact that a significant portion of AngII receptors in the human heart are of the AT<sub>2</sub> subtype, and an increasing number of recent studies have identified a role for this receptor in the cardiovascular effects of AngII, it is no longer prudent to assume the AT<sub>1</sub> receptor is the only subtype involved.

The study of animal models lacking the AT<sub>2</sub> receptor has enhanced our understanding of the biological function of this receptor. AT<sub>2</sub> receptor knock-out mice exhibited behavioural changes such as reduced exploration, impaired thirst response and reduction of spontaneous movement (Hein et al., 1995; Ichiki et al., 1995c). Surprisingly, AT<sub>2</sub> receptor knock-out mice also exhibited a significant elevation in both basal and AngII-stimulated blood pressure that has been partially attributed to upregulation of the AT<sub>1</sub> receptor (Tanaka et al., 1999), as well as the absence of AT<sub>2</sub>-mediated blood volume regulation (Siragy et al., 1999). In addition, AT<sub>2</sub> receptor-mediated nitric oxide production and activation of vascular kinin production provide likely mechanisms for blood pressure regulation (Tsutsumi et al., 1999). Although the AT<sub>2</sub> receptor is not generally associated with the hemodynamic effects of AngII, other data has accumulated that suggests the hemodynamic response to AngII may be determined by the balanced response of both the AT<sub>1</sub> and AT<sub>2</sub> receptors. In mice lacking both the AT<sub>1A</sub> and AT<sub>1B</sub> receptor subtypes, AT<sub>2</sub> receptor mediated blood pressure changes result in lower basal blood pressure (Oliverio et al., 1998). Ablation of the AT<sub>2</sub> receptor

results in hypersensitivity to AngII and increased hypertension and antinatriuresis which suggests a positive counterregulatory role for this receptor in blood pressure regulation (Siragy et al., 1999). Overexpression of the AT<sub>2</sub> receptor gene in vascular smooth muscle of transgenic mice resulted in a vasodilatory response that inhibited AT<sub>1</sub>-mediated pressor effects through nitric oxide synthesis (Tsutsumi et al., 1999). Similarly, cardiac-specific over-expression of the AT<sub>2</sub> receptor gene resulted in decreased sensitivity to AT<sub>1</sub>-mediated pressor and chronotropic actions (Masaki et al., 1998). In addition, a role for the AT<sub>2</sub> receptor in modulating AT<sub>1</sub> receptor-mediated vasoconstriction in rats has been established in SHR that exhibit an exaggerated vasoconstriction response to AngII that correlated with impaired AT<sub>2</sub>-mediated vasodilation (Endo et al., 1998). However, the development of hypertension in this model also correlated with increased levels of AT<sub>2</sub> receptor (Touyz et al., 1999). Other studies support the concept of a vasodilatory role for the AT<sub>2</sub> receptor through the ability of an AT<sub>2</sub> antagonist to enhance AngII-increased blood pressure in rats (Munzenmaier and Greene, 1996) and attenuate the hemodynamic effects of AT<sub>1</sub> receptor blockade in conscious rats (Widdop et al., 1992)

A developmental role for the AT<sub>2</sub> receptor subtype has long been purported due to its high level of expression throughout fetal development, especially in the brain (Gehlert et al., 1991; Grady et al., 1991). A key role for the AT<sub>2</sub> receptor, in addition to the AT<sub>1</sub> receptor, was identified in cardiac development that contributed to cardiac loop inversions and myofibrillar development (Price et al., 1997). The importance of AT<sub>2</sub> receptor function in vasculogenesis was demonstrated through the temporal pattern of expression in the developing aorta that correlated with the decrease in growth rate and the ability of AT<sub>2</sub> receptor blockade to attenuate the reduction in aortic DNA synthesis (Hutchinson et al., 1999; Nakajima et al., 1995). However, mice lacking the AT<sub>2</sub> receptor were viable and exhibited limited defects which suggests the AT<sub>2</sub> receptor either has a limited role in

development or functional redundancy exists in the RAS effector systems. Redundancy in AngII receptors seems to be a favourable explanation, since AT<sub>1</sub> receptor knockout mice were also viable and exhibited few defects (Coffman, 1997)(refer to Section 1.3.4.1). Even with *in vitro* systems, the functional role of the AT<sub>2</sub> receptor has proven difficult to discern. Although expression of the AT<sub>2</sub> receptor correlated with the differentiated or quiescent state *in vitro* in SMC (Kambayashi et al., 1996), PC12W (Meffert et al., 1996), R3T3 cells (Dudley and Summerfelt, 1993), neuronal NIE-115 cells (Reagan et al., 1990) and neuronal NG108-15 cells (Seidman et al., 1996), this phenomena may actually be due to AT<sub>2</sub> receptor regulation by cell-to-cell contact or culture confluency and not differentiation or growth state (Heemskerk et al., 1999).

Conversely, a number of studies have suggested the AT<sub>2</sub> receptor is linked to cellular growth in lymphocytes (Kunert-Radek et al., 1994), cardiac endothelial and fibroblast cells (Kuizinga et al., 1998), astrocytes (Jaiswal et al., 1991b), and neuroblastoma cells (Chen et al., 1993). Although vascular AngII receptors were believed to be exclusively AT<sub>1</sub>, a small but significant population of AT<sub>2</sub> receptors has been observed in the adult rat aorta (Viswanathan et al., 1991; Chang and Lotti, 1991). A new concept suggests that VSMC usually possess an minimal level of AT<sub>2</sub> receptors that are expressed at higher levels during injury or disease processes. While the majority of AngII receptors in normal rat carotid artery are AT<sub>1</sub>, AT<sub>2</sub> receptors become dominant two weeks after injury (Hutchinson et al., 1999; Panek et al., 1992). A recent study of the temporal pattern of receptor expression following injury of rat carotid artery demonstrated an immediate induction of the AT<sub>2</sub> receptor that was followed by later induction of the AT<sub>1</sub> receptor (Hutchinson et al., 1999); however, AT<sub>2</sub> receptor blockade enhanced DNA synthesis in this study. In contrast, a growth-promoting role for the AT<sub>2</sub> receptor in neointimal formation was demonstrated by the efficacy of AT<sub>2</sub> receptor antagonists in blocking vascular hypertrophy and fibrosis (Levy et al., 1996).

Further studies of AngII-induced hypertension in rats demonstrated that AngII-stimulated hypertrophy of the VSMC was mediated by AT<sub>2</sub> receptors, whereas changes in VSMC phenotype were mediated by AT<sub>1</sub> receptors (Sabri et al., 1997). Similarly, Otsuka et al. (1998) demonstrated upregulation of both AT<sub>1</sub> and AT<sub>2</sub> receptors in the aorta of SHR and that AT<sub>2</sub> receptor blockade reduced the medial cross-sectional area. Although CGP42112 has been reported to block neointimal proliferation in response to injury (Janiak et al., 1992), this compound cannot distinguish between a growth or antigrowth role for the AT<sub>2</sub> receptor due to its concentration-dependent antagonistic/agonistic properties. Similarly, an upregulation of the AT<sub>2</sub> receptor has been associated with injury and cellular repair in skin (Viswanathan and Saavedra, 1992) and neuronal cells (Gallinat et al., 1998; Lucius et al., 1998).

Recent studies have also reported anti-growth and pro-apoptotic roles for the AT<sub>2</sub> receptor and indicated a functional antagonism exists between the AT<sub>1</sub> and AT<sub>2</sub> receptors. For example, the growth response to AngII was enhanced in the presence of an AT<sub>2</sub> receptor antagonist in coronary endothelial cells that express both AT<sub>1</sub> and AT<sub>2</sub> receptors (Stoll et al., 1995). These data were supported by several reports of AT<sub>2</sub>-mediated growth inhibition in endothelial cells (Metsarinne et al., 1993), cardiomyocytes (van Kesteren et al., 1997; Booz and Baker, 1996), and R3T3 fibroblasts (Tsuzuki et al., 1996). Gene transfer of the AT<sub>2</sub> receptor *in vivo* into rat carotid artery following balloon catheter injury resulted in attenuated DNA synthesis and neointimal hyperplasia in opposition to the AT<sub>1</sub> receptor, likely via inhibition of MAPK (Nakajima et al., 1995). This anti-growth role has been further supported with *in vivo* data that demonstrated AT<sub>2</sub> receptor blockade reversed the reduction in infarct size by AT<sub>1</sub> receptor antagonism after ischemic injury in pigs (Jaloway et al., 1998), as well as amplified the early left ventricular growth response to AngII in rats (Bartunek et al., 1999). Similarly, activation of the AT<sub>2</sub> receptors produced an anti-fibrotic

effect in opposition to the AT<sub>1</sub> receptor in a model of kidney nephropathy (Morrissey and Klahr, 1999). In addition, apoptosis, a critical event in cardiovascular remodelling, has been linked to AT<sub>2</sub> receptor activation in R3T3 (Horiuchi et al., 1997) and rat ovarian granulosa (Tanaka et al., 1995) cells that exclusively express the AT<sub>2</sub> receptor. A role for the AT<sub>2</sub> receptor in apoptosis was supported by a study of fibroblasts derived from AT<sub>2</sub> knockout mice which showed that these cells did not exhibit AngII-mediated apoptosis in comparison to the wild-type cells (Li W et al., 1998). However, no apoptotic changes occurred with AT<sub>2</sub> receptor overexpression in the heart of transgenic mice (Masaki et al., 1998). Conversely, both the AT<sub>1</sub> and AT<sub>2</sub> receptors mediated apoptosis *in vivo* in rat blood vessels following AngII infusion (Diep et al., 1999).

In conclusion, the information obtained from the various AngII receptor knock-out mice has supplied valuable insight into the functional multiplicity in the AngII receptor system and, as a result, the concept of an integrated biological response to AngII through redundancy and interplay between the different receptor subtypes has emerged (refer to Section 1.4.3.4). Since both AT<sub>1</sub> and AT<sub>2</sub> receptors exhibit the ability to promote and antagonize the proliferative effects of AngII, it is premature to assume that their roles are set with AT<sub>1</sub>-mediated growth and AT<sub>2</sub>-mediated anti-growth. Further caution in the interpretation of results using receptor antagonists or ablation is warranted after the report that ACE activity, and subsequently AngII formation, is significantly increased in AT<sub>2</sub> receptor knockout models or under conditions of AT<sub>2</sub> receptor antagonism (Hunley et al., 2000) which suggests that some of the effects attributed to AT<sub>2</sub> receptor function could be due to increased activation of other receptors in the RAS. Similarly, some of the biological effects attributed to the AT<sub>1</sub> receptor through losartan may actually be due to increased activation of the unopposed AT<sub>2</sub> receptor (Munox-Garcia et al., 1995). As the search for new and improved receptor antagonists continues, the data base on the relative contribution of the

different angiotensin receptor subtypes grows. It is important that a clear understanding of the biological role of each receptor is established in order for these receptor antagonists to be effectively employed in therapeutic interventions.

## **1.4 ANGIOTENSIN RECEPTOR-ASSOCIATED SIGNAL TRANSDUCTION**

### **1.4.1 Overview of Receptor-mediated Signal Transduction**

The ability of each cell to respond to the myriad of signals present in their environment is essential to the survival of the cell and the organism itself. The transduction of signals from the extracellular to intracellular environment of the cell occurs mostly through cell-surface receptors which, upon binding the extracellular ligand, undergo a conformational change of the transmembrane domain that transmits the signal to initiate an intracellular second messenger signalling cascade. Thus, the function of receptors is simply to participate in the flow of information from the cell surface to the nucleus in order to regulate cell growth and metabolic activity. Several different classes of cell surface receptors have been distinguished based on their structural elements. The receptor classes relevant to this thesis are 1) receptors with an intrinsic enzymatic activity (Tyrosine kinase receptors) and 2) receptors linked through intermediate couplers to signal effector enzymes (G-protein-coupled receptors). This distinction between receptor types, however, is not maintained in the downstream signalling systems. The receptors are coupled to many effectors including phospholipases, ion channels, adenylyl and guanylyl cyclases, protein kinases and phosphatases which allow the initial signal to be amplified and diversified. Cross-talk between the signalling pathways coupled to different receptors further modulates the cellular response and this new area of signal transduction overlap is the focus of intense research.

### **1.4.2 G-protein-coupled signal transduction**

**1.4.2.1 Mechanism of G-protein Activation:** Guanine nucleotide-binding protein (G-protein)-coupled receptors encompass a superfamily of cell surface receptors with a wide

range of ligands such as neurotransmitters, vasoactive peptides and growth factors that play key roles in cellular growth, differentiation and specific cellular functions. All members of the G-protein receptor superfamily consist of a conserved seven transmembrane domain structure and conserved residues on the intracellular loop that interact with heterotrimeric G-proteins. Each G-protein consists of three polypeptides: an  $\alpha$  subunit that binds and hydrolyzes GTP and a  $\beta\gamma$  dimer that serves as a functional monomer and membrane anchor. The formation of a heterotrimer complex and the regulation of the intrinsic GTPase activity allows G-protein receptors to act as molecular switches that can be rapidly turned on and off (Hamm and Gilchrist, 1996). When GDP is bound to the  $G\alpha$  subunit, it associates with the  $G\beta\gamma$  dimer to form an inactive heterotrimer. Once the receptor is activated, a conformational change in the G-protein complex leads to a decreased affinity of  $G\alpha$  for GDP, resulting in a rapid exchange of GDP for GTP. The GTP-bound state represents the active conformation and allows the  $G\alpha$  and  $G\beta\gamma$  subunits to dissociate and bind to downstream effector molecules. Activation of the intrinsic GTPase activity of the  $G\alpha$  subunit results in hydrolysis of the GTP to GDP and the reassociation with the  $G\beta\gamma$  subunit (Hamm and Gilchrist, 1996).

**1.4.2.2 Diversity of G-protein Subunits:** Cloning of various members of the G-protein family from both vertebrates and invertebrates has revealed a large degree of diversity, including at least twenty unique  $\alpha$  subunits, five  $\beta$  subunits and 12  $\gamma$  subunits (Hamm and Gilchrist, 1996). Diversity in the G-protein receptor is mostly due to differences in the  $G\alpha$  and  $G\gamma$  subunits. Regulation of the spatial and/or temporal expression of the different subunits contributes to the regulation of the G-protein receptor (Neer, 1995).

Four classes of  $G\alpha$  subunits have been defined based on homology of their amino acid sequences:  $G\alpha_q$ ,  $G\alpha_i$ ,  $G\alpha_s$ ,  $G\alpha_{12}$  (Post and Brown, 1996). The  $G_i$  family have been defined by their sensitivity to pertussis toxin ADP-ribosylation and their ability to inhibit adenylyl cyclase. The hormones with  $G_i$  protein-linked receptors include AngII, thrombin, endothelin,

serotonin and lysophosphatidic acid. Gs proteins, sensitive to constitutive activation by cholera toxin ADP-ribosylation, have been shown to stimulate adenylyl cyclase. Ligands capable of activating Gs proteins include prostaglandin I<sub>2</sub>, glucagon, parathyroid hormone, thyrotropin, and luteinizing hormone. The Gq family generally couples to the effectors PLC and PKC. Some examples of hormone receptors linked to Gq proteins include AngII, bradykinin, prostaglandin F<sub>2</sub> $\alpha$ , serotonin and substance P. The G12/G13 family of G-proteins are pertussis toxin-insensitive and their effector enzymes have not been clearly defined.

The G $\beta$  subunits exhibit a highly conserved amino acid sequence (50-85% identical) and appear to be interchangeable (Hamm and Gilchrist, 1996). In contrast, the G $\gamma$  subunits are more divergent with 25-74% homology at the amino acid level and variations in post-translational modifications. Thus, the G $\gamma$  subunit is thought to determine the functional specificity of the G $\beta\gamma$  subunit and modulate its interaction with the receptor. The G $\beta$  and G $\gamma$  subunits are tightly bound through non-covalent forces to form a functional monomer that dissociates only under reducing conditions.

**1.4.2.3 G-protein-linked Effectors:** Stimulation of G-proteins leads to the activation or inhibition of one or more intracellular effector enzymes such as phospholipases C, A<sub>2</sub> and D (PLC, PLA<sub>2</sub>, PLD), adenylyl cyclase (AC), protein kinase A (PKA) and protein kinase C (PKC) (Birnbaumer et al., 1990). Briefly, AC activation results in the formation of cAMP. Consequently, cAMP-dependent PKA is activated, resulting in the phosphorylation of numerous targets including transcription factors. Phospholipase C activation generates the second messengers inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). IP<sub>3</sub> triggers calcium release from intracellular stores and this increase in cytosolic calcium initiates a variety of cellular responses through calcium-dependent enzymes such as PKC. DAG diffuses within the membrane bilayer and activates the membrane-associated serine/threonine kinases, PKC. The isoforms of PKC have been sorted into three categories, conventional

isoforms ( $\alpha, \beta, \gamma$ ), novel isoforms ( $\delta, \epsilon, \eta, \theta$ ) and atypical isoforms ( $\zeta, \lambda, \mu$ ), that differ in their intracellular localization and downstream targets/substrates (Clemens et al., 1992). In general, activation of protein kinases PKA or PKC initiates a phosphorylation cascade that involves a number of substrates including other protein kinases such as MAPK/ERKs, phosphatases, ion channels, other growth factor receptors, G-proteins, transcription factors and DNA-binding proteins.

**1.4.2.4 Tyrosine Phosphorylation and  $G\beta\gamma$ -mediated Signalling Pathways:** An universal mechanism in cell signalling relies on the reversible phosphorylation and dephosphorylation of second messenger proteins (adapter proteins, kinases, phosphatases) to control the association and dissociation of key effector molecules. In general, phosphorylation of tyrosine residues is a key event in the regulation of growth and differentiation (Barford, 1991). Although G-protein-linked receptors lack an intrinsic tyrosine kinase activity, the body of accumulated evidence supports the concept that G-protein-coupled receptors utilize similar mechanisms and pathways as tyrosine kinase receptors to activate intracellular second messenger proteins. One of the key signalling targets for both tyrosine kinase and G-protein-coupled receptors is the family of serine/threonine kinases termed extracellular receptor-related kinase (Erk) or mitogen-activated protein kinases (MAPK). A recent study demonstrated the both  $G_i$  and  $G_q$  mediate Erk1 and Erk2 activation by a common tyrosine kinase-, Ras- and calcium-dependent pathway that involves calmodulin in some systems (Della Rocca et al., 1997). In addition,  $G_i$ - and  $G_q$ -coupled receptors have been shown to recruit and promote the tyrosine phosphorylation of Pyk2 and Src, which results in Src-mediated activation of MAPK (Dikic et al., 1996) as well as other Src-related kinases such as Syk and Lyn (Wan et al., 1996).

One of the mechanisms for cross-talk between G-protein receptors and tyrosine kinase phosphorylation pathways occurs through an interaction of  $G\beta\gamma$  subunits with tyrosine

kinase intermediates. The importance of the  $G\beta\gamma$  subunits, independent of the  $G\alpha$  subunit, in transducing the downstream signal is a relatively new area of research. Activation of the  $G\beta\gamma$  subunits occurs through GTP binding to the  $G\alpha$  subunit and subsequent dissociation of the trimeric G-protein. Relatively little is known about regions in  $G\beta$  or  $G\gamma$  that interact with effector molecules. Some evidence suggests that a conserved sequence for  $G\beta\gamma$  binding may be present in its various target effector molecules (Chen et al., 1995). The  $G\beta\gamma$  subunits have been shown to directly associate with several effector molecules such as adenylyl cyclase (Chen et al., 1995),  $PLC\beta$  (Camps et al., 1992),  $PLA_2$  (Jelsema and Azelrod, 1987), PI3K (Morris et al., 1995), a newly discovered isoform of PI3K $\gamma$  (Stephens et al., 1994; Stoyanov et al., 1995), Ras (van Biesen et al., 1995) and intracellular tyrosine kinases such as Pyk2 (Lev et al., 1995), Tsk and Btk (Langhans-Rajasekaran et al., 1995). Similarly, the  $G\beta\gamma$  subunits have been shown to mediate the translocation of receptor kinases such as GRK ( $\beta$ ARK) which modulate receptor function through phosphorylation of the receptor itself (Pitcher et al., 1992). The ability of the  $G\beta\gamma$  subunit to recruit adapter proteins such as Shc and stimulate its phosphorylation (Touhara et al., 1995), possibly through the non-receptor tyrosine kinase Src (Luttrell et al., 1996), may represent a key point of convergence between G-protein-mediated and tyrosine kinase receptor-mediated signalling pathways (Chen et al., 1996). Specifically,  $G_i$  protein-coupled receptors stimulate MAPK through several  $G\beta\gamma$ -mediated pathways such as  $G\beta\gamma$ -recruitment of p85-regulated PI3K and a Src family kinase (Hawes et al., 1996) as well as  $G\beta\gamma$ -activation of Ras through a subset of the signalling proteins Shc, Grb2, Sos or Ras-GRF (Kranenburg et al., 1997; Shou et al., 1995; van Biesen et al., 1995; Crespo et al., 1994; Koch et al., 1994). The interaction of  $G\beta\gamma$  with p85 $\alpha$ /p110 $\beta$  PI3K has been shown to be enhanced by IRS-1 binding (Kurosu et al., 1997). In addition, stimulation of muscarinic  $G_q$  protein-coupled receptors results in  $G\beta\gamma$ -dependent recruitment of PI3K $\gamma$  to the plasma membrane and subsequent activation of MAPK through a pathway requiring Shc,

Grb2, Sos, Ras and Raf (Lopez-Illasaca et al., 1997) and possibly a direct interaction of PI3K $\gamma$  and Ras (Rubio et al., 1997). The ability of Gq proteins to stimulate MAPK may be due to G $\beta\gamma$  interactions with Raf-1 protein kinase (Pumiglia et al., 1995). Interestingly, the association between Raf-1 and G $\beta\gamma$  may provide a mechanism for inhibiting the interaction of G $\alpha$  with G $\beta\gamma$  subunits or conversely Raf-1 with GTP-Ras and, thus, modulate their downstream signalling.

### 1.4.3 Angiotensin Receptor-mediated Signalling Pathways

AngII has been established as a key biopeptide involved in many normal and pathological functions. On a single cell level, the response to AngII stimulation results from the integration of the many parallel signalling pathways activated through at least two cell-surface receptor subtypes, AT<sub>1</sub> and AT<sub>2</sub>. Research has focussed on unravelling the complex signal transduction mechanisms associated with the receptors for AngII in order to open new perspectives for the development of therapeutic tools.

**1.4.3.1 AT<sub>1</sub> Receptor:** The AT<sub>1</sub> receptor subtype has been identified as a G-protein-coupled receptor organized into a seven transmembrane domain structure that includes a ligand binding pocket and three cytoplasmic loops. The AT<sub>1</sub> receptor signalling pathways include those classically associated with Gq and Gi receptors that activate downstream events through the effectors phospholipase C, phospholipase D, phospholipase A<sub>2</sub>, calcium channels and adenylate cyclase inhibition (see review Hughes 1998).

The generation of phospholipid-derived second messengers represents one of the major signal transduction pathways of AngII. In cultured SMCs, PLC is activated immediately to produce inositol 1,4,5-triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), important second messengers that initiate the release of calcium from internal stores in the sarcoplasmic reticulum and activate the downstream effector PKC, respectively (Smith, 1996). The specific isoform of PLC activated by AngII has not been clearly established. For instance, the AT<sub>1</sub> receptor has been shown to stimulate the traditional G-protein-coupled

PLC $\beta$  (Schelling et al., 1997; Booz and Baker 1995). On the other hand, the PLC $\gamma$  isoform accounted for the majority of IP $_3$  generation in response to AngII in another study (Marrero et al., 1994). Interestingly PLC $\gamma$  has been linked to the AT $_1$  receptor through a tyrosine kinase-dependent mechanism that involves Src (Marrero et al., 1995c; Ishida et al., 1995). However, in another system AngII-mediated IP $_3$  production was reported to be resistant to tyrosine kinase inhibitors (Leduc et al., 1995a). A novel protein, ATRAP, has been recently identified that specifically interacts with the AT $_{1A}$  receptor to modulate PLC activation (Daviet et al., 1999).

The next important downstream effector activated in this pathway is PKC. Multiple isoforms of PKC have been identified; however, the function of the various PKC isoforms and their subcellular localization remain to be clarified (Natarajan et al., 1994b). In VSMCs, four isoforms of PKC ( $\alpha, \beta, \epsilon, \zeta$ ) have been identified and AngII stimulation resulted in the activation of PKC $\epsilon$  (Malarkey et al., 1996), as well as the translocation of PKC $\alpha$  and PKC $\beta$  to the nucleus (Haller et al., 1994). Similarly, five PKC isoforms ( $\alpha, \delta, \zeta, \lambda, \mu$ ) have been identified in canine pulmonary arterial SMCs and AngII-stimulation increases the levels of PKC $\zeta$  and PKC $\alpha$  in the nucleus while PKC $\delta$  translocates to the myofilaments (Damron et al., 1998). AngII has also been shown a potent and rapid activator of a novel Ser/Thr protein kinase, PKD, that is structurally distinct from the PKC isoforms but may represent a component of PKC-independent signal transduction (Abedi et al., 1998). Downstream signal transduction leads to activation of various protein kinases, including MAPK (Eguchi et al., 1996), a novel FAK homologue termed the calcium-dependent tyrosine kinase (CADTK) (Brinson et al., 1998), calcium-calmodulin-dependent protein kinase (Lu et al., 1994) and calcineurin (Baukal et al., 1994), as well as the induction of early growth-related proto-oncogene such as *c-fos* in aortic SMCs (Taubman et al., 1989) and Fos-regulating kinase (Huang et al., 1998).

Subsequent to PLC, activation of the AT $_1$  receptor results in stimulation of PLD, an

enzyme that cleaves phosphatidylcholine to produce phosphatidic acid which, in turn, undergoes a rapid conversion to DAG (Freemann et al., 1994). This second, more prolonged, DAG production may result in a sustained activation of PKC in SMCs (Lassègue et al., 1993; Griendling et al., 1986). Thus, the prolonged activation of PLD, and its independent regulation from PLC, may be a significant factor in the long-term responses to AngII. A recent study proposed that the full mitogenic response to AngII required both MAPK and PLD activation (Wilkie et al., 1996). Also, PLD activation involved PKC activity and  $Ca^{2+}$  mobilization in VSMCs (Freeman et al., 1995). Recently,  $G\beta\gamma$  subunits were shown to be important in the coupling of the  $AT_1$  receptor to PLD in VSMC (Ushio-Fukai et al., 1999a).

$AT_1$  receptor activation has also been linked to  $G_i$  protein stimulation which results in the inhibition of AC (Jard et al., 1985) and the stimulation of  $PLA_2$  (Pfeilschifter and Bauer, 1986). AngII-treatment of vascular SMCs has been shown to result in the activation of  $PLA_2$  which hydrolyzes membrane phospholipids to produce free fatty acids such as arachidonate, a precursor to prostaglandins and leukotrienes (Rao GN et al., 1994). A cytosolic form of  $PLA_2$  is activated via the  $AT_1$  receptor in SMCs and translocates to the nucleus in a  $Ca^{2+}$ -dependent manner (Freeman et al., 1998)

Another key signal transduction mechanism for the  $AT_1$  receptor involves the autocrine production of other growth factors and/or activation of their receptors. A recent study of the mitogenic effects of AngII provided convincing evidence that epiregulin, an EGF-related growth factor, is the main autocrine factor involved in VSMCs (Thomas et al., 1999). Alternatively, PDGF has been identified as a key autocrine factor since  $AT_1$  receptor activation resulted in PDGF-A and -B mRNA expression, tyrosine phosphorylation of PDGF- $\alpha$  and - $\beta$  receptors and the resultant intimal thickening in an *in vivo* rat balloon-injured carotid artery system (Abe et al., 1997). Previously, the growth response of VSMCs to AngII has been attributed to the autocrine production of bFGF and TGF $\beta$ 1 (Itoh et al., 1993;

Koibuchi et al., 1993). The ability of AngII to stimulate a hypertrophic rather than hyperplastic growth response in some cell types has been attributed to the level of autocrine production of TGF $\beta$ 1 (Gibbons et al., 1992; Koibuchi et al., 1993); however, recent evidence suggests TGF $\beta$ 1 is not involved and instead, the inability of AngII to regulate key cell cycle regulatory molecules such as p27<sup>Kip1</sup> (Servant et al., 2000). Similarly, AT<sub>1</sub>-mediated TGF $\beta$ 1 production may exert a modulatory effect on other cellular functions such as migration (Liu G et al., 1997). An autocrine/paracrine mechanism of endothelin-1 production has also been demonstrated for AT<sub>1</sub>-mediated proliferation of SMCs (Sung et al., 1994).

Alternatively, a novel mechanism for AT<sub>1</sub>-mediated growth involves the transactivation of other growth factor receptors. Recently, AngII was shown to directly phosphorylate the epidermal growth factor (EGF) receptor through a calcium-dependent mechanism that acts as a scaffold for recruitment and activation of Src, pyk2, Shc and Grb2 in VSMCs (Eguchi et al., 1998). Similarly, activation of the AT<sub>1</sub> receptor by AngII in cardiac fibroblasts resulted in the calcium/calmodulin-dependent transactivation of EGF receptor, and the subsequent stimulation of MAPK, *c-fos* expression and DNA synthesis in a PKC-independent manner (Murasawa et al., 1998). AngII has also been shown to phosphorylate the PDGF- $\beta$  receptor, in the absence of PDGF release, in rat aortic SMCs which may indicate a cross-talk mechanism exists between these distinct signalling pathways (Linseman et al., 1995). Interestingly, AngII has also been shown to increase PDGF and TGF $\beta$ 1 production through an intracrine mechanism (Weber et al., 1994).

**1.4.3.2 Tyrosine phosphorylation in AngII-stimulated signal transduction:** The AngII receptors represent G-protein-coupled receptors for which protein tyrosine phosphorylation has been identified as crucial signal transduction component (Sauro et al., 1996; Marrero et al., 1995a). Since G-protein receptors lack intrinsic tyrosine kinase activity, the mechanisms by which these receptors couple to kinase cascades remains to be elucidated. Although the

precise role of this signalling pathway remains to be established, evidence is mounting to suggest that tyrosine phosphorylation plays an important role in the growth-promoting effect of G-protein receptor agonists like AngII. Numerous studies have established AngII stimulates the tyrosine phosphorylation of an extensive list of cellular proteins in VSMC, including several key protein kinases such as Src (Ishida et al., 1995), ERK1, ERK2 (Tsuda et al., 1992), FAK (Polte et al., 1994), JAK2 and Tyk2 (Giasson et al., 1997; Marrero et al., 1995b), Pyk2 (Eguchi et al., 1999), c-CRK II and its substrate p130Cas (Takahashi et al., 1998), Rho-kinase (Yamakawa et al., 2000), PLC $\gamma$  (Marrero et al., 1995c), adapter proteins such as Shc (Linseman et al., 1995; Molloy et al., 1993) and IRS-1 (Ali et al., 1997b; Du et al., 1996b), several STAT transcription factor isoforms (Marrero et al., 1995b; Bhat et al., 1994), and the protein tyrosine phosphatase PTP1D (Ali et al., 1997b). AngII treatment also results in the rapid phosphorylation of paxillin (Leduc and Meloche, 1995b; Okuda et al., 1995; Turner et al., 1995) and FAK (Polte et al., 1994) which translocate to focal adhesions and subsequently phosphorylate talin (Giasson et al., 1997; Turner et al., 1995) and tensin (Ishida et al., 1999).

In the majority of these studies, activation of the AT<sub>1</sub> receptor resulted in the tyrosine phosphorylation of downstream signal effectors; however, the involvement of tyrosine phosphorylation in the AT<sub>2</sub>-mediated signalling pathways remains to be defined. A key role for protein tyrosine phosphatases in AT<sub>2</sub>-mediated signalling pathways has been established (refer to Section 1.4.3.3). The AT<sub>1</sub> receptor itself may play a direct role in the tyrosine phosphorylation signalling cascade, since activation, phosphorylation, and internalization will modulate the association of signalling molecules with the carboxyl-terminus. Although the AT<sub>1</sub> receptor is tyrosine phosphorylated (Venema et al., 1998; Paxton et al., 1994), there are conflicting results as to whether AngII stimulates an increase in the level of receptor tyrosine phosphorylation (Marrero et al., 1995c; Kai et al., 1994), possibly due to problems

with the antibodies used for detection (Thomas et al., 1999). Recent studies have conclusively demonstrated a time- and agonist-dependent phosphorylation of the AT<sub>1</sub> receptor (Smith et al., 1998; Thomas et al., 1998; Oppermann et al., 1996). The kinases responsible for phosphorylation of the AT<sub>1</sub> receptor have been identified as MAPK in neuronal cells (Yang et al., 1997b), as well as specific G-protein receptor kinases (GRKs) and PKC in HEK293 cells (Oppermann et al., 1996); however, the sites of phosphorylation are not yet defined. An interesting mechanism for concentration-dependent regulation of AT<sub>1</sub> receptor phosphorylation was reported, where low concentrations of AngII (1 nM) resulting in PKC-mediated phosphorylation and high concentrations of AngII (100 nM) resulted in a GRK-mediated phosphorylation (Balmforth et al., 1997b). A putative "YIPP" motif has been identified in the AT<sub>1</sub> receptor which may function as an SH2 targeting sequence. This motif is involved in the recruitment of PLC- $\gamma$ 1 (Venema et al., 1998). In addition, Jak2 kinase has been shown to directly interact with the carboxyl-tail of AT<sub>1</sub> receptor via the YIPP motif (Ali et al., 1997a), likely through an adapter protein such as SHP-2 (Venema et al., 1998). A direct association of eNOS with the AT<sub>1</sub> receptor carboxyl-terminus was also recently reported (Ju et al., 1998).

The activation of Src kinase (pp60<sup>src</sup>) represents a key element in AngII-mediated growth. Several of the proteins tyrosine phosphorylated in response to AngII stimulation have been identified as downstream targets of Src kinase, including two key GTPase-activating proteins in the Ras-Raf-1 complex, Ras-GAP and Rho-GAP (Schieffer et al., 1996), as well as PLC $\gamma$  (Ishida et al., 1995; Marrero et al., 1995c). Recently, activation of Rho and Rho-kinase via the AT<sub>1</sub> receptor was shown to play an important role in AngII-stimulated hypertrophy of VSMC (Yamakawa et al., 2000). AngII-dependent activation of PLC $\gamma$ , the isoform typically associated with tyrosine kinase receptors, has been shown to account for the majority of IP<sub>3</sub> formation in VSMC (Marrero et al., 1994). Interestingly, a recent study

demonstrated a biphasic activation of PLC with the first phase comprising coupling of the AT<sub>1</sub> receptor to PLCβ1 through its Gβγ subunit and the second phase involving activation of PLCγ by tyrosine phosphorylation (Ushio-Fukai et al., 1998a).

AngII-induced tyrosine phosphorylation cascades also result in the recruitment of MAPKs, a family of serine/threonine kinases that function as key integrators of mitogenic signals from a variety of receptors. AngII has been shown to be a potent activator of MAPK pathway in VSMC *in vivo* (Adam et al., 1995) and *in vitro* (Duff et al., 1992). Specifically, AngII stimulates the phosphorylation and activation of MAPKKK (Liao et al., 1996), MEKK and Raf-1 (Butcher et al., 1993; Ishida et al., 1992), MEK (Ishida et al., 1992) and ERK1 and ERK2 (Duff et al., 1995; Tsuda et al., 1992). AngII has also been shown to activate MAPK through the AT<sub>1</sub> receptor via Gq-dependent Ras activation through PLC and Ca<sup>2+</sup>/calmodulin-dependent tyrosine kinase activity (Eguchi et al., 1996). The MAP/ERK pathway has also been shown to be essential in the enhanced level of AngII-mediated contraction and intracellular calcium in SHR (Touyz et al., 1999)..

Thus, in addition to the classical signal effectors of G-protein-linked receptors, significant overlap exists with the signalling pathways previously associated with tyrosine kinase receptors. As demonstrated by the AngII signalling pathways, these protein phosphorylation cascades play a key role in mediating its growth effects.

**1.4.3.3 AT<sub>2</sub> Receptor Subtype:** The signalling mechanisms of the AT<sub>2</sub> receptor are not well defined in comparison with the AT<sub>1</sub> receptor. Until the last few years, very few studies focused on the function and/or downstream signalling pathways of this receptor since it was assumed that its contribution to the physiological effects of AngII was minimal. Although many processes have been linked to AT<sub>2</sub> receptor activation in various experimental *in vivo* and *in vitro* systems, further clarification has been hampered by the lack of "classical" signal transduction pathways associated with this receptor. The past 5 years have resulted in

numerous studies of AT<sub>2</sub> receptor function and associated signalling pathways, but inconsistencies have clouded our ability to make broad generalizations.

The AT<sub>2</sub> receptor exhibits structural homology with the superfamily of G-protein-coupled receptors through its putative seven-transmembrane domain structure, its post-translational modification by N-terminal glycosylation, the conservation of critical sites for G-protein binding and IP<sub>3</sub> signalling similar to the AT<sub>1</sub> receptor (Mukoyama et al., 1993). Within the carboxy-terminal tail, several consensus sequences for protein kinase phosphorylation such as PKC have been identified (Griendling et al., 1996), but no role for PKC in AT<sub>2</sub> receptor signalling pathways has yet been identified. However, this receptor subtype lacks the signal transduction mechanisms that are hallmarks of G-protein-coupled receptors since it does not appear to couple to adenylate cyclase or phospholipid hydrolysis and lacks sensitivity to analogues of GTP (Mukoyama et al., 1993). Until recently, the apparent absence of coupling to G-proteins was used to define AT<sub>2</sub> receptor binding (Pucell et al., 1991; Dudley et al., 1990). The debate over G-protein-coupling of the AT<sub>2</sub> receptor was clearly demonstrated by the groups that initially cloned this receptor. The AT<sub>2</sub> receptor cloned from the rat PC12W cell line exhibited a pertussis toxin-sensitive G-protein-coupled mechanism for inhibition of PTP activity (Kambayashi et al., 1993b). Despite the similarity of the cloned sequences, the AT<sub>2</sub> receptor cloned from rat fetal cDNA library exhibited no G-protein coupling (Mukoyama et al., 1993). However, several reports have demonstrated AT<sub>2</sub> receptor coupling to pertussis toxin sensitive G-proteins or Gi proteins is involved in the activation of protein phosphatase 2A (Huang et al., 1996b), MAP kinase phosphatase (MKP-1) (Horiuchi et al., 1999), and delayed rectifier K<sup>+</sup> channels (Kang et al., 1995), as well as the inhibition of serum-stimulated cell growth (Ozawa et al., 1996) and bFGF-induced proliferation (Liakos et al., 1997). Conclusive evidence of G-protein involvement in AT<sub>2</sub> receptor signal transduction was recently provided by the direct association of Gi $\alpha_2$  and Gi $\alpha_3$

with the AT<sub>2</sub> receptor in the rat fetus (Zhang and Pratt, 1996). In summary, the cumulative evidence does indicate that the AT<sub>2</sub> receptor is coupled to the pertussis toxin-sensitive G-protein Gi. The conflicting reports of the absence of G-protein-coupling to the AT<sub>2</sub> receptor may reflect differences in the cell model or the capacity of the AT<sub>2</sub> receptor to mediate some of its downstream effects through G-protein-independent signalling pathways.

To date, one of the signal transduction pathways clearly associated with AT<sub>2</sub> receptor activation involves the modulation of protein phosphatases. Initially, there were conflicting reports as to whether AngII activates or inhibits protein tyrosine phosphatases (PTP), even in the same cell system (Brechler et al., 1994; Takahashi et al., 1994; Kambayashi et al., 1993b). Several reports have demonstrated that the AT<sub>2</sub> receptor mediates the rapid activation of PTPs in many different cell types (Tsuzuki et al., 1996a; Nahmias et al., 1995). The anti-growth effects of AT<sub>2</sub> receptor activation may be due to the activation of PTPs (Liakos et al., 1997; Tsuzuki et al., 1996b) and the inhibition of MAPK pathways through the PTP activity of SHP (Bedecs et al., 1997), as well as the ability to dephosphorylate AT<sub>1</sub>-mediated phosphorylation of STATs (Horiuchi et al., 1999). Another key phosphatase in AT<sub>2</sub> receptor signalling is serine/threonine phosphatase 2A (PP2A) which was stimulated in a time- and dose-dependent manner in neuronal cells (Huang et al., 1995; Huang et al., 1996b; Kang et al., 1995). A functional consequence of AT<sub>2</sub>-stimulated PP2A activity was demonstrated in the stimulation of apoptosis in neuronal cells (Shenoy et al., 1999). In addition, an okadaic acid-sensitive protein phosphatase, the MAP kinase phosphatase-1 (MKP-1), plays a key role in AT<sub>2</sub> receptor signalling pathways to mediate apoptosis or growth inhibition (Horiuchi et al., 1999). AT<sub>2</sub>-stimulated MKP-1 activity resulted in the dephosphorylation of Bcl-2 and upregulation of Bax to induce apoptosis in PC12W cells (Horiuchi et al., 1999; Yamada et al., 1996). In contrast, AT<sub>2</sub> receptor stimulation inhibited the MAP kinase pathway through the okadaic acid-insensitive SHP-1 phosphatase in N1E-

115 cells (Bedecs et al., 1997). The AT<sub>2</sub> receptor-mediated regulation of MAP kinase activity may have a crucial role in fetal vasculogenesis, since AT<sub>2</sub> receptor-null mice exhibited an exaggerated growth response that was attributed to higher basal activity of MAPK (Akishita et al., 1999).

Guanylyl cyclase is one of the downstream effector enzymes often linked to AT<sub>2</sub> receptor activation which mediates cGMP production. The first report of a putative signalling pathway for the AT<sub>2</sub> receptor involved depression of basal cGMP levels in neonatal rat neuron cultures (Sumners et al., 1991). Later, both the AT<sub>1</sub> and AT<sub>2</sub> receptors were linked to stimulation of cGMP levels in N1E-115 cells (Zarahn et al., 1992). However, conflicting results have been reported in the pheochromocytoma cell line PC12W, since AT<sub>2</sub> receptor stimulation resulted in the activation (Brechler et al., 1993) or the inhibition (Bottari et al., 1993) or the absence (Kambayashi et al., 1993b) of guanylate cyclase activity. Activation of the AT<sub>2</sub> receptor reduced cGMP levels in the neointima of balloon-injured rat aorta (Moroi et al., 1997). Conversely, the AT<sub>2</sub> receptor increased cGMP levels in rat renal tissues *in vivo* via a nitric oxide signalling pathway that targets guanylyl cyclase (Siragy and Carey, 1996; 1997b). A role for nitric oxide has been identified in AT<sub>2</sub> receptor-mediated regulation of neuronal differentiation (Cote et al., 1998), renal function (Siragy and Carey, 1997b) and apoptosis in vascular endothelial cells (Dimmeler et al., 1997).

Characterization of the AT<sub>2</sub> receptor signalling pathways has been plagued by the lack of identifiable signal transduction systems and conflicting reports in the literature. Confusion regarding the signal transduction pathways linked to the AT<sub>2</sub> receptor has proven common for all the signalling molecules discussed and may be reflective of a broader mechanism that integrates many extracellular and intracellular factors in controlling AT<sub>2</sub> receptor function and coupling to downstream effectors (refer to Section 1.3.3.2). These complications have led to the investigation of "non-traditional" G-protein-linked signalling

pathways such as lipid-signalling pathways.  $AT_2$  receptors have been linked to activation of  $PLA_2$  and arachidonic acid release (Zhu et al., 1998), as well as the biosynthesis of prostaglandins (Jaiswal et al., 1993a; 1991b). In addition, the  $AT_2$  receptor can stimulate the sphingolipid ceramide as a unique signalling pathway (Gallinat et al., 1999; Lehtonen et al., 1999). Recently, evidence for a highly specific and direct interaction between the cytoplasmic tails of the  $AT_2$  receptor and human ErbB3 receptor, a member of the epidermal growth factor receptors, was reported (Knowle et al., 2000).

**1.4.3.4 Cross-talk between  $AT_1$  and  $AT_2$  Receptors:** There is an emerging awareness that cross-talk between the signalling pathways of the  $AT_1$  and  $AT_2$  receptors exists to modify the downstream effects of AngII. This is an attractive concept, since it would provide a sensitive mechanism for modulating the biological actions of AngII on specific cells. An intriguing division of function may exist between the  $AT_1$  and  $AT_2$  receptors to mediate the effects of AngII in the vasculature. Both the  $AT_1$  and  $AT_2$  receptors mediated vascular remodelling in rats through  $AT_2$ -mediated hypertrophic growth and  $AT_1$ -mediated changes in SMC phenotype to a more immature SMC based on increased non-muscle myosin and fibronectin synthesis (Sabri et al., 1997). However, chronic  $AT_2$  receptor blockade has been shown to effectively prevent the matrix accumulation due to collagen production following AngII infusion (Levy et al., 1996). Interestingly, blockade of both the  $AT_1$  and  $AT_2$  receptors was able to partially reverse the aortic remodelling associated with spontaneously hypertensive rats (SHR) through inhibition of  $AT_1$ -mediated changes in blood pressure in conjunction with  $AT_2$ -mediated SMC hypertrophy (Otsuka et al., 1998). The complexity of this relationship between the  $AT_1$  and  $AT_2$  receptor functions was demonstrated in a recent study of fluid regulation in the kidney (Jin et al., 1998). The data demonstrated that the  $AT_1$  and  $AT_2$  receptors negatively and positively regulate AngII-mediated jejunal sodium and water absorption, respectively, and the relative contribution of these receptor subtypes varied with

sodium intake. A recent study demonstrated cross-talk between these two receptors is highly specific in rat VSMCs that express either endogenous or transfected AT<sub>2</sub> receptor, since activation of the AT<sub>2</sub> receptor inhibited AT<sub>1</sub>-mediated tyrosine and serine phosphorylation of STAT1, 2 and 3, likely via activation of the PTP SHP and inhibition of ERKs, but not the upstream effector Jak (Horiuchi et al., 1999).

Several studies have demonstrated a functional antagonism in which the AT<sub>2</sub> receptor modulates the actions of the AT<sub>1</sub> receptor (Mazzocchi et al., 1997; Stoll et al., 1995)(refer to 1.3.4.2). In addition, blockade of the AT<sub>2</sub> receptor reverses infarct size reduction by AT<sub>1</sub> receptor blockade following ischemic injury in pigs (Jalowy et al., 1998). Gene transfer of the AT<sub>2</sub> receptor *in vivo* into the rat carotid artery following balloon catheter injury attenuated DNA synthesis and neointimal hyperplasia in opposition to the AT<sub>1</sub> receptor, likely via inhibition of MAPK (Nakajima et al., 1995). Similarly, AT<sub>2</sub> receptor overexpression in mice decreased AT<sub>1</sub> receptor-mediated pressor and chronotropic effects (Masaki et al., 1998). This antagonism, however, represents only one aspect of the cross-talk between the AT<sub>1</sub> and AT<sub>2</sub> receptors observed in numerous systems. A functional synergism between the AT<sub>1</sub> and AT<sub>2</sub> receptors has also been documented. Several recent studies have found that both AT<sub>1</sub> and AT<sub>2</sub> receptors inhibit bFGF-induced proliferation of bovine adrenocortical cells (Liakos et al., 1997), mediate apoptosis in rat blood vessels (Diep et al., 1999) and promote the growth effects of AngII *in vitro* in mouse lymphocytes (Kunert-Radek et al., 1994) and neuroblastoma cells (Chen et al., 1993). In addition, a role for both the AT<sub>1</sub> and AT<sub>2</sub> receptors has been established in a wide-range of biological functions such as changes in vascular compliance post-myocardial infarction (Ceiler et al., 1998), AngII-stimulated aldosterone secretion (Mazzocchi et al., 1998), AngII-induced vasopressin release in the CNS (Hogarty et al., 1994) and the regulation of TGFβ1 expression in valvular interstitial cells (Campbell and Katwa, 1997). Similarly, blockade of both AT<sub>1</sub> and AT<sub>2</sub>

receptors provided more efficient inhibition of AngII-mediated epinephrine release during hypoglycemia in rats (Worck et al., 1998). Thus, the broad range of effects and the convoluted and often contradictory results from antagonism of one or both receptors has made it difficult to define the relationship between these two receptor subtypes. Definition of the respective roles of the AT<sub>1</sub> and AT<sub>2</sub> receptors must be within the context of one specific cell type and biological function.

Cross-talk between the AT<sub>1</sub> and AT<sub>2</sub> receptors has been noted in numerous studies of AngII-dependent signal transduction. The prominent role of tyrosine phosphorylation in AT<sub>1</sub> receptor signalling pathways and PTPs in AT<sub>2</sub> receptor signalling pathways would provide an ideal mechanism for cross-talk between these two receptors (refer to Sections 1.4.3.1-2). In fact, AT<sub>2</sub> receptor stimulation of Ser/Thr phosphatase 2A has been shown to counteract the AT<sub>1</sub> receptor stimulation of Erk 1 and 2 in rat neurons (Huang et al., 1996a). A recent study that examined AngII stimulation of the important downstream signalling molecule IP<sub>3</sub> concluded that both the AT<sub>1</sub> and AT<sub>2</sub> receptor pathways were involved in IP<sub>3</sub> production and effective blockade may require antagonism of both receptor subtypes (Goutsouliak and Rabkin, 1998). Similarly, activation of both AT<sub>1</sub> and AT<sub>2</sub> receptors stimulated signalling pathways involved in calcium mobilization and contractile beating in rat cardiomyocytes (Shao et al., 1998) and tyrosine phosphorylation in chick cardiomyocytes (Goutsouliak and Rabkin, 1997). In addition, the stimulation of prostaglandin release by AngII requires activation of both receptor subtypes in porcine aortic SMCs (Jaiswal et al., 1993a), rabbit vas deferens cells (Catalioto et al., 1994), neonatal rat cardiomyocytes (Lokuta et al., 1994) and human astrocytes (Jaiswal et al., 1991). Selective stimulation of different prostaglandin species also provides a unique system for mediating the downstream effects of both AT<sub>1</sub> and AT<sub>2</sub> receptors. A recent study demonstrated both AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes controlled renal PGE<sub>2</sub> production, but only the AT<sub>2</sub> receptor stimulated PGF<sub>2 $\alpha$</sub> .

production in rats (Siragy and Carey, 1997a). Another level for significant modulation of the cellular response to AngII is the number of cell-surface receptors. An interaction between these two receptor subtypes has been demonstrated at the level of receptor regulation with the AT<sub>1</sub> receptor regulating changes in both AT<sub>1</sub> and AT<sub>2</sub> receptor expression in bovine adrenal cells (Oulai et al., 1997). Similarly, both the AT<sub>1</sub> and AT<sub>2</sub> receptor regulate AT<sub>2</sub> receptor expression in vascular endothelial cells (De Paolis et al., 1999).

Thus, the diversity in species and cell types that have demonstrated a dual requirement for both the AT<sub>1</sub> and AT<sub>2</sub> receptors to mediate the biological actions of AngII indicates this phenomenon is universal and cautions against oversimplifying the relative roles of this unique receptor system.

## **1.5 PHOSPHATIDYLINOSITOL 3-KINASE**

### **1.5.1 Function of PI3K**

Phosphatidylinositol-3 kinase (PI3K) was initially discovered through the association of lipid kinase activity with anti-phosphotyrosine immunoprecipitates (Whitman et al., 1985). Further characterization of PI3K revealed it is a lipid kinase that specifically phosphorylates phosphoinositides (PI) on the 3' position of the inositol ring. PI3K activity results in the formation of PI(3)P, PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>. These PI products constitute a small portion of the total inositol-containing lipids (Varticovski et al., 1994) and do not serve as substrates for PLC (Serunian et al., 1989). Thus, PI3K products act as unique second messengers for signal transduction. The levels of PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub>, which are undetectable in quiescent cells, are regulated by growth factor receptor activation whereas, in comparison, the levels of PI(3)P remain relatively constant and likely reflect a housekeeping role in intracellular transport (Varticovski et al., 1994).

The biological importance of PI3K is indicated by the ever-increasing number of functions that have been identified to date. Using specific inhibitors of PI3K and/or

dominant-negative mutations of PI3K, the diverse cellular functions of PI3K can be grouped into four distinct groups: mitogenic signalling, inhibition of apoptosis, cell adherence and motility, and intracellular vesicle trafficking (Carpenter and Cantley, 1996). PI3K activation is a marker for mitogenesis (Cantley et al., 1991; Kaplan et al., 1987) which can be stimulated through both tyrosine kinase-dependent and -independent receptors by PDGF (Severinsson et al., 1990), IGF-I (Petley et al., 1999), EGF (Roche et al., 1994), IL-2 (Brennan et al., 1997; Reif et al., 1997), IL-3 (Sato et al., 1993) and IL-4 (Wang et al., 1993). A central role for PI3K in regulating G1 to S phase cell cycle progression has been established and the ability of the PTEN tumor suppressor protein to regulate G1 phase has been linked to dephosphorylation of the PI3K product PI(3,4,5)P<sub>3</sub> (Ramaswamy et al., 1999). PI3K also has a role in cellular differentiation pathways stimulated by IGF-I (Hayashi et al., 1999; Petley et al., 1999) and NGF (Kraemer et al., 1999). In VSMC, constitutively active PI3K will maintain the differentiated phenotype even in the presence of growth stimulating factors (Hayashi et al., 1998). In addition, PI3K plays a key role in the anti-apoptotic effect of growth factors (Minshall et al., 1999; Yao and Cooper, 1995) through the promotion of matrix adhesion (Khwaja et al., 1997), regulation of Akt/PKB (Kauffmann-Zeh et al., 1997) and upregulation of Bcl-2 (Minshall et al., 1999). In contrast, a role for PI3K activity in promoting apoptosis has also been defined through upregulation of caspase-3 (Godbout et al., 1999). PI3K is also implicated in several specific metabolic functions such as insulin-dependent GLUT4 glucose transporter recruitment (Haruta et al., 1995; Clarke et al., 1994), histamine release and integrin activation (Yano et al., 1993). Recently, PI3K dysregulation has been implicated in the NIDDM (Andreelli et al., 1999) and IDDM (Folli et al., 1993) diabetic states, as well as the cellular mechanism of insulin resistance (Egawa et al., 1999; Tirosh et al., 1999; Virkamaki et al., 1999).

A role for PI3K has been established in cell motility and cell adherence. The ability

of PDGF to stimulate membrane ruffling or chemotaxis requires the interaction of PI3K with a PDGF receptor (Kundra et al., 1994; Wennstrom et al., 1994). In addition, PI3K is involved in microtubule reassembly in response to insulin and PDGF (Kapeller et al., 1995), and actin rearrangement by PDGF (Wymann and Arcaro, 1994). The involvement of PI3K in growth factor regulation of integrins and cell adherence has also been demonstrated (Hemmings 1997; Kinashi et al., 1995). PI3K is an early effector in integrin-mediated signalling pathways such as the MAP kinase and Akt/PKB pathways (King et al., 1997). Specifically, PI3K has been shown to associate with FAK, a key tyrosine kinase in the signalling pathways initiated by integrins (Chen and Guan, 1994b) and participate in PDGF-mediated phosphorylation of both FAK and paxillin (Rankin et al., 1996). A recent study demonstrated that PI3K binding is required for FAK to promote migration on fibronectin, in addition to FAK binding of Src and p130(Cas) (Reiske et al., 1999). In contrast, integrin-mediated migration in macrophages involves a PI3K-dependent, but FAK-independent, mechanism (Danikovitch et al., 1999).

Considerable research has been focussed on defining the specific role of PI3K in intracellular protein trafficking, since this function has implications for many mitogenic and metabolic cellular functions. PI3K involvement in membrane traffic was first suggested by studies of the PI3K homologue in yeast, *VPS34*, which is required for vacuole protein sorting and segregation (Herman and Emr, 1990). A role for PI3K has been implicated in several aspects of intracellular membrane trafficking and protein sorting in mammalian cells. PI3K is a component of the trans-Golgi-endosomal system and its activity is essential in the transport of newly-synthesized proteins (Brown et al., 1995). Although PI3K does not appear to be involved in receptor internalization, its role in the endocytotic pathway was clearly demonstrated by PDGF receptors with mutated PI3K domains that inhibit receptor degradation (Joly et al., 1994; Carlberg et al., 1991). In addition, the involvement of PI3K in

endosome fusion via Rab5 and the recycling of transferrin receptors has been demonstrated by PI3K inhibition and constitutively active PI3K (Spiro et al., 1996; Li G et al., 1995). Also, PI3K appears to be involved in the process of exocytosis and receptor recruitment to the cell membrane (Shephard et al., 1995).

### 1.5.2 Structure of PI3K

Three classes of PI3Ks have been defined based on their structural homology, and lipid substrate specificity. Class I PI3Ks consist of a heterodimer with a regulatory and a catalytic subunit and preferentially phosphorylate PI(4,5)P<sub>2</sub>. Some heterogeneity exists for both subunits; however, the significance of this heterogeneity has not been determined. Specificity in PI3K signalling may occur through differential recruitment of various isomers of the regulatory subunit and the catalytic subunit. To date, distinct genes encoding two isoforms of the p85 regulatory subunit have been identified: p85 $\alpha$  and p85 $\beta$  (Otsu et al., 1991). Although these two isoforms exhibit a high degree of homology, differences in their ability to activate PI3K activity (Baltensperger et al., 1994), regulation (Reif et al., 1993) and their distribution (Volinia et al., 1992) have been noted. The p85 subunit functions as an adapter that positions the p110 catalytic subunit in close proximity to the appropriate substrates. Each p85 contains two SH2 domains that enclose the p110 binding site and two proline-rich domains, as well as an SH3 site which resembles the domain of other adapters such as Grb2 and Shc. In addition, p85 contains a BCR-homologous (BH) domain, but no GTPase activity towards a known GTP-binding protein has been identified. To date, four regulatory subunits for PI3K have been identified. A truncated version of p85, p55<sup>PIK</sup>, is encoded by a gene distinct from p85 $\alpha$  or  $\beta$  (Inukai et al., 1996; Pons et al., 1995). In addition, alternative splicing of the p85 $\alpha$  gene can produce two additional isoforms, p50 $\alpha$  (Inukai et al., 1996) and p55 $\alpha$  (Inukai et al., 1997). In all of these "truncated" adaptor subunits, the p110 binding region and the SH2 domains are conserved; however, the SH3 domain, Bcr-like domain and one of the

proline-rich domains are absent. Regulation of these adapter subunits may provide specificity in PI3K signalling as demonstrated by their differential recruitment into phosphotyrosine complexes during insulin stimulation (Shepherd et al., 1997). Further complexity is indicated by the ability of p85 to dimerize through its SH3 and BH domains, which may facilitate p110 autophosphorylation as well as generate novel binding surfaces for recruitment of other factors (Harpur et al., 1999; Layton et al., 1998).

The p110 subunit also exhibits heterogeneity with four isoforms,  $\alpha$ ,  $\beta$ ,  $\delta$  and  $\gamma$ , identified to-date in mammalian systems (Vanhaesebroeck et al., 1997; Lopez-Illasaca et al., 1997). The p110 $\alpha$ ,  $\beta$  and  $\delta$  isoforms contain several conserved regions including Ras-binding sites, an ATP binding site, the PI-kinase region and the N-terminal inter-SH2 p85-binding site (Vanhaesebroeck et al., 1997). A recent study of p110 $\alpha$  and p110 $\beta$  established distinct roles in signal transduction for PDGF- and insulin-induced actin rearrangement (Hooshmand-Rad et al., 2000). Although both p110 $\alpha$  and p110 $\beta$  are considered p85-dependent isoforms that require an interaction with the p85 regulatory subunit to exhibit any catalytic activity (Hiles et al., 1992), a pool of p110 $\alpha$  PI3K activity that was p85-independent has been reported (Johanson et al., 1999; Woscholski et al., 1994). In addition, p110 $\beta$  may be activated by G $\beta\gamma$  subunits (Murga et al., 2000). Similarly, p110 $\gamma$  (PI3K $\gamma$ ) is a p85-independent isoform that has been shown to interact with G $\beta\gamma$  subunits for catalytic activation (Lopez-Illasaca et al., 1997). This PI3K isoform also contains an amino-terminal Ras-binding site, a PI-kinase region and an N-terminus regulatory region (Stoyanova et al., 1997). Recently, a regulatory p101 subunit capable of a tight association with p110 $\gamma$  was isolated but it did not exhibit any homology with other PI3K regulatory proteins (Stephens et al., 1997).

Two other classes of PI3K have also been defined (Vanhaesebroeck et al., 1997). Class II PI3Ks are structurally distinct from Class I PI3K and contain a distinct C2 domain at their carboxyl terminus. This class of PI3Ks preferentially use PI and PI(4)P as their lipid

substrates. In addition, Class III PI3K are homologous to the yeast Vps34p and exhibit a substrate specificity restricted to PI.

### 1.5.3 Role of PI3K in Signal Transduction

PI3K plays a key role in the signalling pathways leading from tyrosine kinase, cytokine and G-protein-coupled receptors. Through SH2, SH3, PH or other domains for protein-protein interaction and lipid-protein interaction, PI3K is able to interact directly with receptors, adapter molecules and multiple intracellular signalling effectors.

In the past few years, several potential targets of PI3K have been identified. However, the details of how 3-phosphorylated lipids mechanistically affect downstream signalling is just emerging. Recently, a novel protein module called the FYVE finger domain, a special type of RING zinc finger that specifically binds the PI(3)P product of PI3K has been identified. FYVE domain-containing effectors, such as EEA1 (early endosome antigen 1) and Hrs, regulate vacuolar/lysosomal membrane trafficking pathways (Wurmser et al., 1999). Similarly, PI(3,4,5)P<sub>3</sub> also acts as a second messenger through the recruitment of regulatory proteins to the plasma membrane via their pleckstrin homology domains (Varnai et al., 1999) such as Grp1 (General receptor for phosphoinositides. In addition to the direct stimulation of signal effectors, the products of PI3K may regulate the physical interaction of PI3K with other signal transduction components. PI(3,4,5)P<sub>3</sub> has been shown to interact with SH2 domains and modulate the ability of tyrosine phosphorylated proteins such as Src and IRS-1 to associate with PI3K (Rameh et al., 1995).

An important downstream target of PI3K is Akt/PKB (Reif et al., 1997; Burgering and Coffey, 1995), a subfamily of Ser/Thr protein kinases with three isoforms identified to date: PKB $\alpha$ , PKB $\beta$  (Jones et al., 1991) and PKB $\gamma$  (Brodbeck et al., 1999). Akt/PKB activation involves its recruitment to the membrane by PI3K products where phosphorylation of the regulatory sites of PKB occurs (Downward, 1998; Hemmings, 1997; Alessi et al., 1996).

Direct interaction of the PI3K product PI(3,4)P<sub>2</sub> with the pleckstrin homology domain facilitates Akt/PKB dimerization and activation (Franke et al., 1997; Hemmings et al., 1997; Klippel et al., 1997). Recently, the ability of G-proteins to stimulate PKB/Akt was linked to activation of PI3K $\beta$  by G $\beta\gamma$  subunits (Murga et al., 2000). The downstream effects from activation of Akt/PKB include a number of key biological functions such as protein synthesis, glucose homeostasis and regulation of cell survival through activation of a wide-range of proteins including mTOR (Scott et al., 1998), and glycogen synthase kinase 3 (GSK3)(van Weeren et al., 1998). Recently, Pak was identified as a downstream effector of Akt/PKB that suppresses apoptosis through phosphorylation of Bad (Tang et al., 2000). Recent studies also link Akt/PKB with inactivation of members of the forkhead family of transcription factors (Rena et al., 1999) which mediate cell-cycle regulation through modulation of p27<sup>kip1</sup> levels (Medema et al., 2000).

A component of PI3K signal transduction is p70 S6 kinase which plays an important role in the progression of cells from G1 to S phase in response to a wide variety of mitogens (Brown and Schreiber, 1996). Activation of this kinase requires ser/thr phosphorylation at four sites by an unidentified kinase(s). Although the products of PI3K can mediate activation of p70 S6 kinase *in vitro* (Weng et al., 1995), it remains to be clarified whether S6 kinase is activated directly by PI3K products or by an intermediate effector *in vivo* (Burgering and Coffey, 1995). However, a specific pool of PI3K activity associated with p110 $\alpha$  was found to colocalize with p70 S6 kinase on actin stress fibres and shown to influence actin fibre formation (Johanson et al., 1999). A PI3K-dependent signalling complex consisting of p70 S6 kinase, phosphoinositide-dependent kinase-1 (PDK1) and PKC $\zeta$  may facilitate the efficient activation of p70 S6 kinase (Romanelli et al., 1999). Direct activation of several calcium-insensitive PKC isoforms, including  $\delta$ ,  $\epsilon$ ,  $\eta$  and  $\zeta$ , by PI3K products has been demonstrated, although the calcium-dependent PKC isoforms were unaffected (Standaert et al., 1997; Toker

et al., 1994; Nakanishi et al., 1993). Although PI3K has been shown to bind directly to PKC $\delta$  *in vivo*, this association does not appear to be regulated by tyrosine phosphorylation or PI3K products (Ettinger et al., 1996). However, the PKC $\zeta$  isoform associates with PKB as a negative regulator (Doornbos et al., 1999). Another key component of the PI3K signalling cascade that colocalizes with p70 S6 kinase is 3-phosphoinositide-dependent protein kinase 1 (PDK1). PDK1 also contains a PH domain that binds the PI3K products PI(3,4)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> with high affinity (Vanhaesebroeck and Alessi, 2000). PDK1 is a Ser/Thr kinase that may act as a key regulatory checkpoint in PI3K signal transduction, since it is capable of activating divergent pathways downstream of PI3K through its direct activation of three of the key targets of PI3K: Akt/PKB, PKC $\zeta$  and p70 S6 kinase (Dong et al., 1999; Alessi et al., 1998; Alessi et al., 1997). The characterization of a multimeric complex that contains PDK1, PKC $\zeta$  and p70 S6 kinase suggests there is an efficient mechanism for PI3K-dependent p70 S6 kinase activation (Romanelli et al., 1999). In addition, analysis of mouse PDK1 has revealed multiple isoforms that may provide additional complexity to PI3K signalling pathways (Dong et al., 1999).

A role for PI3K in Ras-mediated signalling pathways has been well-established, although there is conflicting evidence as to whether PI3K is upstream or downstream of Ras. Ras has been shown to bind directly to the p110 subunit of PI3K and dominant-negative Ras mutants can prevent growth factor-stimulated formation of PI3K products (Rodriguez-Viciana et al., 1994), thus implying that PI3K is downstream of Ras. However, dominant-negative mutants of Ras or Raf have been shown to block the downstream effects of constitutively active PI3K (Hu et al., 1995) or insulin-stimulated PI3K (Yamauchi et al., 1993) which implies that PI3K is upstream of Ras. In addition, PI3K stimulated DNA synthesis could be blocked by inhibition of Ras and S6 kinase (McIlroy et al., 1997). These conflicting results may be resolved by the proposition that the position of PI3K relative to

other signalling effectors such as Ras is cell type-dependent. This concept was validated by Satoh et al. (1993) who showed that the ability of PDGF receptors with mutated PI3K binding sites to block downstream Ras activation varied in different cell types. Recently, the PI3K product PI(3,4,5)P<sub>3</sub> has been shown to directly bind and recruit GAP1(m), a member of the Ras GTPase-activating proteins, via the pleckstrin homology domain (Lockyer et al., 1999).

PI3K signal transduction also involves FAK, and FAK appears to function both upstream and downstream of PI3K. Specifically, PI3K activity is necessary for tyrosine phosphorylation of FAK through PDGF-stimulated (Rankin et al., 1996) and Rho-dependent pathways (Murakami et al., 1999). However, FAK phosphorylation results in the recruitment of PI3K and subsequently serves as a substrate for FAK, both *in vitro* (Chen and Guan, 1994a) and *in vivo* (Chen and Guan, 1994b). In addition, FAK has been shown to operate upstream of PI3K in the PI3K-induced apoptotic survival pathway (Sonoda et al., 1999). These conflicting results likely reflect both the complexity of the signalling pathways involved in PI3K regulation and variations between different cell types and functional endpoints.

#### **1.5.4 Regulation of PI3K**

Key to understanding how PI3K is regulated is the knowledge that this is a soluble enzyme which uses membrane-associated substrates. Thus, the activation of PI3K can be regulated by a change in its catalytic activity as well as its subcellular localization and proximity to its substrates.

A common mechanism of enzyme regulation involves the phosphorylation of key residues which alters the intrinsic enzyme activity or the association of the enzyme with other modulatory proteins. Although both the p85 and p110 subunits are tyrosine phosphorylated (Gout et al., 1992), there is little evidence that tyrosine phosphorylation of

PI3K itself enhances its catalytic activity (Hiles et al., 1992; Hu et al., 1992). In contrast, serine phosphorylation of the p85 subunit may regulate PI3K activity. Growth factor stimulation has been shown to increase the level of p85 serine phosphorylation (Freund et al., 1995), and association with a serine-threonine kinase has been shown to downregulate PI3K (Carpenter et al., 1993). Alternatively, serine phosphorylation of p110 $\delta$  has been demonstrated to control PI3K activity (Vanhaesebroeck et al., 1999). This phosphorylation event is dependent on PI3K activity *in vivo* and *in vitro*, which suggests PI3K is autoregulated. It has been previously suggested that p110 may possess an endogenous protein serine/threonine kinase activity which phosphorylates the p85 subunit to down-regulate lipid kinase activity (Freund et al., 1995; Dhand et al., 1994; Carpenter et al., 1993). Mutation of Arg-916 in PI3K has been shown to eliminate both the PI3K and serine/threonine kinase activity (Dhand et al., 1994). Although this kinase activity is thought to be largely autoregulatory, some studies have reported that p85/p110 can phosphorylate IRS-1 in a wortmannin-sensitive manner (Freund et al., 1995; Lam et al., 1994; Tanti et al., 1994), although IRS-1 modification could occur via a PI3K/PKB kinase cascade (Li et al., 1999). PI3K $\gamma$  has also been identified as a bifunctional lipid kinase and protein kinase, and protein phosphorylation may be essential for coupling to G-proteins (Stoyanova et al., 1997).

The physical association of PI3K with its signalling intermediates and substrates may be the most crucial mechanism of regulation. The p85 regulatory subunit contains two SH2 domains that can directly bind to a tyrosine phosphorylated pTyr-X-X-Met motif. This sequence is present in many growth factor receptors and adaptor proteins. The regulatory p85 subunit of PI3K has no intrinsic catalytic activity, but instead it associates with the catalytic p110 subunit and forms membrane-associated signalling complexes with activated growth factor receptors either directly (Joly et al., 1994) or via adaptor proteins such as IRS-1 (Giorgetti et al., 1993; Backer et al., 1992a). The resultant association leads to a translocation

of the p85/p110 PI3K heterodimer from the soluble fraction to the plasma membrane and vesicular fractions enriched in Golgi membranes. This adapter-mediated translocation of PI3K helps to position the catalytic subunit near the membrane and their lipid substrates. A specialization in the function of the different p110 isoforms may be reflected in differential subcellular localization that has been documented by Johanson et al. (1999) with p110 $\alpha$  colocalizing with p70 S6 kinase on actin filaments and p110 $\gamma$  colocalizing with the microtubule network. Different subcellular locations were also noted in sympathetic and sensory neurons of mice with p110 $\alpha$  found predominantly in the plasma membrane while p110 $\beta$  and p110 $\gamma$  localized in the perinuclear region (Barlett et al., 1999).

Alternatively, PI3K may be activated through SH2 domain interactions with specific tyrosine phosphorylated proteins. PI3K can be activated directly through an interaction with tyrosine phosphorylated IRS-1 or tyrosine-phosphorylated peptides derived from the PDGF receptor according to *in vitro* assays (Backer et al., 1992b; Layton et al., 1998). In this mode of activation, a peptide that can bind both SH2 domains in p85 that flank the p110 binding site can produce a conformational change and activation of p110 (Layton et al., 1998; Panayotou et al., 1992; Otsu et al., 1991). The SH3 domain of p85 can also mediate PI3K activation. The SH3-mediated interaction of p85 with the microtubule-associated protein dynamin, a large GTPase/GTP-binding protein, results in activation of PI3K (Gout et al., 1993). In addition, the p85 regulatory subunit also exhibits two proline-rich domains that can interact with the SH3 domains of other proteins. An interaction of p85 with the SH3 domains of proteins such as the Src-family kinases has also been shown to significantly enhance PI3K activity (Pleiman et al., 1994). Several proteins that can bind p85 via their SH3 domains include Src-family kinases such as Fyn, Lyn (Pleiman et al., 1994) and Src (Wages et al., 1992). This interaction could account for the direct activation of PI3K by proteins lacking the SH2-consensus sequence pTyr-X-X-Met. The interaction of p85 with ABL, a tyrosine

kinase with anti-apoptotic effects that phosphorylates key proteins such as Shc (Matsuguchi et al., 1994), paxillin (Salgia et al., 1995) and Syp (Tauchi et al., 1994), involves both SH2 and SH3 domains. The association of p85 and ABL involves an adapter protein, p120<sup>CBL</sup>, that binds to both the SH3 and SH2 domains of p85 (Sattler et al., 1996). Interestingly, p120<sup>CBL</sup> also links p85 to the EGF receptor through both the SH2 and SH3 domains (Soltoff and Cantley, 1996).

The catalytic activity of p110 leads to the production of phosphatidylinositol-3-phosphate (PI-3P), phosphatidylinositol-3,4-bisphosphate (PI(3,4)P<sub>2</sub>) and phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P<sub>3</sub>). In addition to their function as downstream signalling intermediates, they may also serve an autoregulatory function. The PI3K product, PI(3,4,5)P<sub>3</sub>, for example, has been shown to modulate the association of p85 with other tyrosine-phosphorylated proteins by directly binding to SH2 domains (Rameh et al., 1995).

## **1.6 INSULIN-DEPENDENT REGULATION OF THE RAS**

### **1.6.1 Insulin and Vascular Disease**

Individuals with either non-insulin-dependent or insulin-dependent diabetes mellitus have a significantly elevated risk of developing cardiovascular disorders such as coronary artery disease, hypertension and atherosclerosis (Koskinen et al., 1992; Tuck et al., 1992). Although no definitive mechanism linking diabetes and heart disease has been identified, several population studies have concluded that insulin resistance, and consequently hyperinsulinemia, are independent risk factors for the development of vascular disease (Sowers, 1992; DeFronzo et al., 1991; Fontbonne et al., 1991). Evidence that hyperinsulinemia may actually precede the development of vascular disease has also been provided in several studies of the development of both hypertension (in Sprague-Dawley and

spontaneously-hypertensive rats) and atherosclerosis (in chicken, dog and rat models)(Hwang et al., 1987; Lais and Brody, 1978). Vascular SMC growth and migration are key events in the initiation and progression of vascular disease which may be directly or indirectly influenced by insulin. For instance, insulin has been shown to influence SMC growth (Stout, 1991; Banskota et al., 1989), alter cholesterol metabolism and modulate vascular reactivity to vasoconstrictors (Kahn et al., 1993). These effects are likely mediated through the IGF-I receptor which is more abundant than the insulin receptor in human adult vascular tissue (Banskota et al., 1989). Vascular remodelling has been associated with a significant induction of IGF-I following balloon catheter injury (Cercek et al., 1990), especially in the medial SMC layer (Khorsandi et al., 1992).

### **1.6.2 Interaction Between Insulin and RAS**

A causal link between hyperinsulinemia and the RAS which culminates in SMC growth and vascular disease has been well established experimentally (Gwinup and Elias, 1991), but the mechanism that connects these processes remains undefined. Numerous studies have reported an interaction between insulin/diabetes and AngII/RAS at both the tissue and the cellular levels. Polymorphism in the ACE gene (I/D) may contribute to the pathology of IDDM and NIDDM (Keavney et al., 1995; Nagi et al., 1995; Panahloo et al., 1995; Wierzbicki et al., 1995; Marre et al., 1994). Both acute and long-term ACE inhibitor treatment significantly improved insulin resistance in several clinical trials (Morris *et al.*, 1994; Torlone *et al.*, 1993; Paolisso et al., 1992; Ferriere et al., 1985) and normalized the high IGF-I plasma levels associated with essential hypertension (Diez and Laviades, 1994). Recent clinical trials have also demonstrated that AT<sub>1</sub> receptor antagonism can also improve insulin sensitivity in patients with essential hypertension (Higashiura et al., 1999). Interestingly, subpressor doses of AngII have also been shown to increase insulin sensitivity in NIDDM patients (Morris et al., 1994) and healthy individuals (Fliser et al., 1993), thus

supporting the concept of a biochemical link between these two hormone systems. Insulin-resistance has been shown to change the vascular response to AngII leading to elevated blood pressure and decreased tissue perfusion (Gaboury et al., 1994). Originally, the sympathoadrenal system was considered to be the most likely link between the RAS and circulating insulin (Gans et al., 1991). However, based on the ability of isolated adipocytes from fructose-fed rats to exhibit insulin resistance (Reaven et al., 1989), the changes associated with insulin resistance appear to involve events that occur at the cellular level.

Several *in vivo* studies have documented an interaction between circulating and tissue-based insulin or IGF-I and the expression of RAS components. The hyperinsulinemic state has been shown to dramatically increase AT<sub>2</sub> receptor expression (by 10-fold) in the aorta of genetically obese Zucker rats (Kambayashi et al., 1996). Similarly, renin but not angiotensinogen mRNA levels were modulated by an initial increase followed by a significant decrease during the progression of diabetes in IDDM-like BioBreeding spontaneously diabetic rats (Everett et al., 1992). STZ-induced diabetes in rats correlated with increased AT<sub>1</sub> and AT<sub>2</sub> receptor numbers in the myocardium (Sechi et al., 1994) as well as increased plasma and lung ACE activity, and these changes were partially reversed by insulin infusion (Erman et al., 1993). Conversely, AngII infusion into rats resulted in reduced plasma IGF-I levels and changed the IGF binding protein profile via a non-pressor mechanism (Brink et al., 1996), and caused a decline in insulin sensitivity (Rao RH, 1994).

At the cellular level, an interaction between insulin or IGF-I and AngII has been reported to influence cell growth, receptor number, signalling pathways and metabolic functions. Insulin and IGF-I have been shown to synergistically increase AngII-stimulated DNA and protein synthesis in rat aortic SMCs (Gustafsson et al., 1999; Ko et al., 1993). This interaction involved AngII-mediated upregulation of IGF-1 receptor mRNA and downregulation of IGF-I, IGFBP-2 and -4 mRNAs. Previously, AngII has been shown to

directly regulate the expression of IGF-I in SMCs via a cycloheximide-sensitive mechanism (Delafontaine and Lou, 1993) and the IGF-I receptor through a PKC-dependent pathway (Ververis et al., 1993) or PKC-independent pathway (Du et al., 1996a). Also, AngII stimulated the phosphorylation of IRS-1 and IGF-I receptor in rat aortic SMCs (Du et al., 1996b). Both AngII and IGF-I synergistically upregulate AT<sub>2</sub> receptor number in R3T3 cells (Li JY et al., 1999), and prolonged exposure to insulin, IGF-I or IGF-II in serum-free differentiation conditions induced AT<sub>2</sub> receptor mRNA in cultured rat VSMCs through the IGF-1 receptor (Ichiki et al., 1996). Conversely, IGF-I overexpression in cardiomyocytes resulted in downregulation of AT<sub>1</sub> receptor, angiotensinogen and AngII levels (Leri et al., 1999). Further evidence of cross-talk between these two independent growth factor systems is provided by the capacity of AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists to inhibit insulin-stimulated DNA synthesis in human neuroblastoma cells (Chen et al., 1993).

Direct crosstalk between the signalling pathways activated by insulin and AngII represents another level of overlap that has been observed. Interaction between insulin signalling through a tyrosine kinase receptor and AngII signalling through a G-protein-coupled receptor is a new area of intense research (refer to Section 1.4.2.4). Examination of signal transduction systems commonly activated by these receptors identified two potential targets for crosstalk: IRS-1 and PI3K. In rat cardiomyocytes, crosstalk between insulin and AngII signalling pathways occurred at the level of both tyrosine phosphorylation of IRS-1 and IRS-2, and modulation of PI3K activity (Velloso et al., 1996). Similarly, both AngII and thrombin stimulation resulted in the rapid tyrosine phosphorylation of IRS-1 and IGF-I receptor  $\beta$  chain in rat aortic SMC (Du et al., 1996b). One plausible explanation of how insulin sensitivity is improved by ACE inhibitors is based on evidence that this intervention reverses the decreases in PI3K activity caused by overactivity of the RAS. Consequently, ACE inhibitors can improve insulin signaling in rat hearts (Folli et al., 1999), liver and muscle

(Carvalho et al., 1997), as well as Zucker fatty rats (Nawano et al., 1999). Modulation of intracellular calcium levels is another potential area of overlapping regulation by AngII and insulin pathways. Insulin significantly reduced AngII-induced intracellular free calcium through a tyrosine kinase-dependent mechanism in rat VSMC (Touyz and Schiffrin, 1996). A prerequisite for insulin has also been noted in the stimulation by AngII of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  ion channels in mesangial cells (Ling et al., 1993).

In conclusion, overlap of AngII- and insulin-mediated processes at the functional, signal transduction and gene expression levels supports the concept that cross-talk between these two growth factor systems occurs at the cellular level. This physiologically important cross-talk likely provides a sensitive mechanism for controlling the cellular response to numerous factors. The flexibility of insulin as a regulator of AngII actions was demonstrated by a study that distinguished between the acute and chronic effects of insulin on AngII-mediated actions in human adrenal glomerulosa cells (Natarajan et al., 1995). Interestingly, short-term insulin pretreatment inhibited AngII-induced aldosterone synthesis; however, chronic insulin exposure potentiated AngII activity. Further studies will be necessary to define the precise mechanism of cross-talk.

## 2. STATEMENT OF THE PROBLEM & HYPOTHESIS

### 2.1 STATEMENT OF THE PROBLEM

Angiotensin II (AngII) is an important endocrine, paracrine and autocrine growth factor that has been associated with cellular stress and injury, as well as the initiation and progression of vascular disease. To date, one of the most effective treatments for vascular diseases has been angiotensin converting enzyme (ACE) inhibitors that interfere with the conversion of angiotensin I to AngII. However, clinical alternatives are currently being investigated based on selective antagonism of AT<sub>1</sub> receptors which may result in increased stimulation of the other receptors in RAS. Although at least two different classes of AngII receptors, AT<sub>1</sub> and AT<sub>2</sub>, have been identified, their relative contribution to the function and pathophysiology of the cardiovascular system remains unclear. The AT<sub>1</sub> receptor has been identified as the principal mediator of the vascular response to AngII in studies of rodent SMC systems that no longer express both AT<sub>1</sub> and AT<sub>2</sub> receptors. The upregulation of the AT<sub>2</sub> receptor in fetal tissues and in response to growth factors, stress or damage has led to speculation that this receptor has a critical role in vascular development and disease. **Thus, a better understanding of the relative contribution of both the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes to AngII-mediated growth in vascular SMCs will be necessary for successful therapeutic intervention in the renin-angiotensin system.** As a result, a comprehensive study was undertaken to clarify the cellular physiology and identify key signalling systems activated by the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes to mediate the SMC growth response to AngII. Based on the novel concept of interplay between these two receptor subtypes, we hypothesized that both the AT<sub>1</sub> and AT<sub>2</sub> receptors mediate a common endpoint such as SMC growth, but through distinct signalling pathways due to the lack of similarity in their signal transduction mechanisms.

## **2.2 HYPOTHESIS**

The proliferative effects of AngII are mediated by both the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes through distinct signalling pathways in vascular smooth muscle cells.

## **2.3 OBJECTIVES**

The focus of this investigation was to delineate the relative contribution of AT<sub>1</sub> and AT<sub>2</sub> receptors to AngII-stimulated growth in vascular SMCS and identify key signalling pathways for each receptor. A comprehensive overview of the mitogenic response to AngII was examined with several assays for early and late markers of cell growth. The specific objectives of this study are as follows:

- 1) To characterize a vascular SMC culture model established by selective migration from porcine coronary artery (PCA) explants.
- 2) To examine the PCA SMC growth response to AngII and define the contribution of the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes using the selective non-peptide receptor antagonists, losartan and PD123319.
- 3) To define the contribution of phosphatidylinositol 3-kinase to AngII-dependent stimulation of vascular SMC growth.
- 4) To evaluate the involvement of prostanoid synthesis in AngII-stimulated growth of PCA SMCs.
- 5) To characterize the requirement for insulin in AngII-stimulated growth in A10 SMCs.

### 3. MATERIALS AND METHODS

#### 3.1 MATERIALS

Tissue culture supplies were obtained from Hyclone (fetal bovine serum (FBS)), ICN Flow (Dulbecco modified Eagle's medium-high glucose (DMEM), penicillin/streptomycin, trypsin), Linbro (multi-well plates), Gibco/BRL (FBS, fungizone (100X), Nunclone plasticware) and Fisher Scientific (Superfrost Plus slides). Chemicals, growth factors and antibodies were purchased from Sigma (AngII, insulin, IGF, transferrin, L-ascorbic acid, selenium, protease inhibitors, indomethacin, Hoescht No.33258, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT), bovine serum albumin-fraction V (BSA), dithiothreitol (DTT), bromodeoxyuridine (BrdU), anti-BrdU, anti-smooth muscle  $\alpha$ -actin, anti-smooth muscle myosin and anti-smooth muscle caldesmon monoclonal antibodies, and Cy3-conjugated anti-mouse and anti-rabbit antibodies) and Marivac Ltd. (paraformaldehyde-EM grade). The following antibodies were used in these studies: anti-von Willebrand Factor monoclonal antibody (Boehringer-Mannheim), anti-PCNA monoclonal antibody (DAKO), anti-paxillin monoclonal antibody (Transduction Laboratories), anti-IRS-1 polyclonal antibody to the human carboxy terminus (Santa Cruz), anti-FAK polyclonal antibody to peptide with human a.a.1033-1052 at carboxy terminus (Santa Cruz) and anti-AT1 receptor polyclonal antibody to peptide of rat a.a. 225-237 conjugated to thyroglobulin (Chemicon). Immobilon-P PVDF membranes were supplied by Millipore, the ECL chemiluminescent detection system was purchased from Amersham and protein G-Sepharose was from Pharmacia-LKB. Anti-phosphotyrosine PY20 antibody (either free IgG or coupled to horse radish peroxidase), anti-PI3K p85 antibody and anti-PI3K p110 antibody were acquired from ICN, Upstate Biotechnology Inc. and Santa Cruz, respectively. Phosphatidylserine, phosphatidylinositol and phosphatidylinositol-4,5-bisphosphate were provided by Calbiochem. LY294002 and wortmannin were purchased from Bio-Mol and Sigma Chemical

Co., respectively. Radiolabelled chemicals ([methyl-<sup>3</sup>H]-thymidine, [5,6-<sup>3</sup>H]-uridine, [ $\alpha$ -<sup>32</sup>P]-dCTP, [ $\gamma$ -<sup>32</sup>P] dATP, [<sup>32</sup>P]-orthophosphate, [<sup>3</sup>H]-arachidonic acid and [<sup>125</sup>I]-SarIle AngII) were purchased from NEN-Dupont. Molecular biologicals were purchased from Perkin-Elmer (GeneAmp PCR kit), 5'-3' Corp. (Inhibit-ACE), Amersham (nick translation kit) and Molecular Probes (SYBR Green I). The AngII receptor antagonists losartan and PD123319 were kindly provided by DuPont-Merck and Warner-Lambert Parke-Davis, respectively.

## **3.2 SMOOTH MUSCLE CELL CULTURES**

### **3.2.1 Coronary Artery Excision**

Whole hearts from 10 month-old neutered male swine were obtained from the local abattoir immediately after removal and stored on ice. The left descending coronary artery was flushed with PBS + 10 $\times$  fungizone, dissected out of the heart and cleaned of adhering fat or cardiac tissue. The coronary artery was cut into segments of 2-5 mm, placed into Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 2 mM glutamine, 86  $\mu$ M streptomycin and 140  $\mu$ M penicillin and incubated at 37°C in 5% CO<sub>2</sub>. To eliminate potential contaminants, artery pieces were incubated in media containing 10 $\times$  fungizone for 2-4 days which was then reduced to 5 $\times$  fungizone for an additional 2-4 days. Fungizone at 1 $\times$  concentration was retained in the media for the remaining time in culture.

### **3.2.2 Primary SMC Culture**

Porcine coronary artery (PCA) SMCs were prepared by migration from free-floating explants of porcine left descending coronary artery segments and propagated in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, 2 mM glutamine, 86  $\mu$ M streptomycin and 140  $\mu$ M penicillin. The cells capable of migration and proliferation during the second week of migration consisted mainly of SMCs (>95%). All experiments

utilized this population of SMCs after two passages (p=2). Cells (at passage 2) were plated and grown to 65-75% confluency, rinsed with PBS and incubated in serum-free DMEM supplemented with 65.8 nM transferrin, 1 nM selenium, 10 mM sodium pyruvate, 200  $\mu$ M ascorbate and 10 nM insulin (Libby and O'Brien, 1983) for 5-7 days to induce a quiescent state. Cell number was established following trypsinization by hemacytometer counts. To characterize the cell phenotype, immunofluorescent microscopy is used with antibodies against SM-a actin, SM-myosin (SM1 and SM2), h- caldesmon, and VonWillebrand's Factor VIII.

### **3.2.3 A10 Cell Culture**

A10 smooth muscle cells (ATCC, Rockville, MD - CRL1476) were originally derived from the thoracic aorta of rat embryo (Kimes and Brandt, 1976). Cultures were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum, 2 mM glutamine, 86  $\mu$ M streptomycin and 140  $\mu$ M penicillin and subcultured at 1/3 dilution every two days. To attain quiescence, A10 cells (65-75% confluency) were washed once with phosphate-buffered saline (PBS) and incubated in serum-free DMEM supplemented with 5  $\mu$ g/ml transferrin, 1 nM selenium, 10 mM sodium pyruvate, 200  $\mu$ M ascorbate and 10 nM insulin (Libby and O'Brien, 1983) for 96 hours. All experiments described used quiescent A10 cells between passages 2-40.

## **3.3 IMMUNOCYTOCHEMISTRY**

Cells were grown on glass slides (Superfrost, Fisher) and incubated in serum-free defined media for 4-5 (A10) or 5-7 days (PCA SMC) to induce quiescence as described above. Following AngII ( $10^{-6}$  M) stimulation for varying time periods (0-30 min), cells were washed twice with PBS, fixed in methanol ( $-20^{\circ}$ C) and air-dried. After rehydrating in PBS containing 0.1% BSA and 0.05% sodium azide, cells were incubated with anti-p85 antibody or anti-p110 antibody overnight at  $4^{\circ}$ C. To stain for phenotype markers actin, myosin or

caldesmon, quiescent SMCs on glass slides were washed with PBS and fixed for 15 min at 4°C in either 1% paraformaldehyde solution (0.1 M Na<sub>2</sub>PO<sub>4</sub>, 0.9% NaCl pH 7.4) and permeabilized with 0.1% Triton X-100 (in PBS or to stain for Vonwillebrand's Factor, cells were fixed in 95% methanol (-20°C). Cells were incubated with primary antibody diluted in 0.1% BSA/0.02% sodium azide/PBS followed by Hoescht No.33258 nuclear stain and Cy3-conjugated secondary antibody to visualize the cells. The primary antibodies were diluted as follows: SM  $\alpha$ -actin (1/200), SM myosin (1/250), SM caldesmon (1/500), Vonwillebrand's factor (1/40), PI3K p85 (1/100), PI3K p110 (1/50), BrdU (1/200), PCNA (1/100). To verify the staining pattern of the vessel wall layers with the chosen antibodies, CA rings were frozen immediately following dissection and cross-sectional sections of 7  $\mu$ m thickness were prepared using a cryostat. Each slice was mounted on glass slides and permeabilized by incubation in 1% Triton X-100/PBS for 30 min. Staining with primary antibody and secondary antibody were completed as described above.

### **3.4 CELL GROWTH ASSAYS**

#### **3.4.1 RNA Synthesis**

To measure RNA synthesis, triplicate sets of SMCs were seeded in 24-well culture dishes at  $5 \times 10^3$  cells/well or 12-well culture dishes at  $8 \times 10^4$  cells/well following trypsinization (0.5% trypsin, ICN) and grown in DMEM containing 20% FBS until approximately 75% confluent (1-2 days). The growth media was removed and replaced with DMEM supplemented with 5  $\mu$ g/mL transferrin,  $10^{-9}$  M selenium,  $2 \times 10^{-4}$  M ascorbate,  $10^{-8}$  M insulin for the specified time.

For analysis of the dose-dependent stimulation of RNA synthesis by AngII, triplicate sets of cells were stimulated with varying concentrations of AngII in the presence of [<sup>3</sup>H]-uridine (1.5  $\mu$ Ci) without changing the media. Following a 6 hour incubation at 37°C, the cells were lysed (0.5% SDS, 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4)) and the

incorporated label was recovered by trichloroacetic acid (TCA)-precipitation (10% final concentration) onto GF/A glass fibre filters. Each filter is washed four times with 5% TCA, once with 95% ethanol and the radioactivity determined by liquid scintillation counting. Inhibitors or receptor antagonists were added 15 min prior to AngII stimulation.

For analysis of the time required for AngII to stimulate RNA synthesis, triplicate sets of cells were treated with AngII ( $10^{-6}$  M) for varying time periods (0 - 6 hours). In each case, the media was removed, cells were rinsed twice and incubated in conditioned differentiation media for the remainder of the time. [ $^3$ H]-uridine (1.5  $\mu$ Ci) was added to the existing media for a total of 6 hours and the incorporated label was recovered by TCA-precipitation (10% final concentration) onto GF/A glass fibre filters and counted by liquid scintillation counting as described above.

To determine the time of efficacy for different inhibitors, triplicate sets of cells were treated with AngII ( $10^{-6}$  M) and [ $^3$ H]-uridine (1.5  $\mu$ Ci) for a total of 6 hours. The inhibitor was added at the specified time after AngII stimulation. At the end of the 6 hour assay, the incorporated label was recovered by TCA-precipitation onto GF/A glass fibre filters and counted by liquid scintillation counting.

### 3.4.2 DNA Synthesis

To measure DNA synthetic activity, triplicate sets of quiescent SMCs were set-up as described for RNA synthesis. Each set was stimulated with varying concentrations of AngII in the presence of [ $^3$ H]-thymidine (2  $\mu$ Ci) without changing the media. Following a 72 hour incubation at 37°C, the cells were lysed and the incorporated label recovered by TCA-precipitation followed by filtration as described above. To evaluate the time required to induce quiescence, triplicate sets of cells were pulsed with 2  $\mu$ Ci of [ $^3$ H]-thymidine for 30 minutes at 12 hour time intervals. To evaluate the time required to reenter the cell cycle, DNA synthetic activity was measured in triplicate sets of SMCs by pulse-labelling with 2

$\mu\text{Ci}$  of [ $^3\text{H}$ ]-thymidine for 30 minutes at varying times after stimulation with 20% FBS or 1  $\mu\text{M}$  AngII. Cells were lysed and analyzed as described above.

To visualize the cells synthesizing DNA in response to AngII stimulation, quiescent SMCs on glass slides were stimulated with AngII in the presence of 10 nM BrdU and incubated at 37°C for 96 hours. The media was decanted, cells were rinsed with PBS and fixed in cold 95% methanol for 15 min. To denature the DNA, the fixed cells were incubated in 0.1 M HCl for 1 hour at 37°C and rinsed repeatedly in 0.1 M borate (pH 8.0) and then PBS. The slides were stained with a monoclonal antibody to bromodeoxyuridine, diluted 1/50 in 1% BSA/0.02% sodium azide/ PBS. A Cy3-conjugated secondary antibody was used to visualize the primary antibody for fluorescent microscopy.

For analysis of the time required for AngII to stimulate DNA synthesis, triplicate sets of cells were treated with AngII ( $10^{-6}$  M) for varying time periods (0 - 48 hours). In each case, the media was decanted, cells were washed and incubated in conditioned differentiation media for the remainder of the time. [ $^3\text{H}$ ]-thymidine (2  $\mu\text{Ci}$ ) was added to the existing media for a total incubation of 48 hours and the incorporated label was recovered by TCA-precipitation (10% final concentration) onto GF/A glass fibre filters and counted by liquid scintillation counting as described above.

### **3.4.3 Cellular Hypertrophy/Hyperplasia**

To monitor changes in cell number or size, the MTT assay was employed. This method measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide (MTT) by active mitochondria which results in a colorimetric change measured at OD<sub>550</sub>. Quiescent SMCs were prepared in 96-well culture plates and stimulated with AngII for 24, 48, 72 or 96 hours. Inhibitors or receptor antagonists were added 15 min prior to stimulation with AngII. Stimulated cells were incubated with MTT (0.1 mg/mL) for 4 hours at 37°C. Media was decanted and the cells were solubilized in acidified isopropanol. Colour

development was quantified using a Molecular Devices ThermoMAX plate reader at 550 nm absorbance.

To complement the MTT assay, increases in cell number were determined by haemocytometer counting. Quiescent SMCs were prepared in 12-well culture plates and triplicate sets were stimulated with AngII ( $10^{-6}$  M) for 48, 72 and 96 hours. Inhibitors or receptor antagonists were added 15 min prior to stimulation. At the end of the stimulation period, media was decanted and the cells were rinsed once with PBS. Each well was trypsinized with 250  $\mu$ l 1x trypsin (Hepes-buffered saline), quenched with 250  $\mu$ l 20% FBS/DMEM and rinsed twice with additional 250  $\mu$ l media. A haemocytometer was used to count each pooled fraction at least three times and the values were averaged.

#### **3.4.4 Proto-oncogene Expression**

Total RNA was extracted with chloroform following lysis with TRIzol or a modified guanidium isothiocyanate method (Chomczynski and Sacchi, 1987) and precipitated with an equal volume of cold isopropanol. The RNA pellet was resuspended in water and transferred to Nytran membrane using a slot blot apparatus, dried and cross-linked with ultraviolet light. RNA concentration was estimated by absorbance at OD<sub>260</sub>. Following prehybridization in 50% formamide, 4x SSC, 0.2 mg/ml BSA, 0.2% PVP, 0.2% Ficoll, 50 mM NaPO<sub>4</sub> (pH 7.4) for 24 hours at 42°C, the blots were hybridized for 48 hours in the same solution containing  $2 \times 10^6$  cpm of [<sup>32</sup>P]-labelled plasmid containing the sequences for *c-fos* (pFOS1, ATCC 41040) or ribosomal protein L32 (p3AR2.8)(Dudov et al., 1984). Densitometric analysis of the autoradiograms was used to quantify the proto-oncogene mRNA relative to ribosomal protein mRNA levels.

#### **3.5 REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION**

Total RNA was isolated from growing and quiescent SMCs using TRIzol or a modified guanidium isothiocyanate method (Chomczynski and Sacchi, 1987) and

precipitated with an equal volume of cold isopropanol. The RNA pellet was washed in 70% ethanol and resuspended in water. Reverse transcription of 1 µg of RNA was conducted using the Perkin-Elmer GeneAmp PCR kit. PCR amplification with 25 ng of sense and antisense primers and 1.25U Amplitaq *Taq* DNA Polymerase was conducted over 35 cycles using a three-step program (1 min at 95°C, 1 min at 55°C, 1 min at 72°C) that concluded with 7 min at 72°C. Samples were analyzed by electrophoresis on 1.7% agarose gel and visualized with SYBR Green I. Primers for GADPH (Zahradka et al., 1993), AT<sub>1A</sub> and AT<sub>1B</sub> (Matsubara et al., 1994), and AT<sub>2</sub> (Nio et al., 1995) were synthesized according to published cDNA sequences with an Oligo1000 DNA Synthesizer.

### 3.6 ANGIOTENSIN RECEPTOR BINDING ASSAY

Quiescent SMCs were plated in 6-well culture dishes and incubated in serum-free supplemented DMEM as described above. Triplicate sets of cells were rinsed with PBS, blocked with 0.1% BSA for 2 hours and incubated with 1 nM [<sup>125</sup>I]-SarIle-AngII and a varying concentration of unlabelled receptor antagonists or AngII in Binding buffer (50 mM Tris-HCl (7.4), 20 mM NaCl, 4 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.25% BSA) with inhibitors (0.3 mM bacitracin, 1 mg/mL aprotinin, 5 mg/mL phosphoramidon, 0.5 mg/mL leupeptin, 1 mg/mL pepstatin) for 1.5 hours at 4°C. Cells were rinsed four times with 1% BSA/PBS, lysed in 0.5% SDS/0.025 M NaOH and quantified by gamma scintillation counter. To maximize binding to the AT<sub>2</sub> receptor, 2 mM DTT was included in the binding buffer for specified experiments. Non-specific binding was determined in the presence of (10<sup>-6</sup> M) AngII.

### 3.7 IMMUNOPRECIPITATION

Quiescent SMCs, in 100-mm culture dishes, were stimulated with AngII (10<sup>-6</sup> M) for varying periods of time and cell lysates were prepared by addition of 1 mL lysis buffer (1% NP-40, 20 mM Tris-HCl pH 7.5, 10% glycerol, 137 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM

phenylmethylsulfonyl fluoride (PMSF), 0.4 mM orthovanadate). The lysates were cleared by centrifugation, and their protein concentration were determined by the BCA method (Pierce). A 100  $\mu\text{g}$  aliquot (1  $\mu\text{g}/\mu\text{L}$ ) was then mixed for 2 hours at 4°C with protein G-Sepharose beads which were subsequently removed by centrifugation at 12,000 $\times$ g for 5 min. Each aliquot was then mixed over 2.5 h at 4°C with 4  $\mu\text{g}$  of anti-phosphotyrosine antibody, anti-p85 antibody or anti-p110 antibody. Protein G-Sepharose beads were added for an additional 2 hours and the beads collected by centrifugation. The beads were washed four times with 1 mL cold lysis buffer and either resuspended directly in 50  $\mu\text{L}$  2 $\times$ SDS/gel loading buffer for Western blot analysis or treated further prior to measuring PI3K activity.

### **3.8 WESTERN BLOT ANALYSIS**

Cell extracts were prepared from quiescent and growing SMCs in 2 $\times$ SDS gel loading buffer (1 $\times$  buffer=62.5 mM Tris-HCl pH 6.8, 1% SDS, 10% glycerol, 0.005% bromophenol blue, 5%  $\beta$ -mercaptoethanol) and heated at 95°C for 5 min.. Following centrifugation, samples were analyzed by gel electrophoresis on a 7.5% polyacrylamide gel and transferred to PVDF membrane at 90 V over 90 min in 20% methanol, 25mM Tris, 130mM glycine. Membranes were blocked by a 60 minute treatment at room temperature with blocking solution (3% BSA in TBS-T (10 mM Tris-HCl pH 7.5, 0.1 M NaCl, 1 mM EDTA, 0.1% Tween 20). Anti-p85 antibody or anti-phosphotyrosine antibody (1:5000 dilution) was added in fresh blocking buffer and incubated for 60 minutes at 37°C. Similar conditions were used for the secondary antibody (HRP-conjugated anti-rabbit IgG). The membranes were washed five times over 30 minutes with TBS-T and HRP detected using the ECL chemiluminescent system. Quantification was based on densitometric analysis of the resulting autoradiogram.

### **3.9 SUBCELLULAR FRACTIONATIONS**

Quiescent SMCs were prepared in 100-mm plates as previously described. Following stimulation with AngII ( $10^{-6}$  M) for various times (0-30 min), the cells were treated with 1

mL of Buffer A (50 mM  $\beta$ -glycerophosphate, 1 mM EDTA, 2 mM EGTA, 0.34 mM  $\text{CaCl}_2$ , 250 mM sucrose, 0.05% digitonin, 1 mM PMSF, 0.1 mM leupeptin) to isolate the cytoplasmic fraction. After removal of Buffer A, the cells were incubated in 1 mL Buffer B (50 mM  $\beta$ -glycerophosphate, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 0.1 mM leupeptin) and the lysate was centrifuged for 15 min at  $12,000\times g$  to separate the membrane fraction (supernatant) and the nuclear fraction (pellet; resuspended in 100  $\mu\text{L}$  2 $\times$ SDS/gel loading buffer). The cytoskeletal fraction was prepared by addition of 1 mL 2 $\times$ SDS/gel loading buffer to the plate followed by vigorous scraping. Note that all steps were performed at  $4^\circ\text{C}$  and fractions were stored at  $-20^\circ\text{C}$  for Western blot analysis. Equivalent amounts of total protein (0.5-0.1 mg) were loaded for each group of samples based on densitometric analysis after gel electrophoresis and staining.

### **3.10 PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY ASSAYS**

#### **3.10.1 *In Vivo* PI3K Assay**

Quiescent SMCs, in 4-well culture dishes, were incubated in phosphate-free DMEM for 4 hours prior to labelling with 200 mCi/L [ $^{32}\text{P}$ ]-orthophosphate. Cells were preincubated for 10 minutes with receptor antagonists or LY294002 prior to stimulation with agonist. At 15 min after addition of AngII, the media was decanted, cells were washed with PBS and precipitated with ice-cold 5% perchloric acid. Phosphatidylinositides were extracted from the cell pellet with acidified chloroform/methanol solution (Balla et al., 1988) and analyzed by thin layer chromatography on Silica G plates (Okada et al., 1994). Dried plates were exposed to autorad film at  $-80^\circ\text{C}$ .

#### **3.10.2 *In vitro* PI3K Assay**

The immunoprecipitates obtained with the anti-p85 antibody or anti-p110 antibody were assayed according to Whitman et al. (1988) with minor modifications (Šuša et al., 1992). The protein G-Sepharose pellets were washed three times with assay buffer (20 mM

Tris-HCl pH 7.6/ 10 mM MgCl<sub>2</sub>/ 100 mM NaCl) and resuspended in 90 μL assay buffer containing 20 μM [<sup>32</sup>P]-ATP. The reaction was initiated by adding 10 μL of a phosphoinositide mixture. The lipid substrates were prepared by sonicating equal quantities of phosphatidylserine, phosphatidylinositol and phosphatidylinositol 4,5-bisphosphate in assay buffer to yield a final concentration of 200 μg/mL. Following a 20 min incubation at 37°C, the reaction was stopped by addition of 1 M HCl-methanol (1:1) and the samples was extracted twice with chloroform. The lipids were recovered from the organic phase by evaporation, suspended with 10 uL chloroform and analyzed by thin layer chromatography as described above (Okada et al., 1994).

### **3.11 ARACHIDONIC ACID RELEASE**

Quiescent SMCs were cultured in 24-well plates as previously described. Prior to stimulation, the cells were labelled with 2 μCi/mL [<sup>3</sup>H]-arachidonic acid for 48 hours at 37°C in 5% CO<sub>2</sub>, and then rinsed four times with serum-free supplemented DMEM. Receptor antagonists were preincubated for 10 minutes prior to stimulation with agonist. Following stimulation with AngII or 2% FBS for 4 hours, the media was removed and counted by liquid scintillation counter with 1 mL of Aquasol (NEN-DuPont) scintillation fluid. Control cells were incubated for an equivalent period of time with no stimulation and the media was counted as described above.

### **3.12 STATISTICAL ANALYSIS**

For all studies, the data from at least three replicates was expressed as mean ± S.E. Statistical differences (set at p<0.05) were identified with the Student's t-test, as indicated by \* at data points.

## 4. CHARACTERIZATION OF SMOOTH MUSCLE CELL MODEL

### 4.1 INTRODUCTION

The normal arterial vessel wall consists of a regular arrangement of endothelial, smooth muscle and fibroblast cells, present in three distinct layers of endothelium, media and adventitia. The response to injury or other stress stimuli can vary between the different cellular components of the vessel. Medial SMCs retain the ability to reversibly modulate their phenotype which allows for their proliferation and/or migration into the intima at the site of injury (Schwartz et al., 1995). It is these characteristics that lead to the adaptive and pathogenic growth of SMCs which is key to vascular remodelling and lesion formation.

To define the SMC response to various stimuli and growth factors *in vitro*, various SMC culture systems have been established (Chamley-Campbell et al., 1979). Ideally, cells grown in culture conditions will retain the majority of characteristics typical of SMCs in the arterial environment such as the ability to express and organize the various components of the contractile apparatus as well as the capacity for phenotypic modulation in response to various stimuli. Although the *in vitro* environment does not mimic the matrix composition or multicellular interactions of the vessel wall, it does provide an isolated and simplified system to study the SMC response to specific stimuli.

A SMC culture model based on selective migration from porcine coronary artery (PCA) explants will be described in this chapter. To characterize the PCA SMC primary cultures, we (i) defined the optimal window of migration for SMCs, (ii) characterized the cell phenotype using immunofluorescence staining, and (iii) defined the parameters to establish quiescence and reinitiate mitogenesis. By utilizing the distinct temporal pattern of migration *in vitro* of the heterogeneous cell populations from the arterial wall, selection for cultures that contain only SMCs is possible.

## **4.2 RESULTS & DISCUSSION**

### **4.2.1 Smooth Muscle Cell Phenotype Markers**

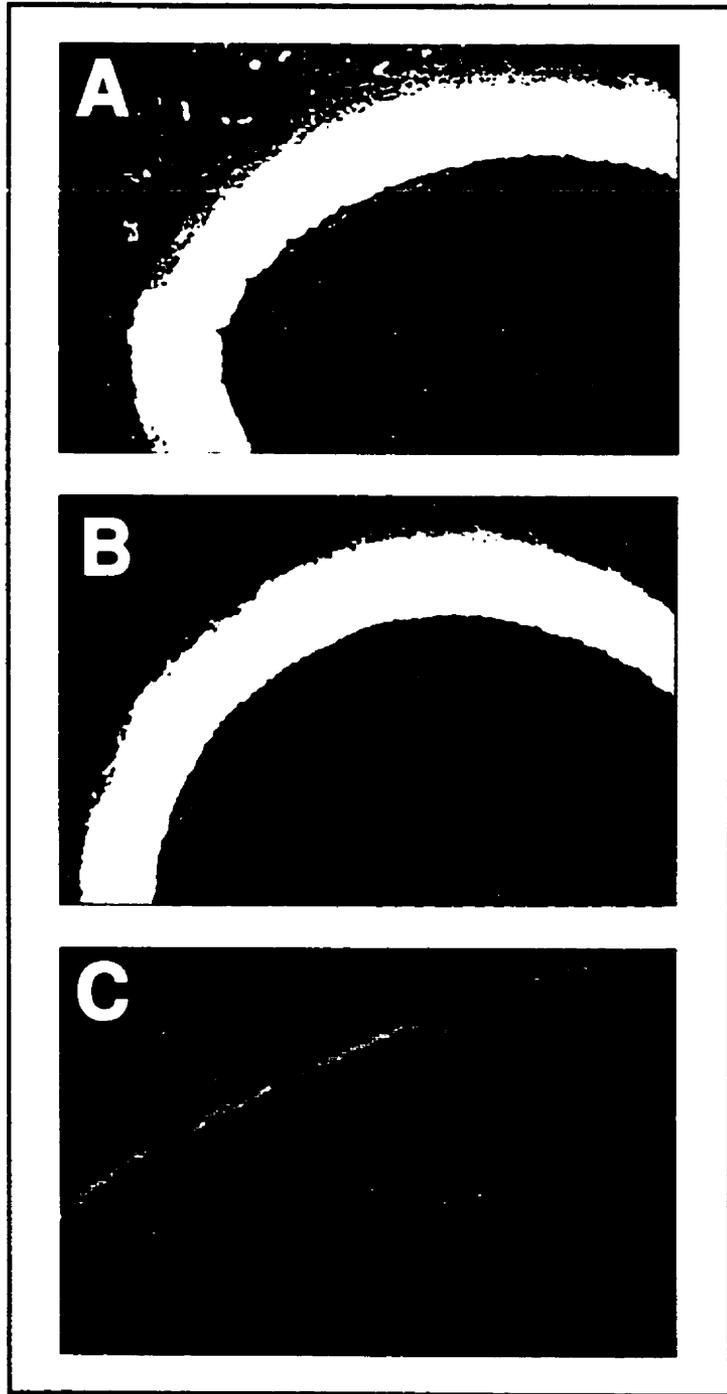
After dissection from whole porcine hearts, the left anterior descending coronary artery (CA) was cut into rings and placed in culture to initiate migration. To verify the specificity of the antibodies used in this study, their ability to stain specific layers of cells was assessed using sections of CA (Figure 1). Antibodies to SM  $\alpha$ -actin (Panel A) and SM myosin (Panel B), both markers of the contractile fibres present in SMCs (Shanahan et al., 1993), stained the cells of the medial layer while the adventitial layer and the endothelial layer remained unstained. As expected, antibody to von Willebrand factor, an endothelial cell marker (Wagner et al., 1984), stained the endothelium exclusively (Panel C). Although no specific marker for fibroblasts has been identified, it is commonly accepted that fibroblast-like cells do not express either SM myosin or von Willebrand factor.

### **4.2.2 Heterogeneity of Migrating Cells**

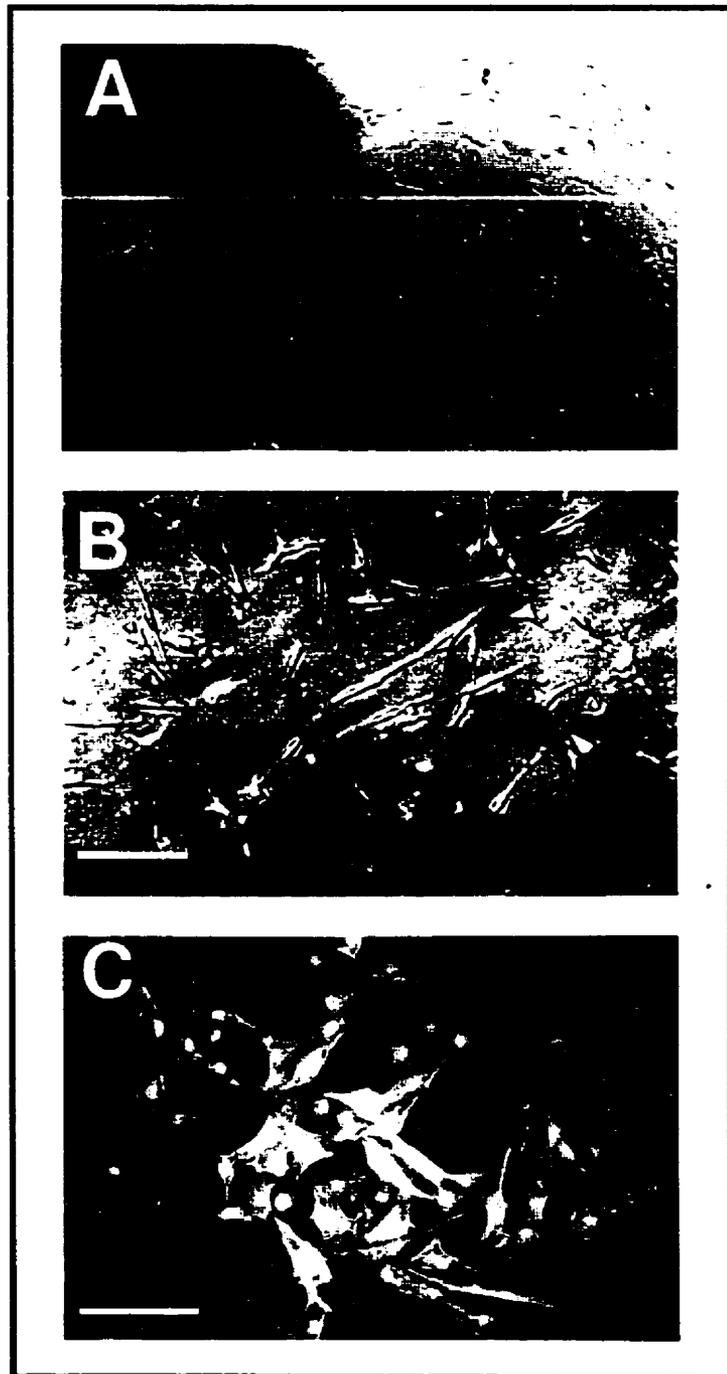
CA rings were incubated in DMEM + 20% FBS and the cells that migrated from the free-floating CA explants were allowed to attach to the culture dish. The first wave of migration was evident after 5-10 days of culture (Figure 2A). Examination of the cells that had migrated and adhered to the culture dishes by phase-contrast microscopy (Figure 2B) showed extensive heterogeneity in cell shape from round to polygonal to spindle shaped. As demonstrated in Figure 2C, many of these cells expressed filamentous SM  $\alpha$ -actin even though several distinct morphologies could be detected. This morphological heterogeneity was maintained through the initial subcultures and may reflect the heterogeneous cell populations that exist within the vessel wall.

It should be noted that a similar pattern of heterogeneous cell shapes was seen in the adherent cells that migrated from the medial layer of the porcine aorta (data not shown). The heterogeneity of the population of SMCs that migrated from the media agrees with the study

**FIGURE 1: *Immunofluorescent staining of Coronary Artery rings.*** Porcine coronary artery ring slices were stained with monoclonal antibodies for cell specific markers and viewed by immunofluorescence microscopy. The medial SMC layer exhibited SM  $\alpha$ -actin (Panel A) and SM myosin (Panel B) immunoreactivity. The endothelial layer clearly stained with anti-von Willebrand factor antibody (Panel C).



**FIGURE 2: *Migration of Heterogeneous cell types from Coronary Artery explants in culture.*** Left descending coronary artery was dissected from porcine hearts, cut into 2-5 mm rings and placed in culture. Cell migration and adherence to the culture dish was observed after 7-10 days (Panel A). Heterogeneity of the cell shapes of the migrated cells is shown in Panel B (bar=25  $\mu\text{m}$ ). SM  $\alpha$ -actin expression was used to identify cells of smooth muscle origin in the total cell population as shown by Hoescht 33258 nuclear stain (Panel C; bar=50  $\mu\text{m}$ ).



by Frid et al. (1997) that demonstrated distinct compartments of the arterial media from pulmonary arteries can produce SMCs with varying expression of SMC markers and growth potential. The physiological significance of the heterogeneity in SMCs is an area of keen interest. Originally it was thought that vascular SMCs were a homogeneous population and, thus, exhibited a homogeneous response to environmental stimuli. However, recent research has demonstrated that heterogeneous SMC populations exist in both injured and normal vessels and these SMC populations exhibit differences in contractility, ECM synthesis and growth properties (Adams et al., 1999; Shanahan et al., 1999; Shanahan et al., 1998). Thus, this heterogeneity in SMC characteristics may have a significant impact on vessel homeostasis and the response to injury.

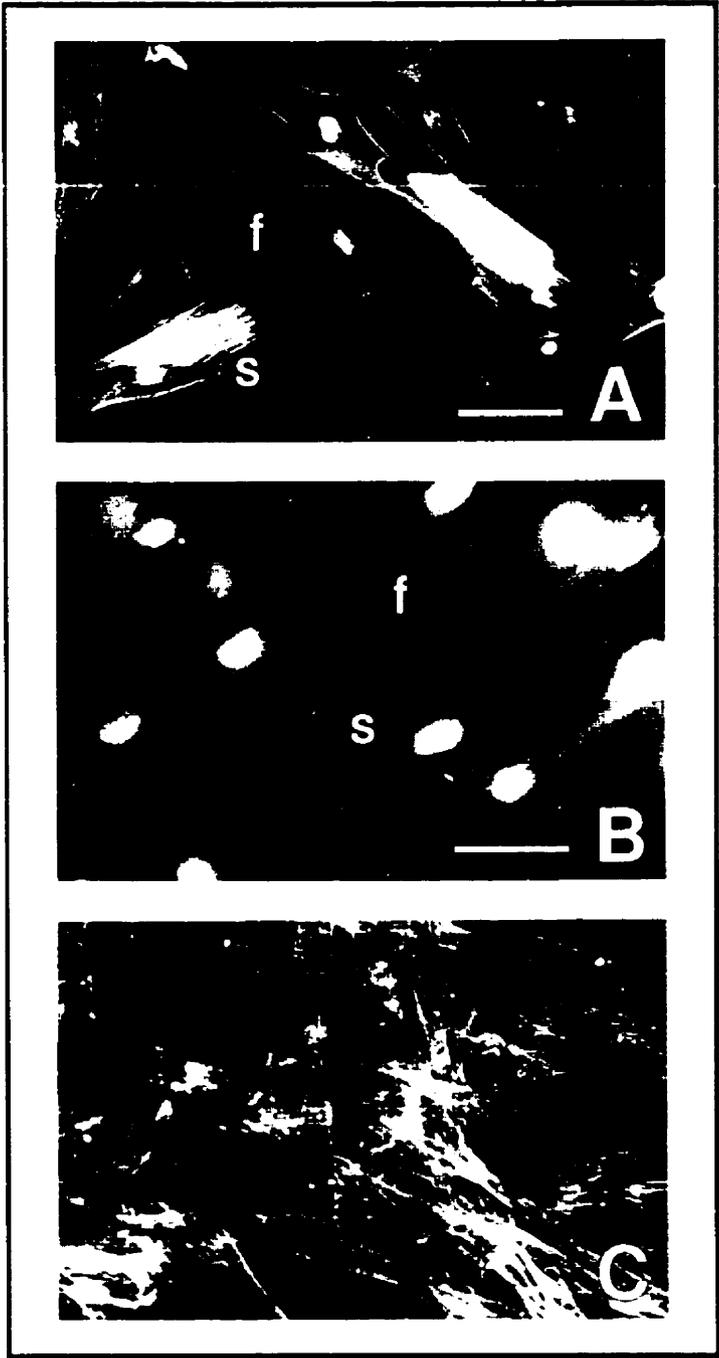
#### **4.2.3 Temporal Migration from Coronary Artery Explants**

##### **4.2.3.1 *Phenotypic Assessment of Cells from the Early Migration Period:***

Immunofluorescent microscopy was used to evaluate the phenotype of individual quiescent cells that migrated from the explants during various periods. The first wave of migration (migration days 1 through 7) consisted of a mixture of fibroblast-like and SMC-like cells as determined by the pattern of immunostaining with antibodies against SM  $\alpha$ -actin, SM myosin (SM1 and SM2), h-caldesmon and von Willebrands factor. A similar temporal pattern of fibroblast-like cell migration has been previously reported for rabbit aorta (Kasai et al., 1964). In Figure 3A, a typical sample of the cells from the first wave of migration is depicted with staining for SM  $\alpha$ -actin clearly distinguishing between SMC-like (s) and fibroblast-like (f) cells (Shanahan et al., 1993). In SMCs, the actin was visible as filaments, whereas in fibroblast cells actin staining was evident only around the periphery of the cell. The presence of SM-myosin organized in filaments confirmed that approximately half of the cells were of SMC origin (Figure 3B). It should be noted that no endothelial cells were present in this population, as confirmed by the lack of anti-von Willebrand factor staining (data not shown).

**FIGURE 3: *Phenotypic Assessment of Cells from the Early Migration Period.***

Immunofluorescence microscopy was used to assess the phenotype of the cells in the first wave of migration (1-7 days) from the coronary artery explants. The pattern of staining with anti-SM  $\alpha$ -actin (Panel A) and anti-SM myosin (Panel B) overlaid with Hoescht 33258 nuclear stain was used to distinguish between SMCs (s) and fibroblasts (f) in this heterogeneous population. Bar=100  $\mu$ m. The pattern of growth of this mixture of cells was multi-layered and irregular (Panel C).

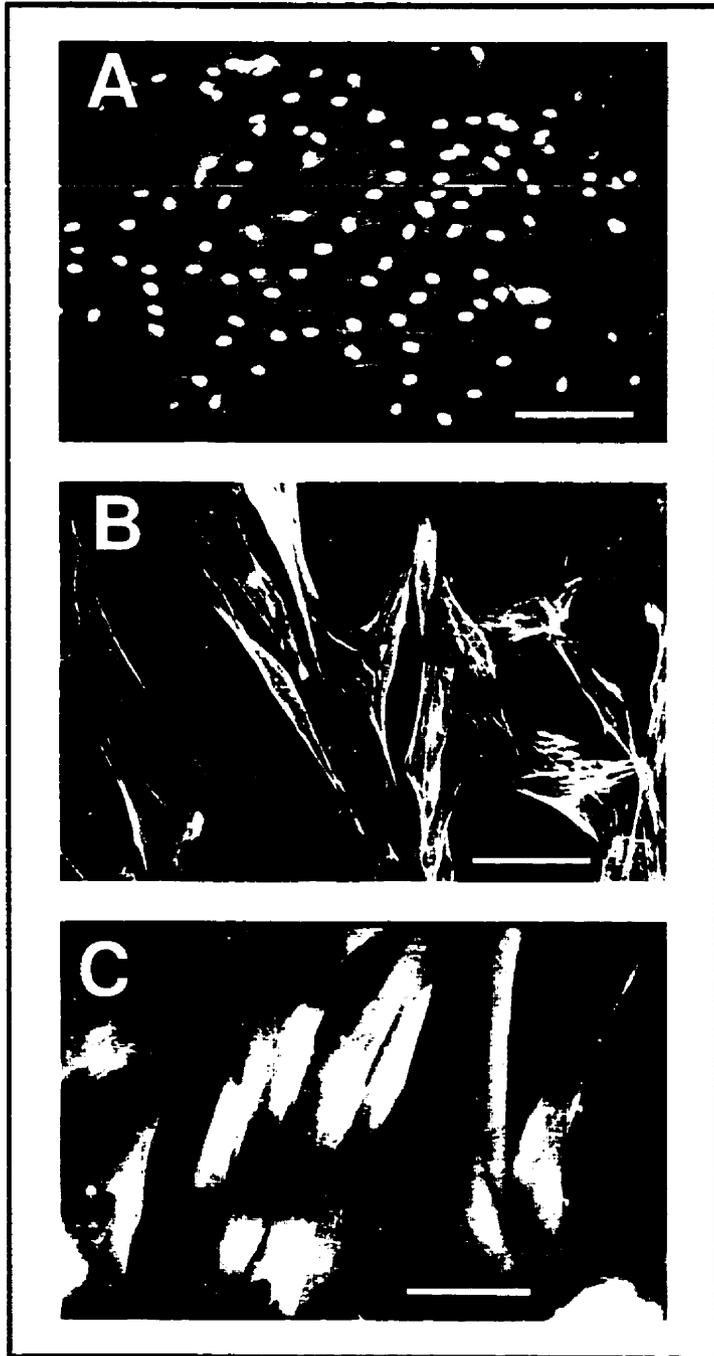


The pattern of growth of these fibroblast and SMC mixed-cultures differed markedly from the typical single-layered, regularly aligned, hill-and-valley pattern characteristic of confluent SMC cultures. Instead, these cultures exhibited an irregular growth pattern with the ability to grow in multiple layers (Figure 3C).

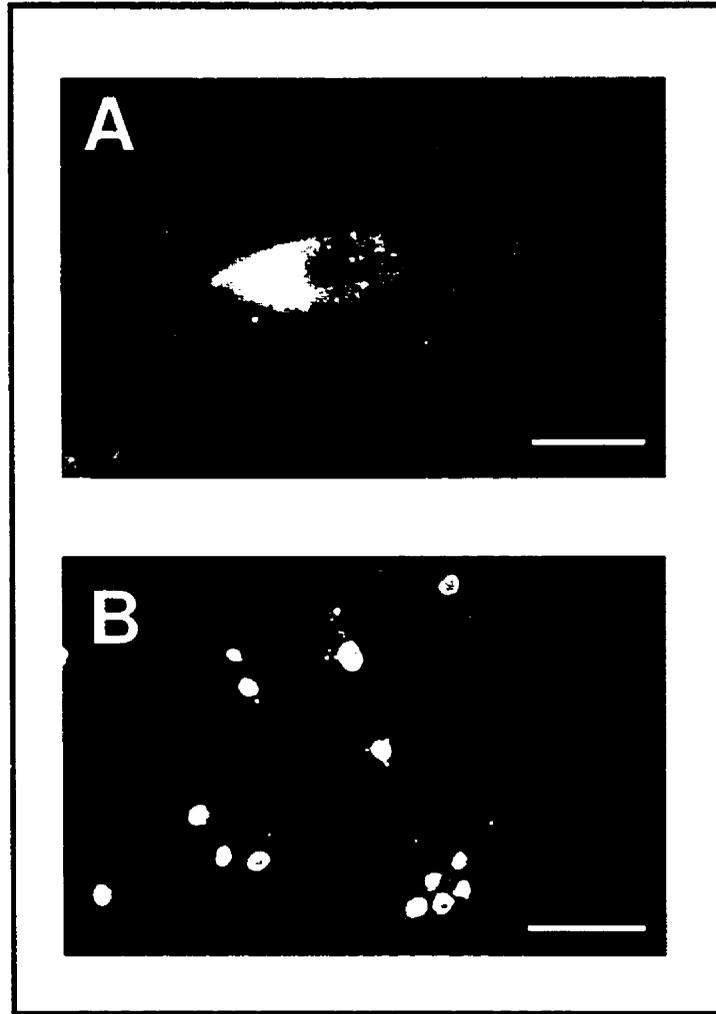
**4.2.3.2 Immunofluorescent Examination of Cells from the Intermediate Migration:** The phenotype of the quiescent cells present in the second wave of migration (migration days 7-14) expressed many of the markers characteristic of SMCs (Shanahan et al., 1993). As shown in Figure 4A, all cells in a typical field exhibited strong staining with a monoclonal antibody specific to SM  $\alpha$ -actin. The SM phenotype of these cells was further confirmed by the filamentous pattern of SM myosin (SM1 and SM2) staining (Figure 4B) as well as the presence of h-caldesmon (Figure 4C). Minimal fibroblast-like cells or endothelial cells were detected in this population of cells based on the presence of SM markers and the lack of von Willebrand factor (data not shown). According to the temporal pattern of cell migration from porcine coronary artery explants, SMCs comprise the second wave of migration (>95% in all fields examined). The absence of fibroblast-like cells in this population of migrating cells may reflect the smaller, more restricted pool present in the vessel wall which may have been exhausted by the first week of migration.

**4.2.3.3 Assessment of Endothelial Cells in the Late Migration:** Examination of quiescent cells involved in the third wave of migration (migration days 14-21) identified a mixture of SMCs and endothelial cells. Endothelial cells could be easily distinguished from SMCs by their distinctive morphology in culture (round, cobble-stone appearance). Their endothelial phenotype was confirmed by their positive staining with anti-von Willebrand factor antibody (Figure 5A) and their poor staining with SM  $\alpha$ -actin (Figure 5B). A plausible explanation for the later migration of endothelial cells could be their inability to adhere to plastic culture plates until sufficient extracellular matrix has been synthesized by SMCs. Thus, the use of

**FIGURE 4: *Immunofluorescent Examination of Cells obtained during Intermediate Migration.*** The phenotype of the cells that migrated out of the coronary artery explants during the second wave of migration (7-14 days) was evaluated by immunofluorescence microscopy. Quiescent cells were stained with antibodies to smooth muscle (SM)-specific isoform of  $\alpha$ -actin (Panel A; bar=50  $\mu$ m), SM myosin heavy chain (Panel B; bar=100  $\mu$ m) and caldesmon (Panel C; bar=100  $\mu$ m) and visualized by epifluorescence microscopy. Each panel is representative of the staining observed in >95% of the cells.



**FIGURE 5: Assessment of Endothelial Cells in the Late Migration.** Endothelial cells were detected in the third wave of migration (14-21 days) from the coronary artery explants by their immunoreactivity with anti-von Willebrand factor that was visualized by epifluorescence microscopy (Panel A; bar=200  $\mu\text{m}$ ). The non-smooth muscle phenotype in this population of cells was shown by the poor reactivity with anti-SM  $\alpha$ -actin overlaid with Hoescht 33258 nuclear stain (Panel B; bar=50  $\mu\text{m}$ ).



**Table 1. Summary of the Cell Types Identified during the Different Migration Periods.**

Migration period (days)	Phenotype Markers			n
	Smooth Muscle Cells SM $\alpha$ -actin, SM myosin, caldesmon, fibrous	Fibroblast-like Cells SM $\alpha$ -actin, diffuse	Endothelial Cells VWBr factor	
0-7 days	50-75 %	25-50 %	0 %	n=5
7-14 days	>95 %	<5 %	<1 %	n=15
14-28 days	75-90 %	0 %	10-25 %	n=3

uncoated culture dishes may facilitate the separation of endothelial cells from SMCs in the migrating population.

**4.2.3.4 Summary of Phenotypic Assessment of Migrating Cells:** Table 1 summarizes the results of our phenotypic assessment of cells that migrated from coronary artery explants in the defined windows of migration. These data demonstrate that the method of selective temporal migration results in the culture of heterogeneous cell types/populations which exist within the coronary artery vessel wall. Characterization using specific phenotypic markers conclusively identified both SMCs and endothelial cells. In addition, a third population of cells was identified by the lack of staining with our panel of SMC-specific and endothelial cell-specific markers. While these cells may be designated fibroblast-like cells, it is a distinct possibility that they represent an immature SMC subpopulation present in the vessel wall that exhibits different migration properties and pathogenic capacity. Future studies with this model should address the heterogeneity of the cell populations with the specific markers that have been recently reported (Adams et al., 1999). The heterogeneity in cell shape observed in SMCs from the same as well as different migration periods may reflect differences that persists in their migratory and proliferative capacity.

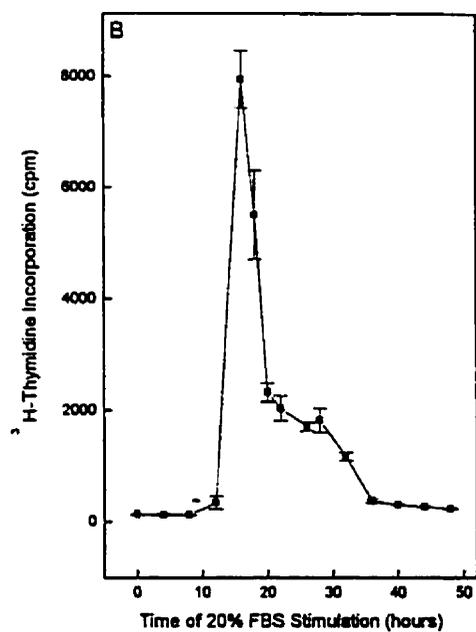
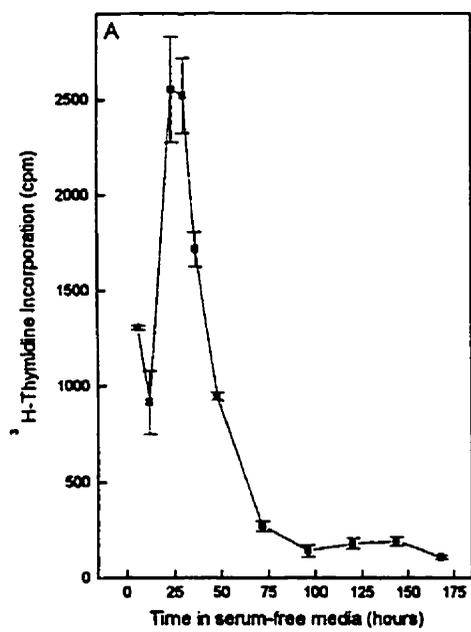
#### **4.2.4 Phenotypic Modulation of *In Vitro* Smooth Muscle Cells**

To characterize the growth properties of the primary SMC cultures obtained from the second wave of migration (days 7-14), cell number was expanded through two cell passages ( $p=2$ ). At confluency, the SMC cultures exhibited the characteristic single-layered, hill-and-valley pattern (Figure 6). The time required for these cells to re-establish a quiescent phenotype was determined by monitoring the rate of DNA synthesis. In this experiment, the amount of [ $^3\text{H}$ ]-thymidine incorporated during a 30 minute pulse was measured at various time points after the growth media (20% FBS) was replaced with a serum-free defined media. The larger variance in the levels of DNA synthesis reflect the asynchrony of this

**FIGURE 6: *Morphology of Confluent Smooth Muscle Cell Cultures.*** Cells obtained from the second wave of migration (depicted in Figure 4) were grown to confluence after two passages. Phase contrast microscopy was used to visualize the growth characteristics of these cells. At confluence, these cells exhibited the single-layered, longitudinally aligned and hill-and-valley pattern characteristic of SMCs.



**FIGURE 7: *Phenotypic Modulation of Smooth Muscle Cell Cultures between Proliferation and Quiescence.*** The time required to establish quiescence after removal of serum was assessed by the rate of [<sup>3</sup>H]-thymidine incorporation into DNA during a 30 minute pulse at the indicated times (Panel A). The time course required for quiescent SMCs to traverse G1 and enter S phase after stimulation with 20% FBS was monitored at the indicated times by the rate of [<sup>3</sup>H]-thymidine incorporation into DNA over a 30 minute period (Panel B). Each data point represents the mean  $\pm$  SE of three independent experiments.



population which would be at various stages in the cell cycle at the time of serum withdrawal. Following a period of 4 to 5 days in serum-free media, the SMCs attained a basal level of DNA synthesis indicative of quiescence and entry into  $G_0$  (Figure 7A). To allow the SMCs to consistently reach a quiescent state and re-establish their contractile apparatus, the standard time for redifferentiation was set at 5-7 days for all subsequent studies of SMC response. These "differentiated" SMCs retained the ability to re-enter the cell cycle in response to mitogens as demonstrated by their synchronous entry into S phase 15-18 hours after stimulation with 20% FBS (Figure 7B). The ability to manipulate the phenotypic state of the cells provides an appropriate *in vitro* system for monitoring changes in SMC phenotype following growth factor stimulation.

#### **4.2.5 Advantages of Selective Temporal Migration Method**

This method of selective temporal migration from coronary artery explants offers the distinct advantages of isolating SMC populations without the use of enzymes. The commonly employed method for establishing primary cultures of SMCs involves the enzymatic dispersion of vessels; however, a generalized digestion of the vessel does not offer any means of separating the various cell types that are present in the distinct cell layers. Alternatively, microdissection of vessels to remove the medial layer is usually limited to larger vessels due to the technical difficulty. This method of selective migration allows the avoidance of enzymes to disperse the cells and, thus, minimizes damage to both cell membranes and membrane-bound receptors which may affect subsequent studies. This observation would explain the lack of proliferative capacity noted for enzymatically-dispersed porcine coronary artery SMCs (Patel et al., 2000). In contrast, the SMCs derived by this method exhibited the capacity to migrate, synthesize matrix, proliferate in response to several growth factors (AngII, bFGF, insulin, data not shown) and survive in culture for extended periods of time ( $\geq 3$  months).

### 4.3 CONCLUSIONS

In this chapter, a coronary arterial SMC culture system has been described that maintains the flexible phenotype of SMCs with the capacity to reversibly alter their phenotype between a proliferative and quiescent state. By monitoring the temporal pattern of migration of distinct cell types, it was possible to define a specific window (migration days 7-14) of time that reproducibly yielded SMCs (>95%). This approach avoids the use of enzymes to fully digest the vessel and thus minimizes the damage to membrane-bound receptors or the release of cells other than SMCs. Therefore, the methodology described in this paper offers an alternative approach for establishing SMC cultures from small vessels such as the coronary artery.

## 5. CONTRIBUTION OF AT<sub>1</sub> AND AT<sub>2</sub> RECEPTORS TO SMC GROWTH

### 5.1 INTRODUCTION

Excessive smooth muscle cell (SMC) growth has been implicated in the development and progression of cardiovascular diseases such as atherosclerosis and hypertension. To date, one of the most effective treatments for vascular diseases has been angiotensin converting enzyme (ACE) inhibitors which interfere with the conversion of angiotensin I to angiotensin II (AngII). However, receptor antagonists are now in use as a clinical alternative to ACE inhibitors for selective interference in the local renin-angiotensin system. At least two different classes of AngII receptors, Type 1 (AT<sub>1</sub>) and Type 2 (AT<sub>2</sub>), mediate the broad range of physiological effects of AngII on the cardiovascular system. Until recently, the AT<sub>1</sub> receptor has been regarded as the principal mediator of the vascular response to AngII. As a consequence, the AT<sub>2</sub> receptor has been studied less intensely and fewer details of its biological functions have been defined. The abundance of the AT<sub>2</sub> receptor in fetal tissue and its acute regulation in response to growth factors, stress or damage has led to speculation that this receptor has a critical role in tissue growth and development. However, recent research has discovered a level of functional redundancy and interplay between the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes that highlights the importance of utilizing *in vitro* systems that express both receptor subtypes. Further understanding of the contribution of both the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes to AngII-mediated effects will have important implications in our understanding of the initiation and progression of cardiovascular diseases.

The contribution of the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes to the cellular processes coupled to AngII-mediated cell growth were examined in the porcine coronary artery SMC culture system that was described in Chapter 4. The experimental data presented in this chapter characterized: (i) AngII receptor populations expressed in quiescent PCA SMCs, (ii) the effects of AngII on early and late markers of cellular growth, (iii) the relative contribution

of the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes to AngII-mediated growth and the interaction between these main receptor subtypes, as well as (iv) the contribution of prostaglandin synthesis to AngII-mediated signalling pathways.

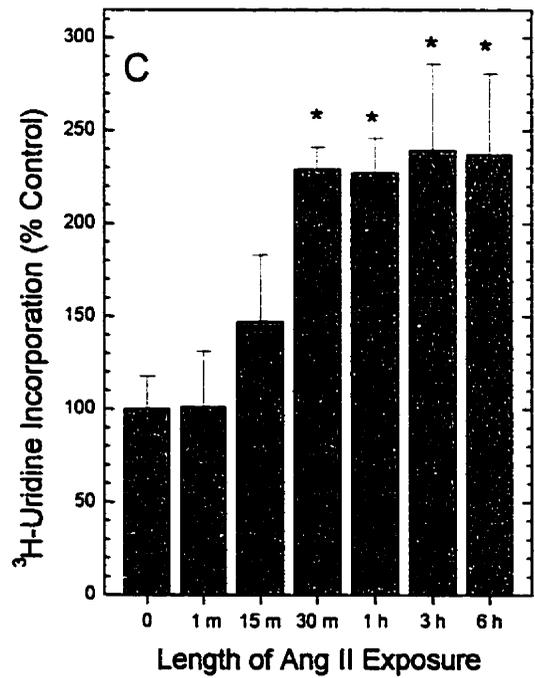
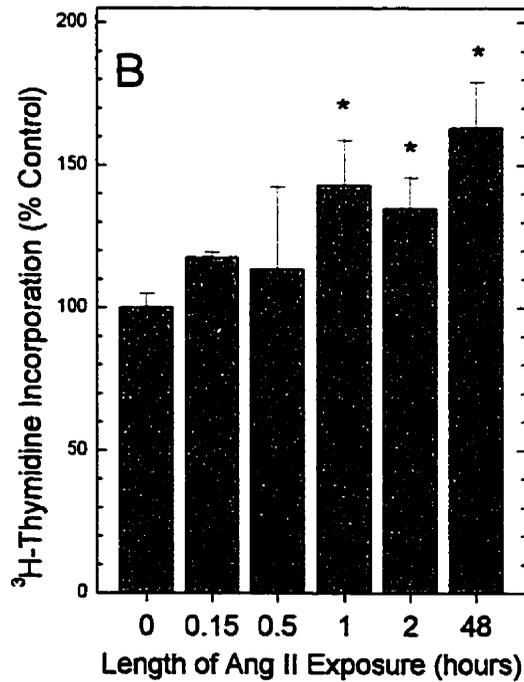
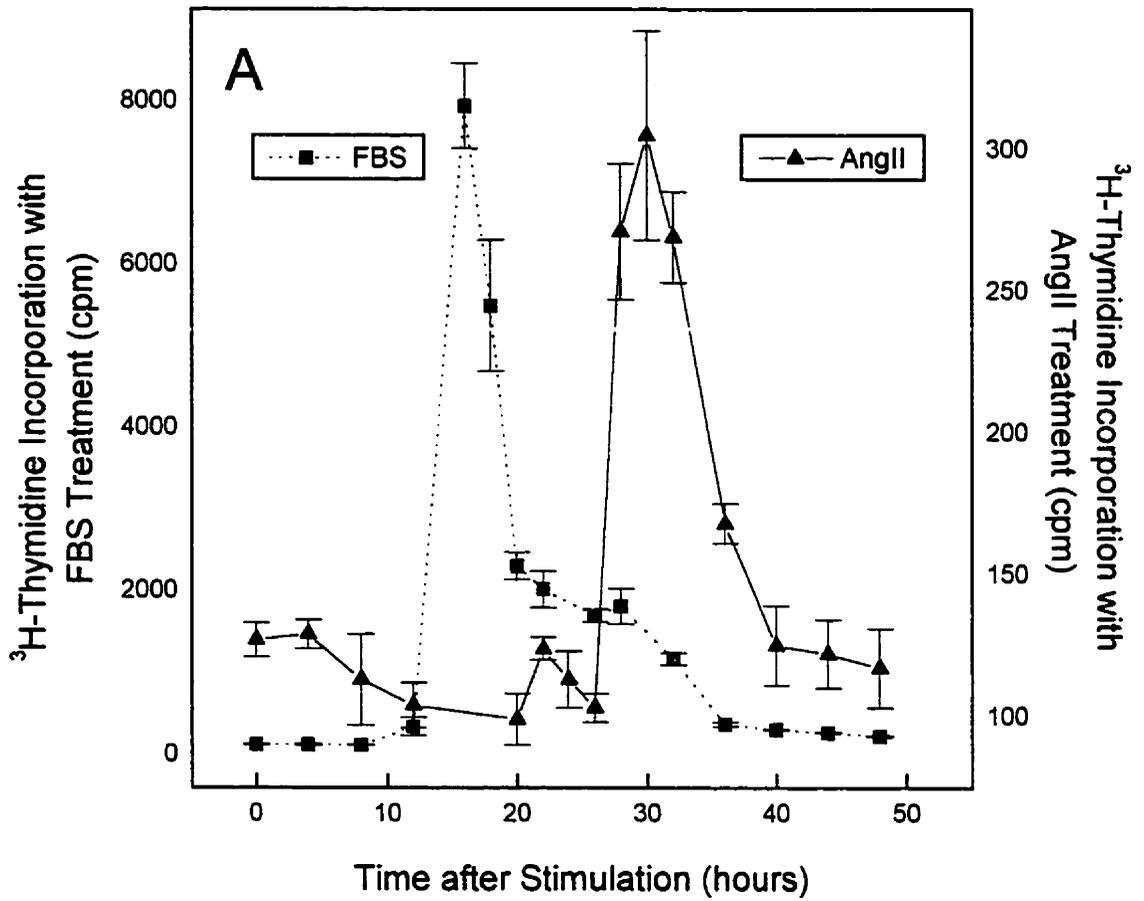
## 5.2 RESULTS

### 5.2.1 Growth Response to AngII in Coronary Artery SMC Cultures.

To characterize the mitogenic response of quiescent PCA SMCs to AngII in comparison to FBS, the rate of DNA synthesis was monitored over an extended period of time (1-48 hours). Based on the amount of [<sup>3</sup>H]-thymidine incorporated during a 30 minute pulse, a time-course for re-entry into the cell cycle was established (Figure 8A). A direct comparison of the growth response to Ang II and the general mitogen FBS was plotted with the common x-axis of time after stimulation and separate y-axes for the amount of [<sup>3</sup>H]-thymidine incorporated. Under the conditions used to establish quiescence, PCA SMCs exhibited a stable level of basal DNA synthesis that significantly increased 28-30 hours following AngII treatment, indicative of a synchronous re-entry into the cell cycle and S phase. In comparison, FBS (20%) treatment of quiescent SMCs resulted in the cells traversing G<sub>0</sub> through S phase within 15-18 hours.

To further characterize the mitogenic response to AngII, we examined the length of AngII exposure required to stimulate a growth response. Quiescent PCA SMCs were exposed to AngII (10<sup>-6</sup> M) for varying periods of time (15 min - 48 hours), after which the AngII was washed out and the level of DNA synthesis was quantified after 48 hours total incubation with [<sup>3</sup>H]-thymidine. As shown in Figure 8B, exposure to AngII for 1 hour was sufficient to increase the rate of DNA synthesis; however, the presence of AngII (and the media that may contain secreted factors) over the entire 48 hour period provided the most significant increase in the rate of DNA synthesis. In comparison, RNA synthesis was monitored as one of the

**FIGURE 8: Mitogenic response of PCA SMCs to AngII.** Panel A: The time course required for quiescent SMCs to traverse G1 and enter S phase after stimulation with AngII in comparison to FBS is shown. The rate of [<sup>3</sup>H]-thymidine incorporation into DNA was measured over a 30 minute period at the indicated time points after stimulation with AngII (10<sup>-6</sup> M)(Right Y axis) in comparison to FBS (20%)(Left Yaxis) as described in "Materials and Methods". The length of exposure to AngII required to stimulate DNA synthesis (Panel B) and RNA synthesis (Panel C) is shown. Panel B: Quiescent SMCs were stimulated with AngII (10<sup>-6</sup> M) for the indicated length of time (0-48 hours) before it was washed out and the total amount of [<sup>3</sup>H]-thymidine incorporated over 48 hours was measured as described in "Materials and Methods. Panel C: Quiescent SMCs were stimulated with AngII (10<sup>-6</sup> M) for the indicated length of time (m=minutes, h=hours) before it was washed out and the total amount of [<sup>3</sup>H]-uridine incorporated over 6 hours was measured. Each data point represents the mean ± SE of at least three independent experiments. Significant differences (\*, p<0.05) are shown relative to time zero conditions.



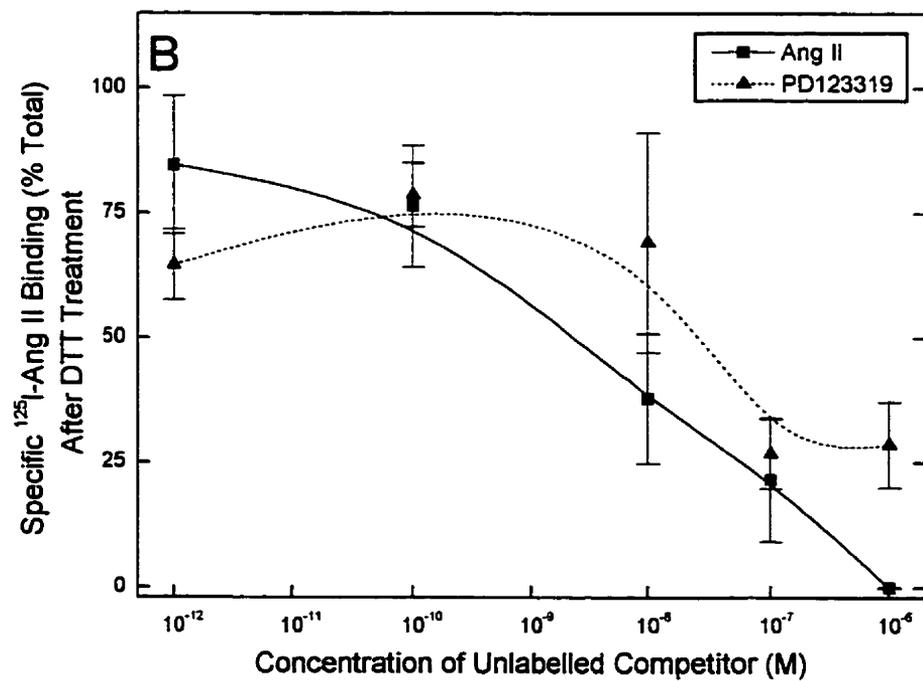
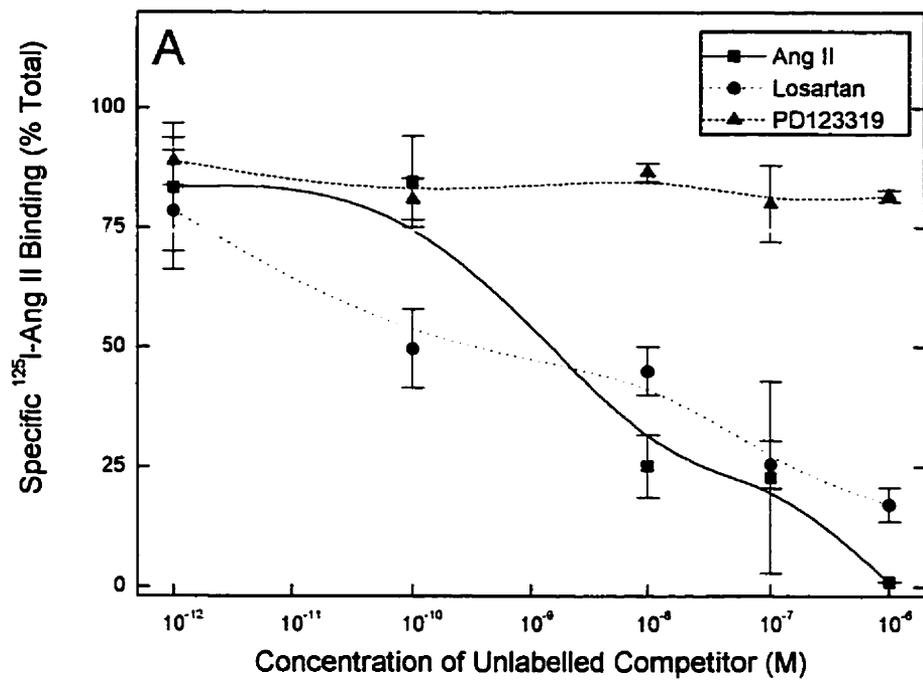
earliest processes activated upon stimulation of cell growth. Due to the relative abundance of rRNA (>80% of total RNA) relative to mRNA, the rate of [<sup>3</sup>H]-uridine incorporation provides an excellent measure of the rate of ribosomal RNA synthesis (Zahradka and Yau, 1994). Quiescent SMCs were stimulated with AngII (10<sup>-6</sup> M) for varying periods of time (1 min - 6 hours), the AngII was washed out and RNA synthesis was quantified after 6 hours. As shown in Figure 8C, AngII rapidly stimulated RNA synthesis with the maximum level of stimulation reached after only 30 min of AngII treatment.

### 5.2.2 Analysis of AngII Receptor Expression in Coronary Artery SMCs.

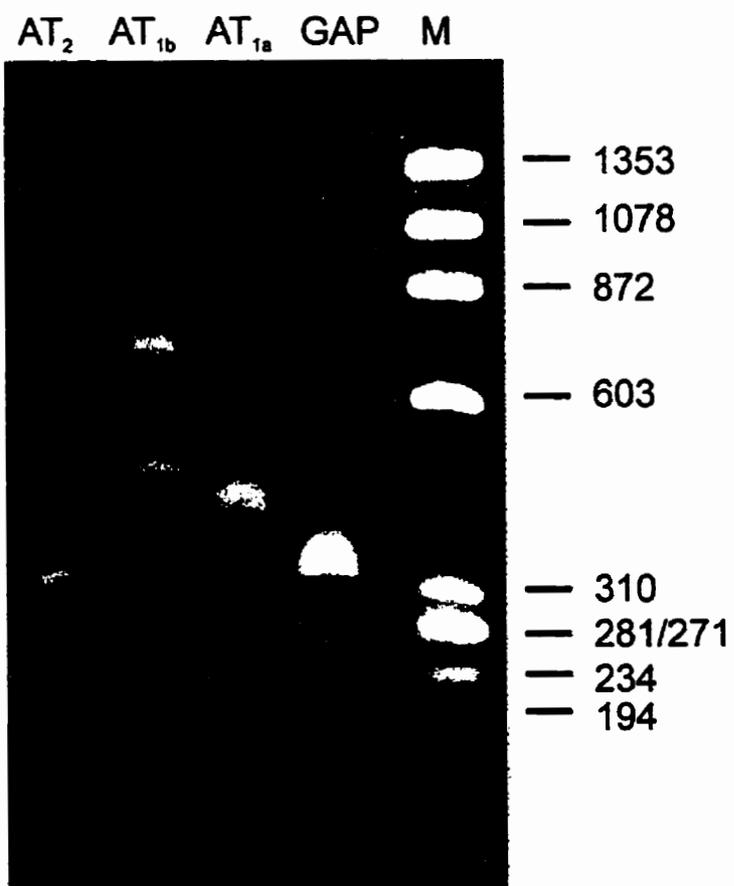
To define the angiotensin receptor subtypes present on quiescent PCA SMCs, binding studies with the AngII analogue, [<sup>125</sup>I]-SarIle AngII, were conducted. The level of non-specific binding was determined by the level of [<sup>125</sup>I]-SarIle AngII bound in the presence of unlabelled AngII (10<sup>-6</sup> M). Under conditions optimized for maximum specific binding, both AngII and losartan, an AT<sub>1</sub> receptor antagonist, displaced [<sup>125</sup>I]-SarIle AngII binding on monolayers of quiescent PCA SMCs in a dose-dependent manner (Figure 9A). The majority of the receptor population consisted of AT<sub>1</sub> receptors based on the ability of losartan to displace 80-85% of the specific binding of [<sup>125</sup>I]-SarIle AngII. Under these conditions, a small population of AT<sub>2</sub> receptors was consistently detected, based on the ability of the AT<sub>2</sub> receptor antagonist PD123319 to displace 15-20% of the specific binding of [<sup>125</sup>I]-SarIle AngII. Treatment with the sulfhydryl-reducing agent DTT (2 mM) reduced binding to the AT<sub>1</sub> receptors and enhanced binding to the AT<sub>2</sub> receptors (data not shown). These conditions facilitated the detection of an AT<sub>2</sub> receptor population on quiescent SMCs based on the ability of PD123319 to displace 80% of [<sup>125</sup>I]-SarIle AngII, in a dose-dependent manner (Figure 9B). Based on this data, the EC<sub>50</sub> of losartan and PD123319 was 1 nM and 1-10 nM, respectively.

To examine the expression of the AngII receptor subtypes, RT-PCR analysis of total

**FIGURE 9: *AngII receptors of PCA SMCs.*** Panel A: The presence of AngII receptors on quiescent porcine coronary artery SMCs was examined by [<sup>125</sup>I]-SarIle AngII binding studies as described in "Materials and Methods. The AT<sub>1</sub> and AT<sub>2</sub> receptor subtype populations were defined by the ability of non-peptide receptor antagonists losartan and PD123319 (10<sup>-5</sup>-10<sup>-12</sup>M), respectively, to displace the specific binding of [<sup>125</sup>I]-SarIle AngII in comparison to unlabelled AngII. Each data point represents the average of 8 independent experiments expressed as mean ± SE. Panel B: Following incubation with 2m M DTT to inactivate binding to AT<sub>1</sub> receptors and enhance binding to AT<sub>2</sub> receptors, the efficacy of the AT<sub>2</sub> receptor antagonist, PD123319 (10<sup>-5</sup>-10<sup>-12</sup>M), to displace [<sup>125</sup>I]-SarIle AngII binding is shown in comparison to unlabelled AngII. Each data point represents the mean ± SE of 7 independent experiments. In all panels, the value of the non-specific binding was set at the value obtained in the presence of 10<sup>-6</sup> M unlabelled AngII. Panel C: RT-PCR analysis of the expression of AngII receptor subtypes AT<sub>2</sub> (lane 1), AT<sub>1b</sub> (lane 2) and AT<sub>1a</sub> (lane 3) with respect to GAPDH (lane 4) in total RNA extracted from quiescent PCA SMCs. DNA size markers (*Hae*III digested φX174) are shown in lane 5 (M).



C



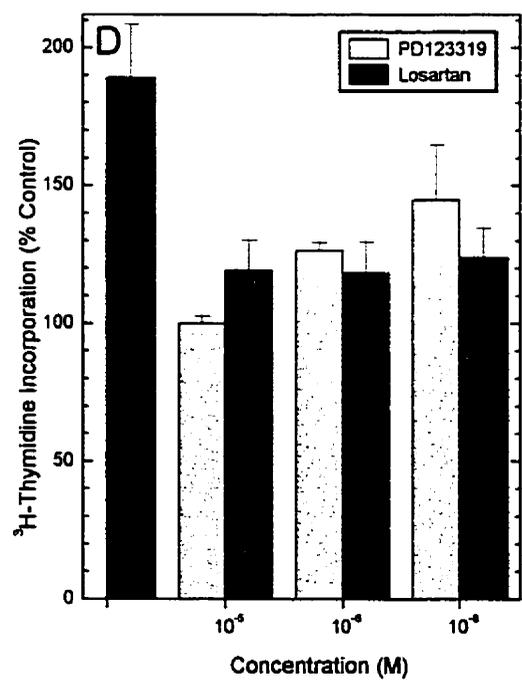
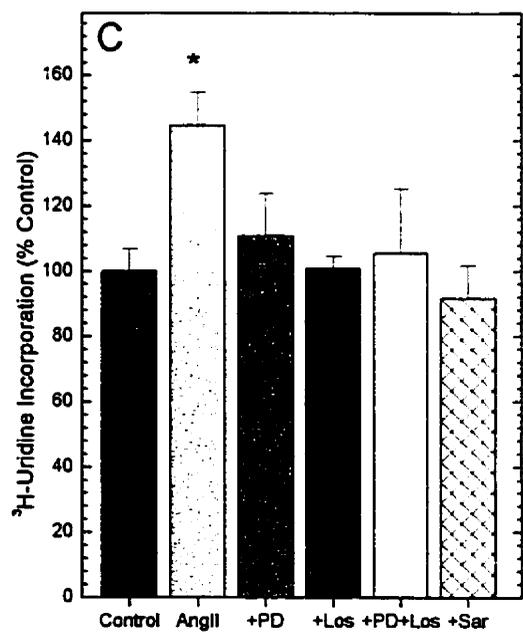
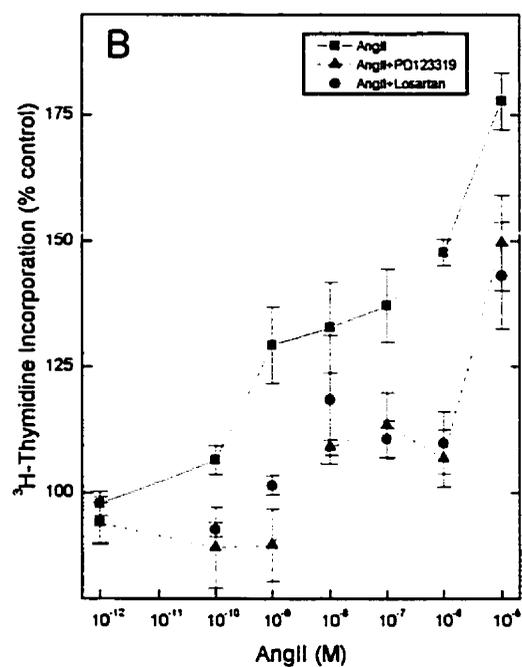
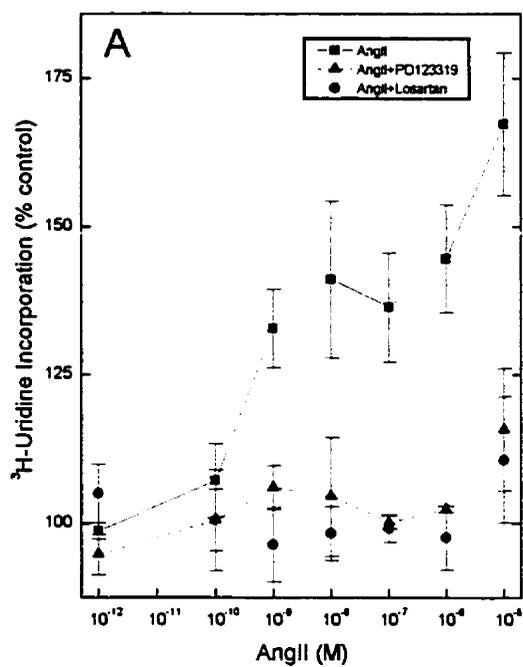
RNA was employed. In quiescent SMCs, mRNA corresponding to the AT<sub>1A</sub>, AT<sub>1B</sub> and AT<sub>2</sub> receptors was detected, and relative band intensities compared relative to the product obtained upon concurrent amplification of GAPDH mRNA (Figure 9C). Negative and positive control lanes were included for each primer set to confirm their specificity and the quality of the RNA (data not shown). The primers to AT<sub>1B</sub> receptor sequence consistently produced multiple bands of specific sizes in several experiments which may indicate some heterogeneity within this receptor class or reflect sequence differences between the porcine and rodent mRNA. It is interesting to note that the same primers for the AT<sub>1B</sub> receptor produced a single band of the expected size in rat aortic SMCs (Saward and Zahradka, 1996; Chapter 7).

### **5.2.3 Contribution of the AT<sub>1</sub> and AT<sub>2</sub> receptors to AngII-stimulated SMC Growth.**

Many studies have attributed AngII's growth effects to AT<sub>1</sub> receptor activation in cell types that do not express the AT<sub>2</sub> receptor. The presence of both AT<sub>1</sub> and AT<sub>2</sub> receptor makes the PCA SMCs an ideal system to evaluate the relative contribution of each receptor subtype to AngII-mediated cell growth. In this study, treatment with non-peptide receptor antagonists for the AT<sub>1</sub> (losartan) and AT<sub>2</sub> (PD123319) receptors was used to selectively block each receptor subtype.

To define the receptor subtype contribution to the early growth response to AngII treatment, the level of RNA synthesis was assessed by the amount of [<sup>3</sup>H]-uridine incorporated over a 6 hour period following AngII treatment of quiescent SMCs. AngII (10<sup>-5</sup>-10<sup>-12</sup> M) stimulation resulted in a significant increase in the level of RNA synthesis (max 167%±14) that could be effectively blocked by either AT<sub>1</sub> or AT<sub>2</sub> receptor antagonists (10<sup>-5</sup> M), losartan or PD123319 (Figure 10A). A standard assay time of 6 hours of [<sup>3</sup>H]-uridine incorporation was employed in all experiments during which the rate of RNA synthesis remained linear (data not shown).

**FIGURE 10: Contribution of the  $AT_1$  and  $AT_2$  receptors to RNA and DNA synthesis.** In Panel A, total RNA synthesis was measured by the rate of [ $^3$ H]-uridine incorporation over a 6 hour time period following stimulation of quiescent SMCs with AngII ( $10^{-5}$ - $10^{-12}$  M) alone or in the presence of either the  $AT_1$  receptor antagonist losartan ( $10^{-5}$  M) or the  $AT_2$  receptor antagonist PD123319 ( $10^{-5}$  M). In Panel B, total DNA synthesis was measured by the total [ $^3$ H]-thymidine incorporation over 72 hours following stimulation of quiescent SMCs with AngII ( $10^{-5}$ - $10^{-12}$  M) alone or in the presence of losartan or PD123319. Each data point in Panel A & B represents the mean  $\pm$  SE of 7 independent experiments. In Panel C, the inhibitory effects of PD123319 ( $10^{-5}$  M), losartan ( $10^{-5}$  M) or both on AngII-stimulated [ $^3$ H]-uridine incorporation over a 6 hours was compared to saralasin ( $10^{-5}$  M). Panel D: The dose-dependent inhibition of DNA synthesis by losartan or PD123319 ( $10^{-5}$ - $10^{-12}$  M) was examined by measuring [ $^3$ H]-thymidine incorporation over 72 hours following stimulation of quiescent SMCs with AngII ( $10^{-6}$  M). Each data point in Panel C & D represents the mean  $\pm$  SE of at least three independent experiments. In all panels, the value of the unstimulated control was set at 100% and used for the determination of statistical difference (\*,  $p < 0.05$ ).



DNA synthesis represents another key parameter of cellular growth that reflects S phase completion in both the hypertrophic and hyperplastic growth response. To provide additional evidence for the involvement of both receptor subtypes in the SMC response to AngII, the overall level of DNA synthesis was monitored by the level of [<sup>3</sup>H]-thymidine incorporation over a 72 hour period. In agreement with the results obtained with the RNA synthesis experiment, the ability of AngII ( $10^{-5}$ - $10^{-12}$  M) to stimulate DNA synthesis (max  $178\% \pm 6$ ) was effectively prevented by either losartan or PD123319 ( $10^{-5}$  M) (Figure 10B). At equimolar concentrations of AngII and receptor antagonists ( $10^{-5}$  M), a partial block of DNA synthesis was observed.

In addition, the efficacy of the receptor antagonists in inhibiting AngII-mediated growth was examined. As shown in Figure 10C, AngII-stimulated RNA synthesis was inhibited with equal efficacy by losartan and PD123319 ( $10^{-5}$  M), individually or in combination, as well as saralasin, a non-specific peptide antagonist. Combined treatment with both AT<sub>1</sub> and AT<sub>2</sub> receptor antagonists was not more effective than either antagonist alone which supports the concept that both pathways are mediating the same biological response. To further examine the effective concentration of receptor antagonists necessary for inhibition of AngII-mediated growth, the level of DNA synthesis was examined after treatment with AngII ( $10^{-6}$  M) in the presence of varying concentrations ( $10^{-5}$ - $10^{-8}$  M) of PD123319 and losartan (Figure 10D). The higher concentrations of PD123319 and losartan at 10-molar excess ( $10^{-5}$  M) completely blocked AngII-stimulated DNA synthesis. The lack of a dose-dependent curve for losartan inhibition may reflect a pseudo-irreversible or insurmountable binding to the AT<sub>1</sub> receptor as previously reported (refer to Section 1.3.2.1, Chapter 1). This concept does not conflict with the competitive binding studies (Figure 9A) since only functional studies can distinguish between insurmountable and surmountable AT<sub>1</sub> antagonism (Panek et al., 1995).

**FIGURE 11: *Time-course of PCNA Expression in AngII-treated SMCs.*** Quiescent SMCs were treated with AngII ( $10^{-6}$  M) and prepared on glass slides as outlined in "Materials and Methods". Using indirect immunofluorescent microscopy, the timecourse of PCNA expression was examined following 12 hours (Panel A), 16 hours (Panel B), 20 hours (Panel C) and 24 hours (Panel D) of AngII ( $10^{-6}$  M) treatment of quiescent SMCs. The results shown were magnified at 33 $\times$  and represent the pattern consistently observed in 3 independent experiments.



#### **5.2.4 PCNA and DNA Synthesis in AngII-responsive SMC Populations.**

Proliferating cell nuclear antigen (PCNA), an accessory molecule to DNA polymerase  $\delta$ , is transiently located in the nucleus of growing cells during DNA synthesis and thus provides an excellent marker for early S phase. Immunofluorescent microscopy was used to monitor PCNA expression in individual cells and define the proportion of quiescent SMCs responsive to AngII stimulation. Quiescent unstimulated SMCs exhibited minimal and diffuse staining for PCNA (Figure 11A). Following Ang II ( $10^{-6}$  M) treatment of quiescent SMCs, increased levels of PCNA staining were evident with a punctate pattern of PCNA within the nucleus of most SMCs within 16 hours after AngII stimulation (Figure 11B). The presence of PCNA was sustained in the nucleus for at least 24 hours following AngII treatment (Figure 11C,D). Blockade of either the AT<sub>1</sub> or AT<sub>2</sub> receptor subtype effectively prevented the induction of PCNA in the majority of SMC populations as shown by the ability of both losartan (Figure 12C) and PD123319 (Figure 12D)( $10^{-5}$  M) to prevent the induction of PCNA expression by AngII ( $10^{-6}$  M)(Figure 12B) in comparison to untreated cells (Figure 12A).

Another assessment of the response to AngII stimulation was obtained by monitoring incorporation of the DNA analogue, bromodeoxyuridine (BrdU). Following a 72 hour incubation with AngII ( $10^{-6}$  M) in the presence of BrdU, newly synthesized DNA could be visualized in the majority of SMCs (Figure 13). Pretreatment with losartan or PD123319 ( $10^{-5}$  M) effectively prevented AngII-stimulated BrdU labelling of all SMCs. It should be noted that no agonistic effects for losartan or PD123319 treatment alone were observed with the growth assays we used (data not shown).

#### **5.2.5 AngII Receptor Subtype Contribution to AngII-mediated Cellular Hyperplasia.**

Cellular growth can occur with the different endpoints of hypertrophy or hyperplasia, as defined by increased cell size in the absence of cell division or increased cell number,

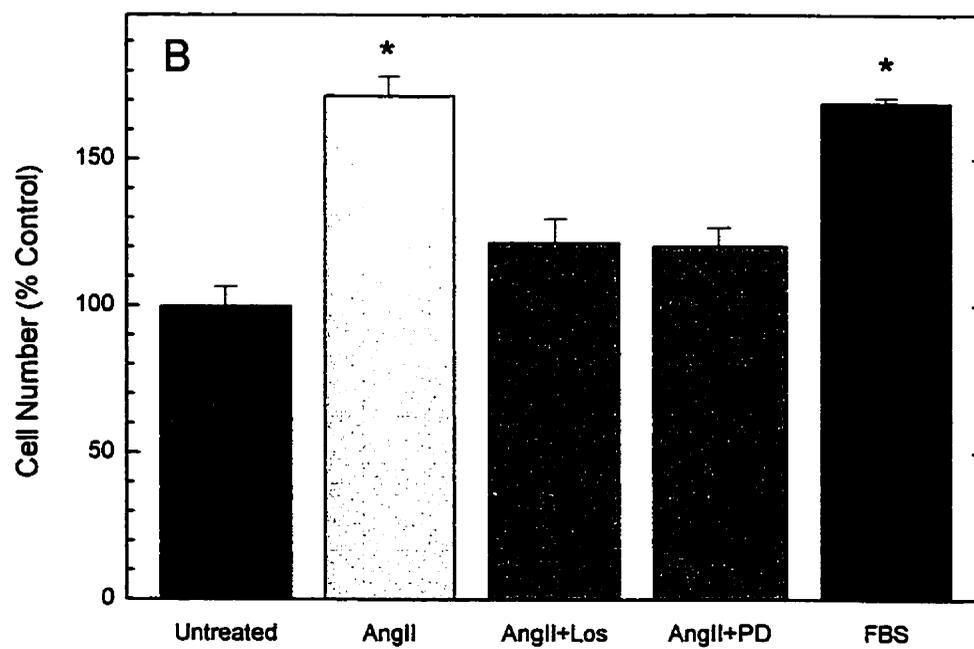
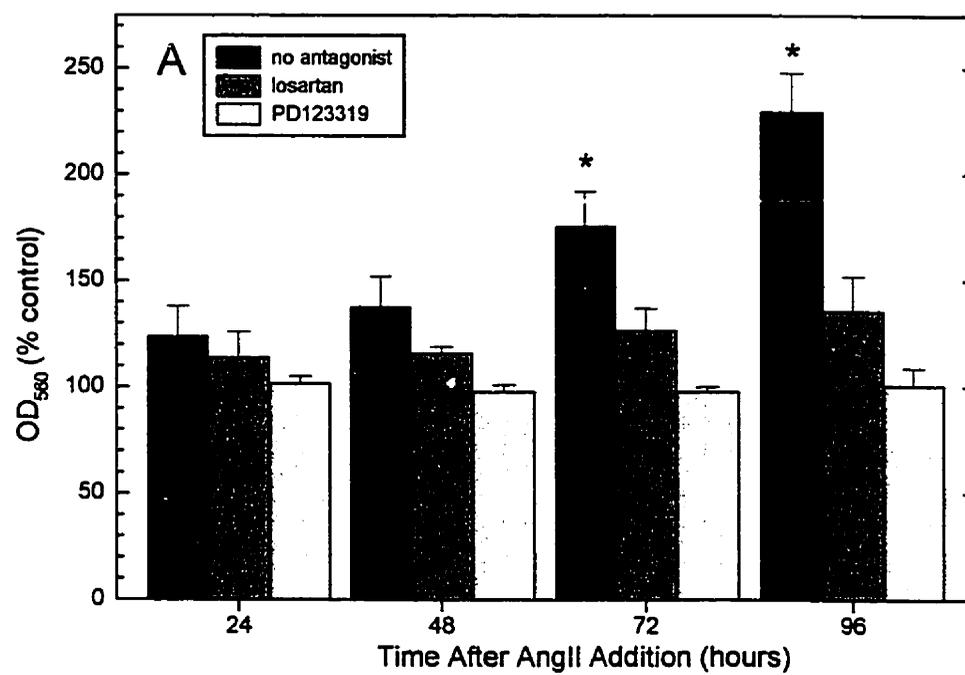
**FIGURE 12: *AT<sub>1</sub>*- and *AT<sub>2</sub>*-mediated PCNA Expression in SMCs.** Quiescent SMCs were treated with AngII ( $10^{-6}$  M) and prepared on glass slides as outlined in "Materials and Methods". PCNA was examined by indirect immunofluorescent microscopy at 33× magnification. The nuclear pattern of PCNA localization is shown in quiescent SMCs (A) in comparison to SMCs treated with AngII ( $10^{-6}$  M) for 20 hours (C). The ability of losartan (B) and PD123319 (D) ( $10^{-5}$ M) to prevent AngII-stimulated PCNA expression is also demonstrated. These results are representative of the pattern consistently observed in 4 independent experiments.



**FIGURE 13: *AngII stimulation of DNA synthesis in SMCs.*** Quiescent SMCs, cultured on glass slides, were treated with AngII ( $10^{-6}$  M) and prepared as outlined in "Materials and Methods". The number of quiescent SMCs responding to AngII stimulation was visualized using indirect immunofluorescent microscopy at  $16\times$  magnification. Following a 72 hour incubation with AngII ( $10^{-6}$  M) in the presence of bromodeoxyuridine, DNA synthesis was induced (B.) compared to control (A.). The ability of the  $AT_1$  and  $AT_2$  receptor antagonists losartan (C.) and PD123319 (D.) ( $10^{-5}$  M), respectively, to prevent the stimulation of DNA synthesis by AngII ( $10^{-6}$  M) is shown. The results shown represent the pattern consistently observed in 4 independent experiments



**FIGURE 14: *AngII Receptor Subtype Contribution to SMC Hyperplasia.*** The MTT assay provides a quantitative analysis of the overall cellular growth response based on the colorimetric assay for MTT reduction by active mitochondria. Panel A illustrates the effect of AngII ( $10^{-6}$  M) stimulation of quiescent PCA SMCs over 96 hours in the absence or presence of losartan or PD123319 ( $10^{-5}$ M). Panel B shows the increase in total cell number following a 96 hour AngII ( $10^{-6}$  M) treatment of quiescent PCA SMCs in comparison to FBS (5%) treatment. The effect of the receptor antagonists losartan and PD123319 ( $10^{-5}$ M) in conjunction with AngII ( $10^{-6}$  M) treatment on cell number is also shown. Each data point represents the mean  $\pm$  SE of at least three separate experiments. In all panels, the value of unstimulated SMCs was set to 100% and used for the determination of statistical differences (\*,  $p < 0.05$ ).



respectively. Although both endpoints have been reported in response to AngII for a variety of cell types, the majority of studies with rodent SMCs have shown a hypertrophic growth response (Koibuchi et al., 1993; Millet et al., 1992; Chui et al., 1991b). To evaluate the growth response to AngII in PCA SMCs, the MTT assay was used. The MTT assay is commonly employed as a quantitative assay for cell growth, cell viability, and apoptosis that is based on the level of reduction of the tetrazolium salt MTT by  $\text{NAD}^+$ -dependent lactate dehydrogenase reaction in active mitochondria (Shi et al., 1993). Treatment of quiescent PCA SMCs with AngII ( $10^{-6}$  M) resulted in an increase in reduced MTT over 96 hours ( $230\% \pm 12$ ) which could be effectively blocked by  $10^{-5}$  M losartan or PD123319 (Figure 14A). It should be noted that control (untreated) cells exhibited a stable values in the MTT assay over the entire 96 hour time period and the inclusion of either  $\text{AT}_1$  or  $\text{AT}_2$  receptor antagonist did not result in a inhibition of MTT values below control values, which implies these conditions did not affect the viability of the cells or induce apoptosis. Also, no additive effect of cotreatment with losartan and PD123319 was observed in this assay (data not shown). In addition, a parallel assessment of cell number after stimulation with AngII in comparison to FBS. AngII ( $10^{-6}$  M) treatment of quiescent PCA SMCs resulted in a significant increase in cell number over 96 hours that was comparable to FBS (5%) treatment (Figure 14B). Pretreatment with either losartan or PD123319 ( $10^{-5}$  M) effectively inhibited the hyperplastic response to AngII. The cell number was stable in the quiescent untreated controls over the 96 hour period, which further supports the conclusion that apoptosis is not stimulated under these conditions. These data provide conclusive evidence that AngII initiates a mitogenic response in PCA SMCs which is sensitive to antagonism of either the  $\text{AT}_1$  or  $\text{AT}_2$  receptor.

To evaluate the specificity of the growth-inhibitory effect of losartan and PD123319, the SMC growth response to both insulin and FBS in the presence of the  $\text{AT}_1$  or  $\text{AT}_2$  receptor

**TABLE 2: Specificity of AngII Receptor Antagonists.**

Time after Stimulation (hours)	Stimulus	Relative Cell Growth (% control)		
		none	losartan	PD123319
24	none	100	111.1±17.0	79.4±1.1
	insulin	129.6±2.0	110.4±11.5	121.7±3.0
	FBS	344.4±13.5	368.2±31.0	320.6±17.5
72	none	100	92.1±2.4	111.1±4.5
	insulin	173.0±15.1	145.2±6.5	158.3±4.5
	FBS	409.5±43.8	403.2±66.7	400.0±25.4

Quiescent SMCs prepared in 96-well dishes were treated with insulin ( $10^{-6}$  M) or FBS (10%) in the absence or presence of losartan or PD123319 ( $10^{-5}$  M). At the indicated times, cell growth was measured with the MTT assay. Each value represents the optical density at 560 nm relative to the unstimulated control which was set to 100%. The data are presented as means  $\pm$  SE of at least three independent experiments.

antagonists was examined using the MTT assay (Table 2). Treatment of quiescent PCA SMCs with insulin ( $10^{-6}$  M) resulted in a significant increase in reduced MTT ( $173\% \pm 15$ ) over 72 hours that was not significantly inhibited by either losartan or PD123319 ( $10^{-5}$  M). Similarly, FBS (10%) stimulated a significant increase in reduced MTT ( $410\% \pm 44$ ) over 72 hours that was not affected by either losartan or PD122319 ( $10^{-5}$  M). Based on these data, neither of the  $AT_1$  or  $AT_2$  receptor antagonists produced a non-specific inhibition of the growth response to mitogens other than AngII.

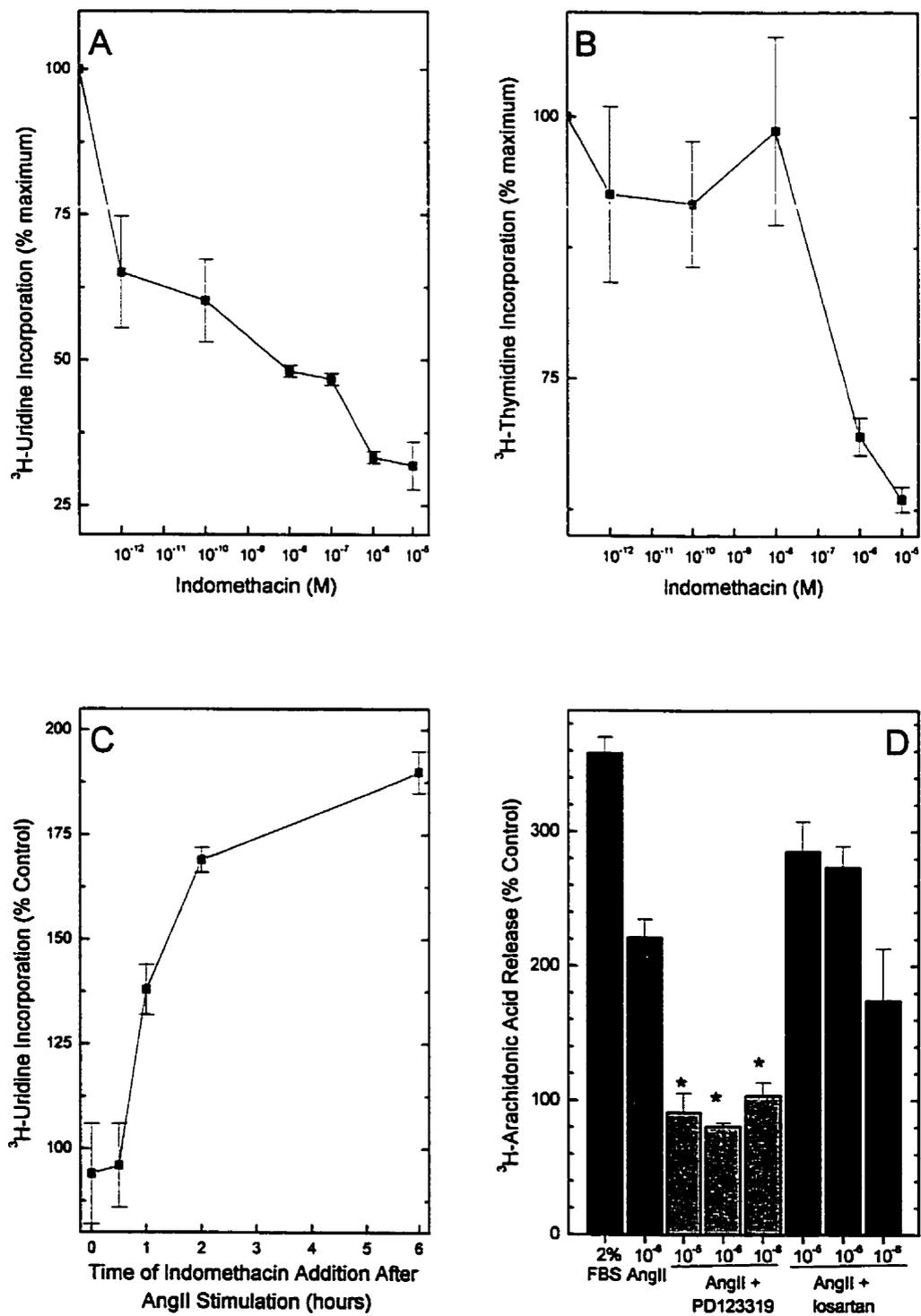
### **5.2.6 Prostaglandin Synthesis in AngII-mediated Signalling Pathways.**

The delayed mitogenic response obtained with AngII relative to FBS suggests secondary growth factors may contribute to part of the growth stimulatory effects of AngII. Since the AngII washout study supports this concept, we considered the possibility that a secreted agent was required as an intermediary. Previously, AngII has been reported to increase the synthesis of arachidonic acid derivatives such as prostaglandins and leukotrienes via activation of the cyclo-oxygenase and lipoxygenase pathways in SMCs (Jaiswal et al., 1993a; Natarajan et al., 1994a). Since these compounds may influence AngII-mediated pathophysiological processes in the vasculature (Jaiswal et al., 1993b; Chatziatoniou and Arendshorst, 1991), the contribution of prostanoid production to the mitogenic effects of AngII was examined.

To investigate the role of prostaglandin synthesis in AngII-stimulated cell growth, the effect of varying concentrations of indomethacin, a general cyclo-oxygenase inhibitor, was assessed in AngII-mediated RNA and DNA synthesis. As shown in Figure 15A, indomethacin effectively inhibited AngII ( $10^{-6}$  M)-stimulated RNA synthesis over a wide range of concentrations ( $10^{-12}$ - $10^{-5}$  M). Similarly, the ability of AngII ( $10^{-6}$  M) to stimulate DNA synthesis was effectively prevented by indomethacin at higher concentrations ( $10^{-6}$ - $10^{-5}$  M) (Figure 15B). In all experiments, AngII-treated cells were incubated with 0.1% ethanol

**FIGURE 15: Contribution of Prostaglandin Synthesis to AngII-stimulated SMC Growth.**

The effect of varying concentrations of the general cyclo-oxygenase inhibitor, indomethacin ( $10^{-5}$ - $10^{-14}$  M), on AngII-stimulated ( $10^{-6}$  M) RNA synthesis (panel A) and DNA synthesis (panel B). Panel C: The effect of indomethacin ( $10^{-5}$  M) addition on RNA synthesis at various times after AngII ( $10^{-6}$  M) stimulation was evaluated. The rate of RNA synthesis was measured by the amount of [ $^3$ H]-uridine incorporated over a 6 hour period. In all panels, the value of the AngII-stimulated control was set at 100%. Panel D: The effectiveness of the AngII receptor antagonists losartan and PD123319 in preventing AngII-mediated release of [ $^3$ H]-arachidonic acid was evaluated. Quiescent SMCs, prelabelled with [ $^3$ H]-arachidonic acid, were stimulated with FBS (2%) or AngII ( $10^{-6}$  M)  $\pm$  losartan or PD123319 ( $10^{-5}$  M) for 4 hours. Each data point represents the mean  $\pm$  SE of at least three separate experiments for all panels. Significant differences (\*,  $p < 0.05$ ) are shown in comparison to AngII stimulated condition.



to control for any vehicle effects of ethanol. No cytotoxicity was evident in cells exposed to indomethacin, even during extended incubation times, based on cellular morphology and the MTT assay (data not shown).

To determine the relative importance of prostaglandin synthesis in the early and late growth events stimulated by AngII, a delayed addition experiment was completed. At varying times following stimulation of quiescent SMCs with AngII ( $10^{-6}$  M), indomethacin ( $10^{-5}$  M) was added and the total amount of [ $^3$ H]-uridine incorporated in 6 hours was measured (Figure 15C). The total level of RNA synthesis stimulated by AngII, in the absence of any inhibition by indomethacin, is shown at 6 hour time point ( $188\% \pm 5$ ). The inclusion of indomethacin for the entire assay period, or 0.5 hours after AngII stimulation, fully inhibited the AngII-mediated increase in RNA synthesis ( $96\% \pm 10$ ). However, the inhibitory capacity of indomethacin on AngII-stimulated RNA synthesis was reduced 1-2 hours after addition of the stimulus. These data implicate prostaglandin synthesis in the early signalling events leading to cell growth by AngII.

To examine the involvement of AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes in the AngII-mediated prostanoid pathway, AngII-dependent stimulation of [ $^3$ H]-arachidonic acid release in the presence of losartan and PD123319 was examined. As shown in Figure 15D, AngII ( $10^{-6}$  M) stimulated significant levels of [ $^3$ H]-arachidonic acid release over 4 hours ( $200\% \pm 12$ ). This response was blocked by the AT<sub>2</sub> receptor antagonist PD123319 ( $10^{-5}$ - $10^{-8}$  M). In comparison, the AT<sub>1</sub> receptor antagonist had no inhibitory effect on the level of arachidonic acid released by AngII treatment. Although no direct agonistic effect of losartan was observed (data not shown), treatment with losartan in conjunction with AngII actually increased the levels of arachidonic acid released which further implicates AT<sub>2</sub> receptor activation in this process.

## 5.3 DISCUSSION

### 5.3.1 PCA SMC Model for AngII-stimulated Cell Growth

Characterization of PCA SMC cultures had previously shown they retained the capacity for reversible phenotypic modulation, since they can enter a quiescent state after prolonged serum withdrawal (4-5 days), but maintain their proliferative response to growth factors (Saward and Zahradka, 1997a; Chapter 4). In this SMC culture system, AngII acts as a mitogen that results in the stimulation of rRNA synthesis and a synchronous re-entry into the cell cycle. It should be noted that the mitogenic capacity of AngII was not as strong as FBS, as demonstrated by the delayed entry into S phase at 28-32 hours in comparison to 15-18 hours after FBS stimulation. In other cell cultures, a delayed mitogenic response has been correlated with the synthesis and/or secretion of secondary factors such as epiregulin, PDGF, TGF $\beta$  and bFGF that participate in the overall growth response (Taylor et al., 1999; Delafontaine, 1998; Koibuchi et al., 1993). Consistent with this concept, maximal levels of DNA synthesis were stimulated in wash-out studies with the prolonged presence of AngII and the media which may contain secreted factors. Further studies have identified PGE<sub>2</sub> as a key autocrine growth factor in this SMC system (Yau et al., unpublished observations).

### 5.3.2 Expression of AngII Receptor Subtypes in PCA SMCs

One key characteristic of PCA SMC cultures demonstrated in this study was the *in vitro* expression of the two main subtypes of receptors for AngII, AT<sub>1</sub> and AT<sub>2</sub>. The relative density of AngII receptors expressed in quiescent PCA SMCs was examined by two independent approaches (Figure 9). Using the non-peptide receptor antagonists losartan and PD123319 to effectively block the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes, respectively, the active cell-surface receptor populations were quantified by [<sup>125</sup>I]-SarIle AngII binding studies. The majority of AngII receptors on quiescent PCA SMCs were AT<sub>1</sub> receptors (80-85% of specific binding), with a minor component of AT<sub>2</sub> receptors (15-20% of specific binding) that could

be more easily detected following treatment with DTT (80% of specific binding). Due to the lack of an antibody to recognize the AT<sub>2</sub> receptor at the time of this study, semi-quantitative RT-PCR was used to confirm the receptor subtypes expressed in these cultures. In agreement with the binding studies, quiescent PCA SMCs did express the AT<sub>2</sub> receptor subtype in addition to the AT<sub>1A</sub> and AT<sub>1B</sub> receptor subtypes. Follow-up work done in this laboratory has confirmed the presence of both AT<sub>1</sub> and AT<sub>2</sub> receptors subtypes in quiescent PCA SMCs using Western blot analysis (Zahradka et al., 1998) and immunofluorescent microscopy that identified both AT<sub>1</sub> and AT<sub>2</sub> receptors distributed uniformly in the plasma membranes of quiescent SMCs (Saward et al., submitted). Similar analysis of human radial arterial SMCs has demonstrated the *in vitro* pattern of expression and localization of both AT<sub>1</sub> and AT<sub>2</sub> is parallel to PCA SMCs (Saward et al., submitted). Based on the results of these complementary experiments, it can be concluded that PCA SMCs express both of the major AngII receptor subtypes *in vitro* and, as a result, this culture system is ideal for studying the interplay and relative contribution of each receptor subtype to cell growth.

Several factors may account for the presence and function of AT<sub>2</sub> receptors in our SMC cultures. It has been reported that the length of time in serum-free media correlates with AT<sub>2</sub> receptor expression by rat aortic SMCs (Kambayashi et al., 1996a) and extended serum deprivation enhances smooth muscle differentiation and reacquisition of the ability to contract (Halayko et al., 1999; Ma et al., 1998). Thus, the conditions used to ensure our SMCs become quiescent (supplemented serum-free media with insulin for 5-7 days), in contrast to the typical 24 hour serum withdrawal employed in many published studies, may be a contributing factor to AT<sub>2</sub> receptor function in these SMC cultures. Although we have demonstrated AT<sub>2</sub> receptors are present in both growing and quiescent populations of both porcine and human SMCs (Saward et al., submitted), the extended differentiation time may also affect the intracellular environment and the receptor coupling to signalling pathways.

A similar concept has been proposed in studies by Liakos et al. (1997) and Tian et al. (1995) that demonstrated a prolonged differentiation period can alter the signalling pathways responsive to AngII receptors in bovine adrenal cells. Alternatively, our use of only second passage SMCs may ensure that extensive dedifferentiation and downregulation of AT<sub>2</sub> receptors has not occurred. Another possibility is that the explant culture method preferentially selects for a subpopulation of SMCs capable of migration and proliferation that expresses the AT<sub>2</sub> receptor at high levels.

### **5.3.3 Both AT<sub>1</sub> and AT<sub>2</sub> Receptors Mediate SMC Growth**

A variety of cell growth assays were employed to provide a comprehensive analysis of the SMC response to AngII as well as insight into the relative contribution of the AngII receptor subtypes. The mitogenic response to AngII involved a dose-dependent increase in the rate of RNA and DNA synthesis within 0.5-1 hour of exposure (Figure 8 & 10). To characterize the growth response to AngII as hypertrophic or hyperplastic, both the MTT assay and cell counts were used (Figure 14). These data clearly indicate that AngII treatment resulted in a mitogenic response involving cell division, since no evidence of cell death or apoptosis was observed in any of these assays. In all assays for early and late growth events, both the AT<sub>1</sub> and AT<sub>2</sub> receptors mediated the mitogenic response to AngII based on the effects obtained with the selective non-peptide receptor antagonists, losartan and PD123319. It is important to note that co-treatment with losartan and PD123319 had the same growth inhibitory effect of either antagonist alone, which further implicates the signalling pathways mediated by both receptors are directly involved in AngII-mediated growth. Immunofluorescent microscopy demonstrated that the majority of PCA SMCs responded to AngII treatment with the induction of nuclear PCNA and incorporation of BrdU during S phase DNA synthesis and the growth response could be blocked in the entire population with either losartan or PD123319 (Figure 11-13). These data support the important finding that

the AT<sub>2</sub> receptor is positively coupled to growth of SMCs and confirm the previously established link between the AT<sub>1</sub> receptor and these endpoints.

#### 5.3.4 AT<sub>2</sub> Receptor Stimulates SMC Growth

It is significant that in PCA SMCs which express both the AT<sub>1</sub> and AT<sub>2</sub> receptors, the AT<sub>2</sub> receptor is clearly linked to the induction of hyperplastic growth by AngII. These results support the report by this laboratory that neointimal formation in porcine coronary artery rings is inhibited by PD123319 following balloon-catheter injury *in vitro* (Wilson et al., 1999), in addition to corroborating the *in vivo* role for the AT<sub>2</sub> receptor in vascular hypertrophy reported in both normotensive (Sabri et al., 1997; Levy et al., 1996; Janiak et al., 1992) and spontaneously hypertensive (Otsuka et al., 1998) rats. Although vascular AngII receptors were initially believed to be exclusively AT<sub>1</sub>, subsequent research supports the concept that adult aorta express a small but significant population of AT<sub>2</sub> receptors (Viswanathan et al., 1991; Chang and Lotti, 1991) that are upregulated following injury and disease processes (Hutchinson et al., 1999; Kambayashi et al., 1996; Panek et al., 1992). A key role for the AT<sub>2</sub> receptor in cardiovascular pathophysiology is indicated by several studies that correlated enhanced AT<sub>2</sub> receptor expression or activation with cardiovascular disease states such as diabetes (Sechi et al., 1994), post-myocardial infarction (Nio et al., 1995; Kuizinga et al., 1998), ischemia (Wiemer et al., 1993), hypertension (Wu et al., 1994), heart failure (Rogg et al., 1996) and cardiac hypertrophy due to pressure-overload (Lopez et al., 1994) or AV-shunt (Iwai et al., 1995; Pieruzzi et al., 1995). Previously, a direct link between the AT<sub>2</sub> receptor and cell growth has been demonstrated in several different cell types (refer to Section 1.3.4.2; Li JY et al., 1999; Kuizinga et al., 1998; Kunert-Radek et al., 1994; Chen et al., 1993; Jaiswal et al., 1991b). Thus, further clarification of the role of the AT<sub>2</sub> receptor in the biological effects of AngII is necessary.

### 5.3.5 Effective Concentrations of AngII and Receptor Antagonists

In these growth assays, high concentrations of AngII ( $10^{-5}$ ,  $10^{-6}$  M) stimulated maximum growth. The high concentration of AngII required to consistently mediate a growth response in these PCA SMCs likely reflects the accumulation of proteases during the prolonged differentiation time (based on Western blot data, unpublished observation, Litchi and Zahradka, 2000) since assays were conducted in this conditioned medium. As a result, high concentrations of the competitive receptor antagonists were used ( $10^{-5}$  M; 10× molar excess). Under the defined conditions, the selectivity of these receptor antagonists was confirmed by the [ $^{125}$ I]-AngII binding studies which demonstrated PD123319 ( $10^{-5}$  M) only displaced binding to a losartan-insensitive site that was enhanced by DTT treatment, while losartan displaced binding from a site that was inhibited by DTT treatment. In addition, the possibility of cross-reactivity of the receptor antagonists is contraindicated by the inhibition of AngII-mediated growth at equimolar concentrations of both losartan and PD123319 as well as the ability of only losartan to selectively inhibit the PI3K pathway (Chapter 6) and only PD123319 to selectively inhibit arachidonic acid release (Figure 15). Both receptor antagonists used in this study have been shown to exhibit a very high degree of selectivity based on losartan binding to the AT<sub>1</sub> receptor with a 10 000-fold greater affinity than for the AT<sub>2</sub> receptor and a  $K_D$  of 6 nM (Chiu et al., 1990) as well as PD123319 binding to the AT<sub>2</sub> receptor with a 3,500-fold greater affinity than for the AT<sub>1</sub> receptor and a  $K_D$  of 8 nM (Dudley et al., 1990). Other reports in the literature have utilized similarly high concentration of both losartan and PD123319 ( $10^{-4}$  -  $10^{-5}$  M) in human kidney and arterial vasculature and confirmed their selectivity in this concentration range by *in situ* autoradiography (Zhuo et al., 1996; Levy et al., 1996; Chiu et al., 1990). Although both receptor antagonists have been classified as competitive inhibitors, previous reports have noted non-competitive binding of losartan which can be attributed to the internalization of this receptor as well as the presence

of non-AT<sub>1</sub> interactions in some cell types (refer to Chapter 1.3.2.1). Analysis of the concentration-dependent inhibition of growth with these receptor antagonists demonstrated that PD123319 competitively inhibited AngII binding but losartan did not exhibit a concentration-dependent pattern of inhibition. At the high concentrations employed in this investigation, no agonistic effects were noted in any of the assays. In addition, non-specific effects of these antagonists at these concentrations were ruled out by the lack of effect on both FBS- and insulin-stimulated SMC growth (Table 2).

### 5.3.6 Role of Prostanoid Synthesis in AngII-stimulated SMC Growth

Prostaglandins may act as key effectors of the mitogenic actions of AngII. Previous studies have shown that AngII stimulate SMC synthesis of arachidonic acid derivatives such as prostaglandins and leukotrienes via activation of the cyclo-oxygenase and lipoxygenase pathways (Natarajan et al., 1994a; Jaiswal et al., 1993ab), as well as of cPLA<sub>2</sub> (Rao et al., 1994). Previously, AngII has been reported to stimulate the leukotriene 12- and 15-HETE (Natarajan et al., 1994a) in addition to the prostanoids thromboxane, PGH<sub>2</sub> (Badahman and Wilson, 1994), PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and PGI<sub>2</sub> (Jaiswal et al., 1993a) which could act as the downstream effectors of the mitogenic actions of AngII. In support of this premise, our data implicates prostaglandin synthesis as an early mediator of AngII's mitogenic actions based on the ability of indomethacin, a general cyclo-oxygenase inhibitor, to inhibit growth in response to AngII treatment in the PCA SMCs (Figure 15).

The respective contribution of AT<sub>1</sub> and AT<sub>2</sub> receptors to prostanoid synthesis in PCA SMCs was examined by arachidonic acid release. This experiment indicated involvement of only the AT<sub>2</sub> receptor, but not the AT<sub>1</sub> receptor (Figure 15). Although, the signal transduction mechanisms of the AT<sub>2</sub> receptor remain poorly defined, prostaglandin synthesis may represent a key signalling pathway of this receptor due to the AT<sub>2</sub>-mediated prostaglandin synthesis and release demonstrated in several cell types that express both the AT<sub>1</sub> and AT<sub>2</sub>

receptor subtypes (Jacobs and Douglas, 1996; Darimont et al., 1994; Lokuta et al., 1994; Jaiswal et al., 1993a; 1991). Follow-up studies have identified AngII-stimulated release of PGI<sub>2</sub> and possibly PGE<sub>2</sub> in PCA SMCs (Saward et al., submitted). In addition, the role of prostaglandins in growth is supported by evidence that prostaglandins can directly stimulate growth in this SMC system (Yau et al., unpublished data) and indomethacin can directly inhibit neointimal formation in an *in vitro* model of restenosis (Yau et al., unpublished data). In conclusion, the stimulation of prostaglandin synthesis through activation of the AT<sub>2</sub> receptors may be an important consideration for the potential role of AT<sub>2</sub> receptors in therapeutic blockade of AT<sub>1</sub> receptors.

### **5.3.7 Requirement for Simultaneous Activation of Both AngII Receptor Subtypes**

The results of this investigation support the hypothesis that activation of both AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes is required for the cellular growth response to AngII. In agreement with these data, this laboratory has reported that blockade of either AT<sub>1</sub> or AT<sub>2</sub> receptors inhibited AngII-mediated migration in Boyden chamber assays (Saward et al., submitted) and neointimal formation in porcine coronary artery rings following balloon-catheter injury (Wilson et al., 1999). Further characterization of the cross-talk between AT<sub>1</sub> and AT<sub>2</sub> receptors in these PCA SMCs revealed a role for the AT<sub>1</sub> receptor in the regulation of AT<sub>2</sub> receptor levels (Zahradka et al., 1998).

It is significant that the PCA SMC culture system, which expresses both AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes in the differentiated phenotype, exhibited a hyperplastic growth response to AngII. The possibility exists that the expression of both AT<sub>1</sub> and AT<sub>2</sub> receptors facilitates a full growth response to AngII through activation of the entire proliferative program, whereas culture systems that express only AT<sub>1</sub> receptors may exhibit a partial or hypertrophic growth response to AngII. This hypothesis fits the hypertrophic growth response observed for AngII treatment of many rodent SMC cultures that express only the AT<sub>1</sub> or AT<sub>2</sub> receptor

(A10 fetal SMCs in Chapter 7; Koibuchi et al., 1993; Millet et al., 1992; Chui et al., 1991; Naftilan et al., 1989). Thus, the stimulation of both AT<sub>1</sub>- and AT<sub>2</sub>-associated pathways may enhance the magnitude of the SMC response to AngII. This finding highlights the importance of studying the relative function of these receptors in systems that express endogenous AngII receptors of both subtypes. The PCA SMC culture system employed in this investigation offers an ideal *in vitro* model to further define the level(s) of interplay and point of convergence of the signalling pathways activated by the AT<sub>1</sub> and AT<sub>2</sub> receptors.

Recent data from a number of independent researchers support the concept that the involvement of both the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes modulates the downstream biological effects of AngII (refer to section 1.4.3.4, Chapter 1). Similar to the results in PCA SMCs, simultaneous activation of both receptor subtypes was also shown to be necessary for AngII-stimulated growth *in vitro* with spleen lymphocytes (Kunert-Radek et al., 1994) and neuroblastoma cells (Chen et al., 1993). In addition, a dual requirement for the AT<sub>1</sub> and AT<sub>2</sub> receptors *in vivo* has been demonstrated in the vascular compliance changes in rats following myocardial infarction (Ceiler et al., 1998), infarct size in pigs following ischemic injury (Jaloway et al., 1998), as well as the pressor effects of AngII administered to the spinal cord in rats (Horiuchi et al., 1997). An intriguing division of function may exist between the AT<sub>1</sub> and AT<sub>2</sub> receptors as a means of separating the biological effects of AngII in the vasculature. Both AT<sub>1</sub> and AT<sub>2</sub> receptors mediated vascular hypertrophy in rats through AT<sub>1</sub>-mediated changes in SMC phenotype and AT<sub>2</sub>-mediated hypertrophic growth (Sabri et al., 1997). Also, AngII-mediated vascular changes involved AT<sub>1</sub>-mediated changes in blood pressure and AT<sub>2</sub>-mediated SMC hypertrophy in hypertensive rats (Otsuka et al., 1998; Levy et al., 1996) and normotensive rats (Cao et al., 1999). In the adaptive changes following myocardial infarction in rats, AT<sub>2</sub> receptors mediated the interstitial DNA synthesis and decreased cardiac function whereas the AT<sub>1</sub> receptors mediated the decreased blood pressure and total

peripheral resistance (Kuizinga et al., 1998). Functional interplay between AT<sub>1</sub> and AT<sub>2</sub> receptors was clearly demonstrated by AT<sub>2</sub> receptor overexpression in mice that resulted in decreased AT<sub>1</sub> receptor-mediated pressor and chronotropic effects (Masaki et al., 1998).

It is important to note that the interaction between the AT<sub>1</sub> and AT<sub>2</sub> receptors is not always synergistic, since the AT<sub>2</sub> receptor can modulate or antagonize the response to AT<sub>1</sub> receptor activation in many cell types (Yamada et al., 1998; Goto et al., 1997; Mazzocchi et al., 1997; Ohkubo et al., 1997; van Kesteren et al., 1997; Booz and Baker, 1996; Laflamme et al., 1996; Stoll et al., 1995; Nakajima et al., 1995). In addition to AT<sub>2</sub>-mediated anti-growth or apoptotic effects, it has been reported that activation of the AT<sub>1</sub> receptor can also mediate anti-growth effects (Li D et al., 1999b; Diep et al., 1999; Cigola et al., 1997; Kajstura et al., 1997; Liakos et al., 1997). Thus, further clarification of the relative roles of both the AT<sub>1</sub> and AT<sub>2</sub> receptors is necessary to decipher the complexity of the biological response to AngII.

The level of interplay between the AT<sub>1</sub> and AT<sub>2</sub> receptors remains to be defined in PCA SMCs. Numerous studies have established a requirement for the activation of both receptors due to crosstalk at the signal transduction level (Refer to 1.4.3.4). For example, both AT<sub>1</sub> and AT<sub>2</sub> receptors mediate calcium mobilization (Shao et al., 1998), tyrosine phosphorylation (Goutsouliak and Rabkin, 1997), IP<sub>3</sub> production (Goutsouliak and Rabkin, 1998), TGFβ1 synthesis (Campbell and Katwa, 1997) and prostaglandin or arachidonic acid release (Catalioto et al., 1994; Lokuta et al., 1994; Jaiswal et al., 1993a; 1991b) in a variety of cell types. Thus, a broad generalization of the biological role of the AngII receptors is not appropriate since the mechanism of cross-talk and the impact of many auxillary factors (species and cell type, culture conditions) remain to be defined. However, the previous assumption that all effects of AngII are mediated through the AT<sub>1</sub> receptor and independent of the AT<sub>2</sub> receptor can no longer be considered legitimate. Many studies in the literature

have utilized only an AT<sub>1</sub> receptor antagonist in their analysis and, thus, vital information on the possible effects of AT<sub>2</sub> receptor in these systems is absent. In addition, the definition of the functionality and regulation of the different receptor subtypes in *in vitro* systems that express only a single population of AngII receptors or contain transfected receptors may be misleading (Wang et al., 1998). The emerging concept of crosstalk between AngII receptors adds an additional layer of complexity to the regulatory mechanisms for AngII's action. The diversity in species and cell types that show a dual requirement for both the AT<sub>1</sub> and AT<sub>2</sub> receptors indicates that this phenomenon may be universal and not just isolated to the PCA SMC culture system. The requirement for activation of both the AT<sub>1</sub> and AT<sub>2</sub> receptors in the mitogenic actions of AngII that was demonstrated in this study stresses the need for studying the relative contribution of each receptor subtype in a cell system that expresses both subtypes.

### **5.3.8 Dual AngII Receptor Model**

Based on the data presented in this chapter and corroborative evidence in the literature, a new model for the cellular response to AngII can be outlined. The overall response to AngII results from the downstream integration of signals from multiple AngII receptor subtypes such as the AT<sub>1</sub> and AT<sub>2</sub> receptors. Although both AT<sub>1</sub> and AT<sub>2</sub> receptors belong to the seven-transmembrane domain superfamily, little similarity in the signalling pathways coupled to these receptor subtypes has been found. Furthermore, evidence supports a model wherein each receptor signals through parallel and distinct pathways with a high degree of crosstalk that converge to contribute to a common cellular response. As a result, the net biological response to AngII would depend on the presence and cellular ratio of the AngII receptor subtypes. Thus, interference with activation of either receptor could disrupt the balance and alter the resultant phenotypic end-point of growth, apoptosis, or anti-growth/differentiation. From this perspective, the system of multiple receptor subtypes would

provide a sensitive mechanism for controlling the cellular response to this multifaceted growth factor.

In this study, the apparent redundancy in receptor function was demonstrated with the end-point of cell growth; however, further analysis of the associated signal transduction pathways is needed to identify the underlying crosstalk mechanism(s). It is plausible that each receptor signals through parallel, but independent, pathways with a high degree of crosstalk and, as a result, interference with either receptor subtype may block an event critical for cell growth. An analogous mechanism of AT<sub>1</sub> and AT<sub>2</sub> receptor crosstalk was recently identified in adrenal cells where an AT<sub>2</sub>-activated protein tyrosine phosphatase cascade inhibits cyclin D1 expression and AT<sub>1</sub>-stimulated prostaglandin synthesis inhibits cyclin D1-associated CDK activity (Liakos et al., 1997) to produce a block of bFGF-mediated proliferation. The PCA SMC system provides an ideal model to delineate the signal transduction pathways linked to both the AT<sub>1</sub> and AT<sub>2</sub> receptors due to their endogenous expression of both receptor subtypes. In agreement with this model, we have identified two independent signal transduction mechanisms with AT<sub>1</sub>-linked phosphatidylinositol-3 kinase (Saward and Zahradka, 1997; Chapter 6) and AT<sub>2</sub>-linked prostaglandin synthesis (refer to Section 5.2.6) that represent subtype-specific signalling pathways critical for the growth response to AngII in PCA SMCs. Identification of the cellular targets of these distinct signalling pathways will define a mechanism for their convergence on the processes involved in AngII-mediated growth.

### **5.3.9 Functional Implications of Dual Receptor Model**

The complexity in function of the different AngII receptor subtypes is evident from recent analysis of knock-out mice lacking specific receptors. Although this receptor is highly regulated throughout development, especially in vasculogenesis (Hutchinson et al., 1999; Nakajima et al., 1995), AT<sub>2</sub> receptor knock-out mice exhibited no significant developmental

defects except abnormal drinking and motility responses (Ichiki et al., 1995c). Similarly,  $AT_{1A}$  receptor knock-out mice (*Agtr1A*) grew and developed normally despite the absence of  $AT_1$  binding sites in major organs such as the kidneys (Coffman, 1997). Indeed, ablation of both  $AT_{1A}$  and  $AT_{1B}$  receptors led to a viable phenotype with reduced growth but uncovered additional roles for  $AT_2$  receptor in blood pressure regulation (Oliverio et al., 1998). Thus, the surprising lack of severe developmental defects in AngII receptor knock-out models has highlighted the functional redundancy in the renin-angiotensin system and interplay between the  $AT_1$  and  $AT_2$  receptor subtypes (Harada et al., 1999; Tanaka et al., 1999; Oliverio et al., 1998; Coffmann, 1997; Ichiki et al., 1995c).

At a functional level, it will be necessary to consider the contribution of both receptors to AngII's biological actions, as well as the effect of antagonism of only one receptor subtype, for effective intervention in the RAS's role in cardiovascular disease. Since treatment with  $AT_1$  receptor antagonists as well as ACE inhibitors causes a marked elevation of circulating AngII levels and its proteolytic products, the activation of other receptors in the renin-angiotensin cascade may exert undefined effects (van der Meulen et al., 1999; Iyer et al., 1998; Pitt et al., 1997; Capasso et al., 1994; Smits et al., 1992). Already some of the cardioprotective benefits of ACE inhibition and  $AT_1$  receptor antagonists have been attributed to  $AT_2$  receptor activation (Iyer et al., 1998; Liu YH et al., 1997). The implications of antagonism of only one receptor in RAS needs to be addressed in light of recent reports that ablation or antagonism of the  $AT_2$  receptor was shown to increase  $AT_1$  receptor levels and ACE activity (Gross et al., 2000; Hunley et al., 2000). Therefore, further understanding of receptor subtype function will help us identify their individual contributions to the cardiovascular pathophysiology. Ultimately, this information may permit the selective inhibition of the mitogenic effects and/or vasoactive actions of AngII and thus provide alternative therapies based on RAS interference for cardiovascular disease.

## 5.4 CONCLUSIONS

The results described in this chapter present conclusive evidence that the vascular SMC AT<sub>2</sub> receptor, in addition to the AT<sub>1</sub> receptor, plays a key role in mediating the mitogenic response to AngII, a hormone associated with cellular stress/damage and vascular disease. Based on the results of this investigation, a model was proposed in which activation of both AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes is required to mediate the downstream growth response to AngII in PCA SMCs. Subsequently, a number of reports have documented a similar dual requirement for both the AT<sub>1</sub> and AT<sub>2</sub> receptors in mediating the biological actions of AngII which suggests this phenomenon represents a functionally significant mechanism that is not just isolated to the PCA SMC culture system. This study stresses the importance of examining the relative contribution of AngII receptors in cell systems that express both receptors. PCA SMCs provide an ideal *in vitro* model for delineating the signal transduction pathways linked to both the AT<sub>1</sub> and AT<sub>2</sub> receptors and further define the level of crosstalk. Currently, AT<sub>1</sub>-linked PI3-kinase and AT<sub>2</sub>-linked prostanoid synthesis have been identified, and these will provide a starting point for future analysis of signalling crosstalk. The future of therapeutic intervention in RAS will depend on further definition of the roles of each receptor subtype *in vivo* and new advances in interference in their associated signalling pathways.

## **6. ROLE OF PI3K IN ANGIOTENSIN II-STIMULATED SMC GROWTH**

### **6.1 INTRODUCTION**

The involvement of AngII in events associated with vascular disease has focussed attention on associated signal transduction systems as potential targets for the development of novel therapeutic interventions. Although both AngII receptor subtypes, AT<sub>1</sub> and AT<sub>2</sub>, belong to the G-protein-coupled receptor superfamily, recent research has identified several non-traditional signal transduction mechanisms such as prostaglandin synthesis (Chapter 5). Tyrosine phosphorylation cascades have also been characterized as a key part of the proliferative response of vascular smooth muscle cells (SMCs) to AngII that involves the recruitment of several proteins such as p125<sup>FAK</sup> (Polte et al., 1994), Stat91 (Sadoshima et al., 1995) and PLC $\gamma$  (Marrero et al., 1994)(refer to Section 1.4.3.2). Phosphatidylinositol 3-kinase (PI3K) is a key component of the tyrosine kinase receptor signal transduction that has been shown to also respond to G-protein receptor-dependent agonists (Stoyanova et al., 1997; Stephens et al. 1994). Thus, PI3K could serve as the crossover point for these distinct systems.

To define the role and regulation of PI3K in AngII-stimulated growth of SMCs, we investigated the effect of AngII treatment on: (i) PI3K activity *in vitro* and *in vivo*, (ii) tyrosine phosphorylation of PI3K regulatory p85 subunit, (iii) subcellular localization and association of p85 and p110 subunits, (iv) the correlation between PI3K and cell growth, and (v) the involvement of AngII receptor subtypes. The results of this investigation provide conclusive evidence that PI3K is an important signal transduction intermediate for AngII-mediated cell growth.

### **6.2 RESULTS**

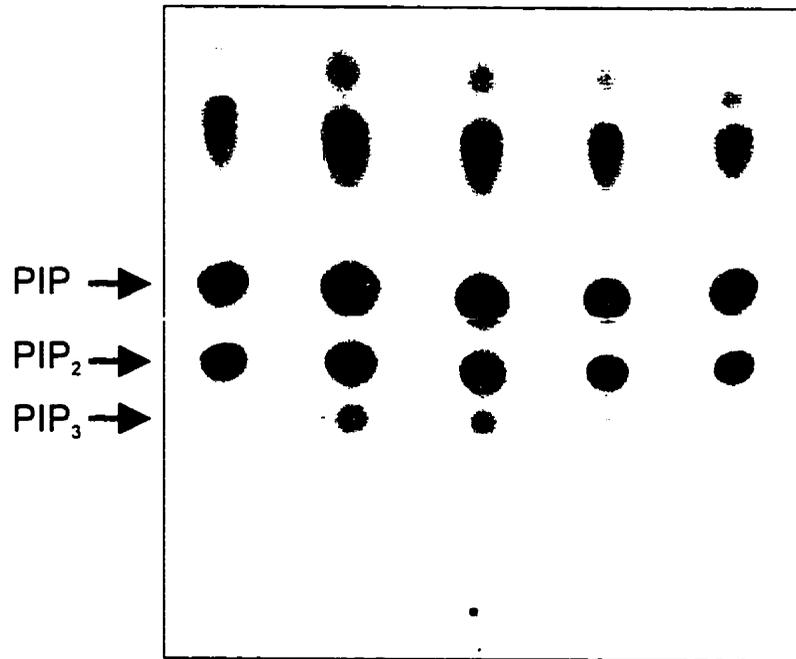
#### **6.2.1 AngII-dependent Stimulation of SMC PI3K Activity.**

To explore the role of PI3K in the signal transduction pathways activated by AngII, we measured PI3K activity through the production of phosphoinositides modified at the 3-position (Figure 16). Metabolic labelling with [ $^{32}\text{P}$ ]-orthophosphate was used to evaluate the activity of this enzyme *in vivo* and the labelled phosphoinositides were extracted and analyzed by TLC. Due to the inability of TLC analysis to resolve PI3P and PI4P as well as PI(3,4)P<sub>2</sub> and PI(4,5)P<sub>2</sub>, the most accurate measure of PI3K activity correlates with the level of PI(3,4,5)P<sub>3</sub> (or PIP<sub>3</sub>) (Okada et al., 1994). Panel A displays the autorad of a TLC analysis of quiescent PCA SMCs treated with AngII (10<sup>-6</sup> M) in the presence of absence of the PI3K inhibitor LY294002 (10<sup>-5</sup>-10<sup>-8</sup> M). In unstimulated cells, minimal amounts of PIP<sub>3</sub> were evident; however, stimulation with AngII resulted in increased levels of PIP<sub>3</sub> that could be inhibited by varying concentrations LY294002. A similar analysis of AngII-stimulated PIP<sub>3</sub> levels is summarized in Panel B based on densitometric quantification of the labelled phosphoinositides. Treatment of quiescent PCA SMCs with AngII (10<sup>-5</sup>-10<sup>-10</sup> M) for 15 min stimulated a dose-dependent increase in PIP<sub>3</sub>.

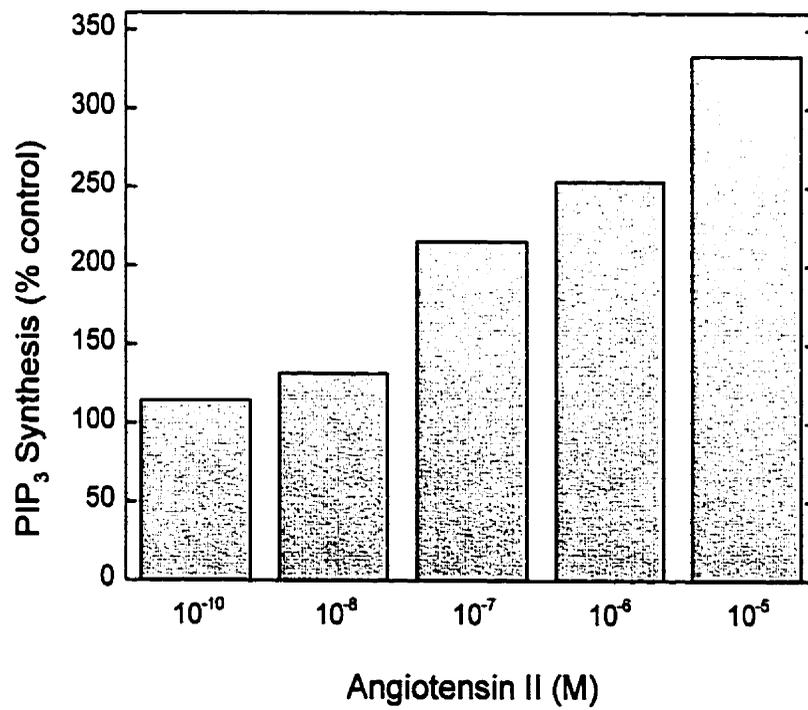
### 6.2.2 Regulation of p85-associated PI3K Activity by AngII.

Multiple isoforms of Class I PI3K have been characterized, including both p85-dependent and p85-independent catalytic activity (refer to Section 1.5.2). *In vitro* analysis of PI3K activity permits the direct evaluation of AngII regulation of p85-dependent PI3K activity. Quiescent SMCs were stimulated with AngII (10<sup>-6</sup> M), lysed and PI3K immunoprecipitated with both anti-p85 and anti-p110 antibodies (Figure 17). PI3K activity in the immunoprecipitates was subsequently measured by the amount of labelled phosphate transferred from [ $\gamma$ - $^{32}\text{P}$ ]-ATP to the substrates PI and PI(4,5)P<sub>2</sub> to form PI3P and PI(3,4,5)P<sub>3</sub>. Due to the substrate preference of Class I PI3K for PIP<sub>2</sub> over PI, higher levels of PIP<sub>3</sub> in comparison to PIP would be expected. Also, the formation of labelled PIP<sub>2</sub> in the assay represents the phospholabelling of PIP contaminants in the substrates.

**FIGURE 16: *AngII stimulation of phosphatidylinositol 3-kinase activity.*** Quiescent vascular SMCs were prelabelled with [<sup>32</sup>P]-orthophosphate and then treated with AngII (with or without inhibitor) for 15 minutes. Phosphoinositides were extracted from the cell lysates and the phosphorylated forms of phosphoinositol were resolved by thin layer chromatography as described in "Materials and Methods". In panel A, a full autoradiogram of the TLC plate is shown for quiescent smooth muscle cells (SMCs) treated with AngII ( $10^{-6}$  M) in the presence of increasing amounts of LY294002 ( $10^{-5}$ - $10^{-8}$  M). Migration of PIP, PIP<sub>2</sub> and PIP<sub>3</sub> is indicated. Panel B illustrates dose-dependent stimulation of PIP<sub>3</sub> formation by AngII ( $10^{-5}$ - $10^{-10}$  M) in SMCs. Data were quantified by densitometric analysis of the autoradiogram and normalized to control (no stimulation). These results were confirmed in 3 independent experiments with different SMC isolations.

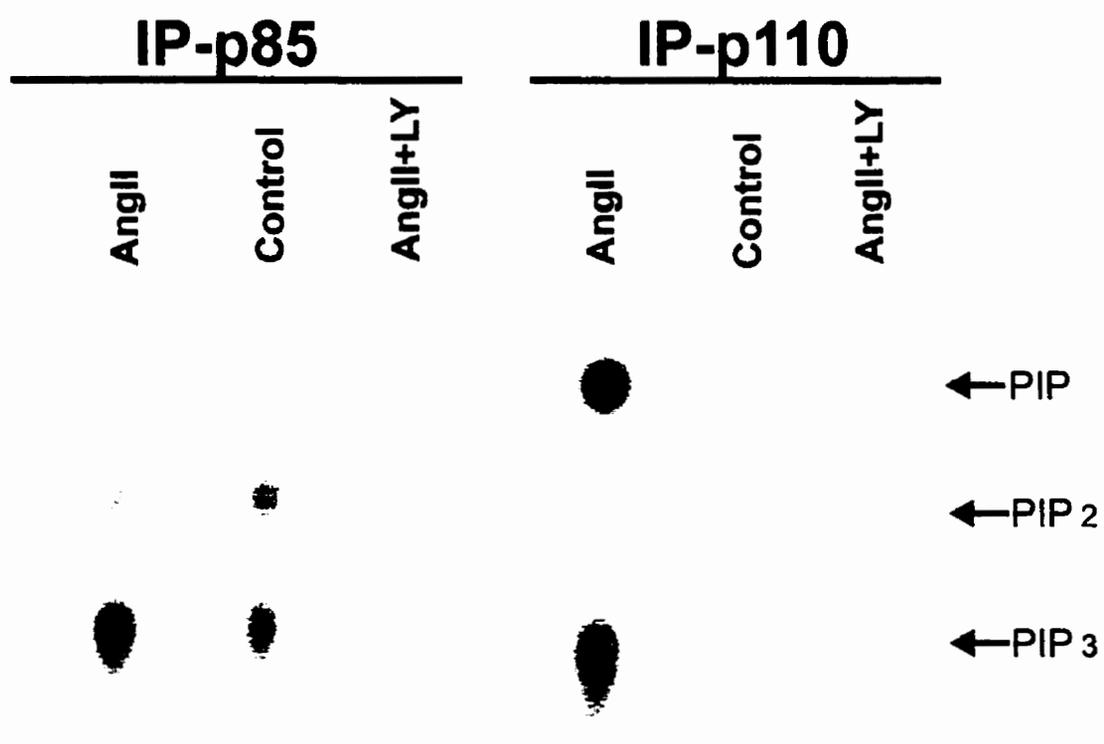
**A**

AngII	-	+	+	+	+
LY294002	-	-	10 <sup>-8</sup>	10 <sup>-6</sup>	10 <sup>-5</sup>

**B**

**FIGURE 17: *Enzymatic analysis of PI3K activity in vitro following immunoprecipitation.***

Cell lysates of SMCs following AngII ( $10^{-6}$  M) treatment were immunoprecipitated with either anti-p85 antibody or anti-p110 antibody and assayed for PI3K phosphorylation of PI and  $PIP_2$  as described in "Materials and Methods". LY294002 ( $10^{-5}$  M) was added to the assay reaction containing the AngII-stimulated immunoprecipitate prior to addition of the lipid substrates. Migration of PIP,  $PIP_2$  and  $PIP_3$  is indicated. These results were confirmed in 4 independent experiments with different SMC isolations.



As shown Figure 17, AngII ( $10^{-6}$  M) treatment for 15 minutes resulted in a rapid increase in labelled PIP<sub>3</sub> in both p85 and p110 immunoprecipitates in comparison to the activity levels in unstimulated cells. The PIP<sub>3</sub> detected in control (unstimulated) cells likely reflects the activity of a pool of PI3K activity required for various growth-independent processes active in quiescent cells (Susa et al., 1992). The inclusion of the PI3K inhibitor LY294002 effectively prevented the stimulation of p85- and p110-associated PI3K activity by Ang II. This data confirms the efficacy of LY294002 as an inhibitor of PI3K activity, since only PI3K is expected in the immunoprecipitated sample. This complementary *in vitro* assay of PI3K activity in cell lysates further substantiates that AngII stimulates PI3K activity as well as provides additional insight into the isoform of PI3K as p85/ p110 isoform.

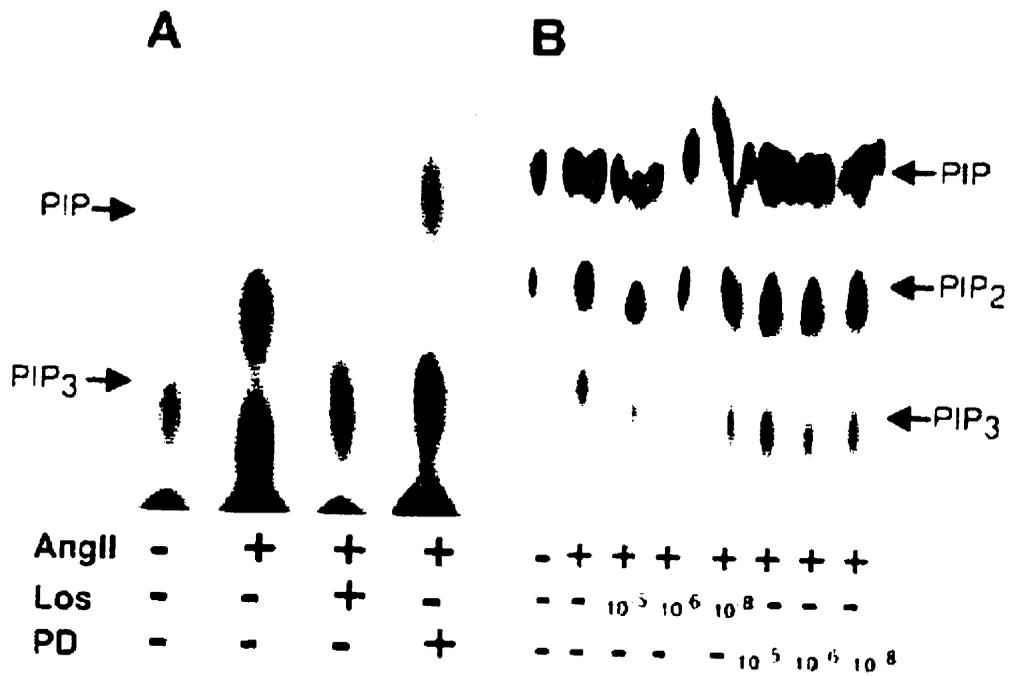
### **6.2.3 AngII Receptor Subtype Contribution to PI3K Activation.**

To verify that the activation of PI3K by AngII was receptor-dependent and to determine which AngII receptor subtype mediates this stimulation, the ability of AngII to stimulate PI3K was evaluated in the presence of the selective non-peptide receptor antagonists, losartan (AT<sub>1</sub>-specific) or PD123319 (AT<sub>2</sub>-specific). In agreement with the data in Figure 16 & 17, treatment of quiescent SMCs with AngII ( $10^{-6}$  M) for 15 min enhanced PI3K activity. Pretreatment with losartan ( $10^{-5}$ - $10^{-6}$  M) significantly inhibited the synthesis of PIP<sub>3</sub> *in vivo* and in p85 immunoprecipitates *in vitro* (Figure 18). In contrast, PD123319 did not inhibit AngII stimulation of PI3K *in vivo* or *in vitro*. These results demonstrate that AngII-dependent stimulation of PI3K is mediated by the AT<sub>1</sub> receptor.

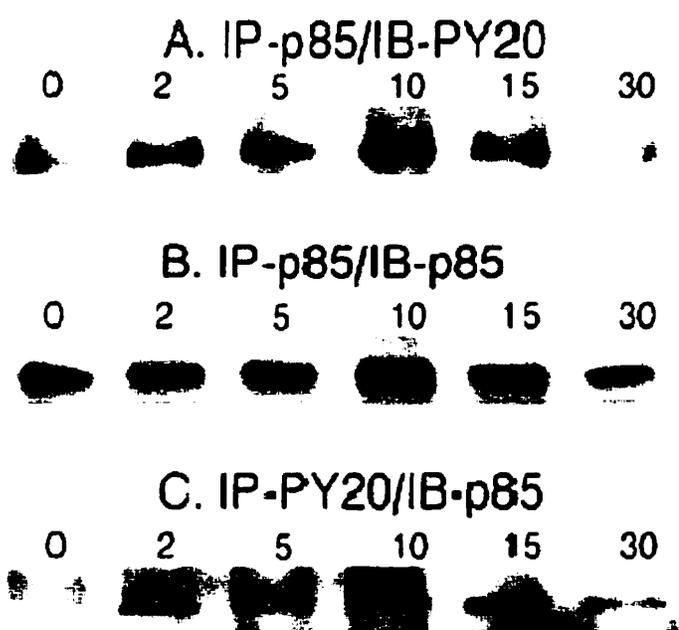
### **6.2.4 AngII-induced Tyrosine Phosphorylation of p85 PI3K Regulatory Subunit.**

One of the possible mechanisms for the activation of PI3K involves tyrosine phosphorylation of the p85 regulatory subunit or its association with tyrosine phosphorylated polypeptides in a signalling complex that contains after growth factor stimulation (Varticovski et al., 1994). Based on the identification of tyrosine phosphorylation

**FIGURE 18: *AngII* receptor subtype contribution to PI3K activation.** In Panel A, an autoradiogram of the TLC plate is shown for the *in vitro* analysis of PI3K activity following p85 immunoprecipitation. Anti-p85 immunoprecipitates from quiescent SMCs after stimulation with AngII ( $10^{-6}$  M) in the presence of the losartan or PD123319 ( $10^{-5}$  M) were assayed for PI3K phosphorylation of PI and PIP<sub>2</sub> as described in "Materials and Methods". In Panel B, an autoradiogram of the TLC plate is shown for the *in vivo* analysis of PI3K activity. Quiescent SMCs, prelabelled with [<sup>32</sup>P]-orthophosphate for 4 hr, were treated with AngII ( $10^{-6}$  M) in the presence of varying concentrations of losartan or PD123319 ( $10^{-5}$ - $10^{-8}$  M) and the phosphoinositides were extracted and analyzed as described in "Materials and Methods". These results were confirmed in 3 independent experiments with different SMC isolations. Migration of PIP, PIP<sub>2</sub> and PIP<sub>3</sub> is indicated.



**FIGURE 19: Assessment of p85 subunit tyrosine phosphorylation.** Quiescent SMCs were treated with AngII ( $10^{-6}$  M) for the indicated times and cell lysates were prepared for immunoprecipitation and/or Western blot analysis. Panel A: Quiescent SMCs were treated with AngII for varying periods of time (0-30 min) and cell extracts were visualized by Western blot analysis with anti-phosphotyrosine PY20 antibody. Panel B: Extracts were incubated with anti-p85 antibody and Western blot analysis of the immunoprecipitated proteins was performed with anti-phosphotyrosine PY20 antibody. In Panel C: The relative amounts of p85 immunoprecipitated in each sample is shown by Western blot analysis with anti-p85 antibody. Panel D: Immunoprecipitation was conducted with anti-phosphotyrosine antibody (PY20) and the blot analyzed with anti-p85 antibody to verify the time-course of phosphorylation. These results were confirmed in 3 independent experiments with different SMC isolations.

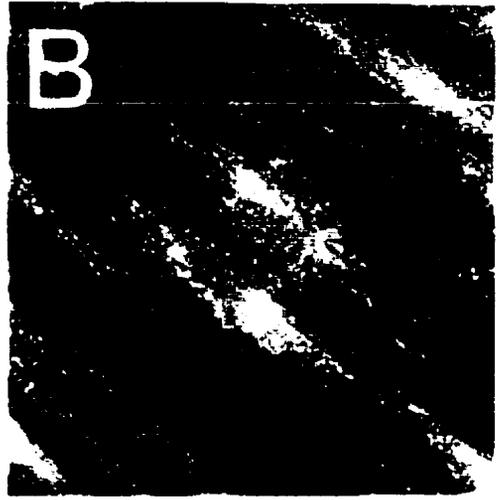
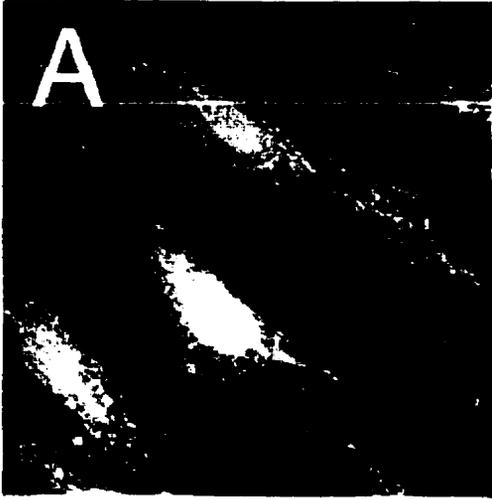


events as a key part of the signalling transduction mechanism for AngII (refer to Section 1.4.3.2), the regulation of tyrosine phosphorylation of the p85 regulatory subunit of PI3K by AngII was investigated in PCA SMCs. Quiescent SMCs were incubated with Ang II ( $10^{-6}$  M) for various times (0-30 min) and cell extracts were immunoprecipitated with an anti-p85 antibody. Western blot analysis with anti-phosphotyrosine antibody demonstrated a rapid and transient phosphorylation of the PI3K subunit p85 in response to AngII treatment, peaking at 10-15 min and returning to control levels by 30 min (Figure 19A). Subsequent Western blot analysis with anti-p85 antibody was used to normalize the levels of p85 in all samples (Figure 19B) and demonstrate that the increase in tyrosine phosphorylation of p85 mediated by AngII was approximately 2-fold after 10 minutes. Confirmation that the target of this antibody was p85 was established by the similar pattern of staining reproduced with a different polyclonal antibody to p85 (data not shown). A complementary analysis of anti-phosphotyrosine antibody immunoprecipitates followed by Western blot analysis with an anti-p85 antibody demonstrated an identical time course for phosphorylation with 2.5-fold increase after 10-15 min (Figure 19C). Thus, the stimulation of quiescent SMCs with AngII results in the tyrosine phosphorylation of the regulatory p85 subunit of PI3K.

#### **6.2.5 Subcellular Localization of p85 in AngII-stimulated PCA SMCs.**

It has been proposed that changes in the subcellular localization PI3K can operate as an additional mechanism for regulating PI3K activity. Indirect immunofluorescent microscopy was used to monitor the intracellular distribution of p85 before and after treatment with AngII. In quiescent SMCs, p85 exhibited a diffuse pattern of staining concentrated in the nuclear region (Figure 20A) with distinct focal areas of p85 scattered throughout the cytoplasm. Following a 5 min stimulation with AngII ( $10^{-6}$  M), a shift in p85 localization is evident as shown by the change in staining pattern from the diffuse pattern in nuclear region to a punctate staining pattern throughout the entire cytoplasm (Figure 20B).

**FIGURE 20: *Examination of the subcellular localization of p85 following AngII stimulation.*** Quiescent SMCs, cultured on glass slides, were treated with AngII ( $10^{-6}$  M) for the indicated times and prepared as outlined in "Materials and Methods". The p85 subunit was detected using indirect immunofluorescent microscopy at a magnification of  $33\times$ . This figure depicts the pattern of p85 staining in untreated cells (Panel A) and following 5 min (Panel B), 15 min (Panel C) or 30 min (Panel D) after AngII treatment. The results show a pattern consistently observed in 5 independent experiments with different SMC isolations.

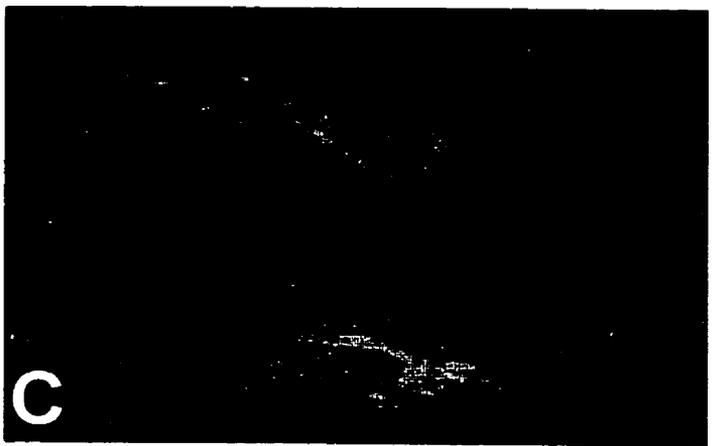
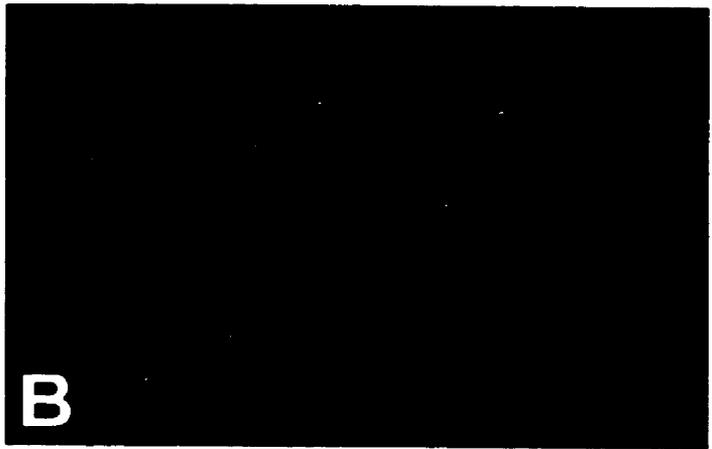
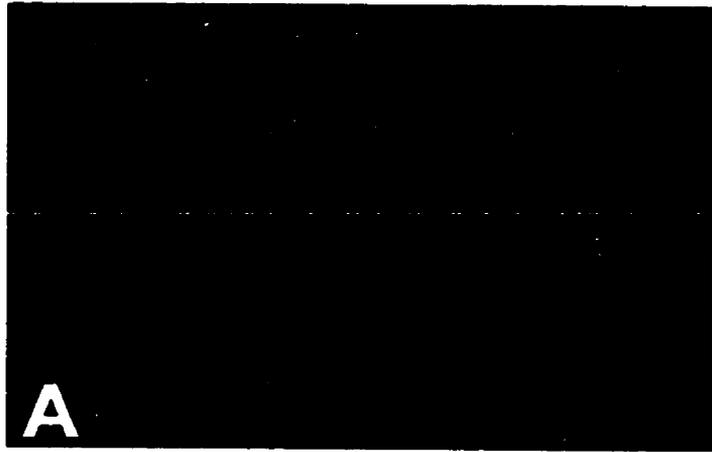


**FIGURE 21: *Pattern of p85 distribution and translocation following AngII stimulation.***

The pattern of p85 distribution in quiescent untreated SMCs (Panel A) and following 5 min (Panel B) and 15 min (Panel C) of AngII treatment ( $10^{-6}$  M) was monitored by indirect immunofluorescent microscopy as described in "Materials and Methods". Samples were examined at a magnification of 100 $\times$ .



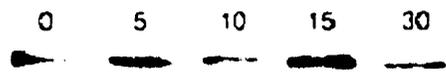
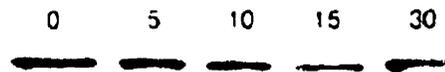
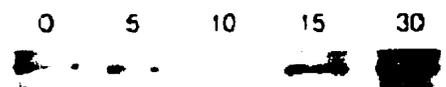
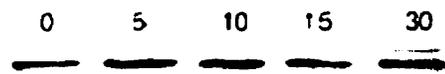
**FIGURE 22: *Analysis of p85 association with the nucleus and the cytoskeleton.*** The pattern of p85 distribution in quiescent SMCs (Panel A) relative to the nucleus (Panel B) was examined by indirect immunofluorescence microscopy as outlined in "Materials and Methods". In Panel C, the pattern of p85 and SM  $\alpha$ -actin is shown following AngII ( $10^{-6}$  M) treatment for 15 min. Samples were examined at 66 $\times$  magnification.



After 15 minutes of AngII treatment, p85 staining has completely vacated the nuclear region (Figure 20C); however, the concentration of p85 in the nuclear region has returned after 30 minutes (Figure 20D). Examination at a higher magnification confirmed that AngII induces a rapid (5-15 min) translocation of p85 from the nuclear region to specific foci throughout the cytoplasm and possibly the cytoskeleton or microtubule network (Figure 21). As shown in Figure 22A & B, double staining of quiescent SMCs suggested the pattern of p85 was not confined to the nucleus but also existed in the perinuclear cytoplasm. In addition, double staining of AngII-stimulated SMCs illustrated that a portion of p85 likely associates with the actin filaments, although the majority of p85 did not overlap (Figure 22C). The anti-PI3K polyclonal antibody used to obtain the information presented in Figures 20-22 has been previously used to establish the subcellular distribution of p85 by other laboratories (Kapeller et al., 1993; Neri et al., 1994). Further confirmation that this antibody's target is p85 was established by the ability to reproduce the pattern of staining with a different anti-p85 polyclonal antibody (Santa Cruz)(data not shown). Furthermore, cells treated with Cy3-conjugated anti-rabbit antibody or anti-p85 antibody alone as controls exhibited no detectable staining (data not shown).

To complement the visual analysis of p85 translocation in response to AngII stimulation, a subcellular fractionation of quiescent SMCs at various time points (0-30 min) following Ang II ( $10^{-6}$  M) stimulation was performed and the relative content of p85 was assessed by Western blot analysis (Figure 23). AngII treatment resulted in an increase in the p85 content of the membrane fraction at 15 min and a parallel decrease in p85 levels in the cytoskeletal and nuclear fractions at 15 min. Also, a noticeable increase in the nuclear p85 content was observed after 30 min AngII exposure. No changes in the levels of p85 were evident in the cytoplasmic fraction.

**FIGURE 23:** *Translocation of p85 to distinct cellular compartments after AngII treatment.* . Quiescent SMCs were stimulated with AngII ( $10^{-6}$  M) for varying periods of time (0-30 minutes), lysed and separated into Cytoplasmic, Membrane, Nuclear and Cytoskeletal fractions as described in "Materials and Methods". Equivalent amounts of protein were loaded in each well and the relative amounts of p85 in each fraction was determined by Western blot analysis with anti-p85 antibody.

**A. Membrane Fraction****B. Cytoskeletal Fraction****C. Nuclear Fraction****D. Cytoplasmic Fraction**

### 6.2.6 Subcellular Localization of the PI3K p110 Catalytic Subunit

PI3K consists of two non-covalently bound subunits that are p85, the regulatory subunit, and p110, the catalytic subunit. Although it has been generally assumed that the two subunits remain bound in both resting and proliferating cells, the subcellular localization of the p110 subunit has not been studied in detail. Indirect immunofluorescent microscopy was used to examine the cellular localization of p110 in quiescent and AngII-stimulated PCA SMCs. In quiescent SMCs, p110 appears to be scattered throughout the cytoplasm with a distinct punctate pattern in addition to perinuclear staining (Figure 24A). Similar to the p85 pattern, AngII stimulation ( $10^{-6}$  M) resulted in a rapid translocation of p110 from the perinuclear region after 15 min (Figure 24B). In contrast with p85 localization, p110 remains absent from the perinuclear region 30 min following AngII treatment (Figure 24C). However, p110 returns to the nuclear and perinuclear region 24 hours following AngII treatment (Figure 24D). Interestingly, AngII stimulation (15 & 30 min) leads to translocation of p110 to specific foci within the nucleus that likely represent the nucleoli.

The differences in p85 and p110 cellular localization following AngII stimulation for 15 min is shown in Figure 25. The localization of p110 to the putative nucleoli following AngII stimulation (15 min) is exhibited clearly in Figure 25C. The nucleoli were identified by their lack of staining with the nuclear stain Hoescht 53228 (Figure 25D). In contrast, no nucleolar p85 is evident (Figure 25A & B).

To examine more precisely the association between the p85 and p110 subunit of PI3K, cell extracts from quiescent SMCs stimulated with Ang II ( $10^{-6}$  M) for various times (0-30 min) were immunoprecipitated with an anti-p85 antibody. Western blot analysis with anti-p110 antibody clearly demonstrated that the association between p85 and p110 decreases following AngII treatment (Figure 26A). In untreated control cells, the p85 and p110 coimmunoprecipitated; however, 15-30 min of AngII stimulation resulted in the absence of

**FIGURE 24: Examination of the subcellular localization of p110 following AngII stimulation.** Quiescent SMCs, prepared on glass slides, were treated with AngII ( $10^{-6}$  M) and p110 was detected using indirect immunofluorescence microscopy at a magnification of 100 $\times$ . The pattern of p110 staining is shown for untreated cells (Panel A) and following 15 min (Panel B), 30 min (Panel C) and 24 hours (Panel D) of AngII treatment. The results represent a pattern consistently observed in 3 independent experiments.

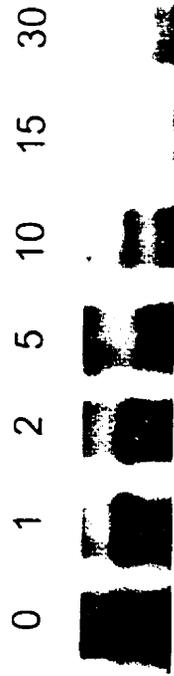


**FIGURE 25: Comparison of p85 and p110 distribution after AngII treatment.** Quiescent SMCs were treated with AngII ( $10^{-6}$  M) for 15 min., prepared on glass slides and stained with anti-p85 or anti-p110 antibodies for examination by indirect immunofluorescent microscopy as outlined in "Materials and Methods". Panel A depicts the pattern of p110 staining with the complementary nuclear stain in Panel B. In comparison, the pattern of p85 staining is shown in Panel C with the accompanying nuclear stain in Panel D. Samples were examined at 66 $\times$  magnification.



**FIGURE 26: Association of the PI3K subunits p85 and p110.** Quiescent SMCs were treated with AngII ( $10^{-6}$  M) for the indicated times (0-30 min) and cell lysates were prepared for immunoprecipitation and Western blot analysis as described in “Materials and Methods”. Panel A: Lysates were incubated with anti-p85 antibody and Western blot analysis of the immunoprecipitated proteins was performed with anti-p110 antibody. Panel B shows the relative amounts of p85 immunoprecipitated in each sample after Western blot analysis with anti-p85 antibody. Panel C: An identical set of immunoprecipitations was conducted with the anti-p110 antibody and the blot analyzed with anti-p85 antibody. Panel D shows the relative amount of p110 immunoprecipitated in each sample after Western blot analysis with anti-p110 antibody.

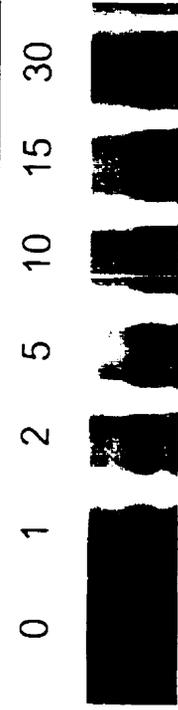
**A. IP-P85 / IB-P110**



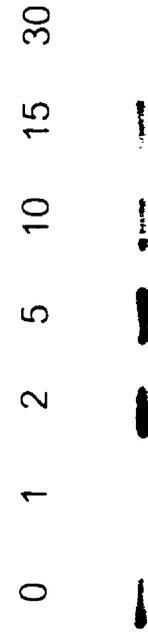
**C. IP-P110 / IB-P85**



**B. IP-P85 / IB-P85**



**D. IP-P110 / IB-P110**



p110 in p85-immunoprecipitates. A subsequent Western blot analysis with anti-p85 antibody was used to visualize the levels of p85 in all samples (Figure 26B). Similarly, immunoprecipitation of p110 from cell extracts of SMCs treated with AngII (0-30 min) and subsequent Western blot analysis with anti-p85 antibody confirmed that AngII stimulates a dissociation between the two PI3K subunits (Figure 26C). The relative levels of p110 in the immunoprecipitates is shown (Figure 26D).

To complement the visual analysis of p110 translocation in response to AngII stimulation, a subcellular fractionation of quiescent SMCs at various time points (0-30 min) following Ang II ( $10^{-6}$  M) stimulation was performed and the relative content of p110 was assessed by Western blot analysis (Figure 27). AngII treatment resulted in an increase in p110 content of the cytoskeletal fraction at 15 and 30 min and the nuclear fraction at 15 and 30 min; however, no change in p110 levels in the cytoplasmic or membrane fractions was observed.

#### **6.2.7 AngII-induced p85 Signalling Complex.**

In this study, the components of the p85 complex formed following Ang II ( $10^{-6}$  M) treatment (0-30 min) of quiescent SMCs was examined by immunoprecipitation with an anti-p85 antibody (Figure 28). Subsequent Western blot analysis with an anti-FAK antibody demonstrated AngII regulates the association of FAK with p85 that peaks within 1 minute and dissociates 5-10 min after stimulation. AngII stimulation resulted in the rapid association of IRS-1 (2-15 min) and Paxillin (5-15 min) with p85 complex. In addition, the p85 signalling complex was associated with the AT<sub>1</sub> receptor (1-15 min). The anti-AT<sub>1</sub> receptor antibody used has been characterized in this SMC system (Zahradka et al., 1998). The dissociation of this signalling complex was observed in all immunoblots by 30 minutes after AngII stimulation with AT<sub>1</sub> receptor, IRS-1 and paxillin returning to control levels. Preliminary data also identified Shc and Src as additional components of this p85 signalling

**FIGURE 27: *Translocation of p110 to distinct cellular compartments after AngII stimulation.*** Quiescent SMCs were stimulated with AngII ( $10^{-6}$  M) for varying periods of time (0-30 minutes), lysed and separated into Cytoplasmic, Membrane, Nuclear and Cytosolic fractions as described in "Materials and Methods". Equivalent amounts of protein were loaded in each well and the relative amounts of p110 in each fraction was determined by Western blot analysis with anti-p110 antibody.

### A. Membrane Fraction

0 5 10 15 30



### B. Cytoplasmic Fraction

0 5 10 15 30



### C. Cytoskeletal Fraction

0 5 10 15 30

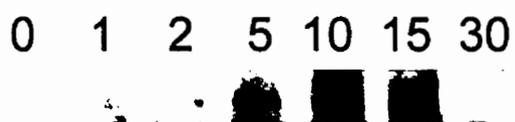
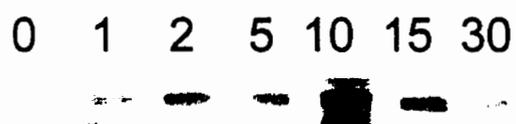
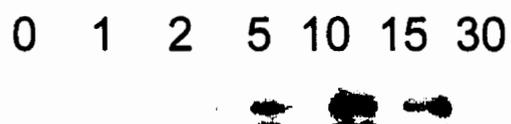
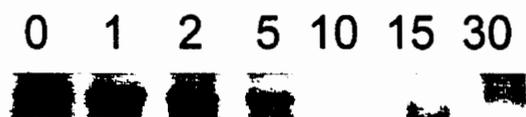


### D. Nuclear Fraction

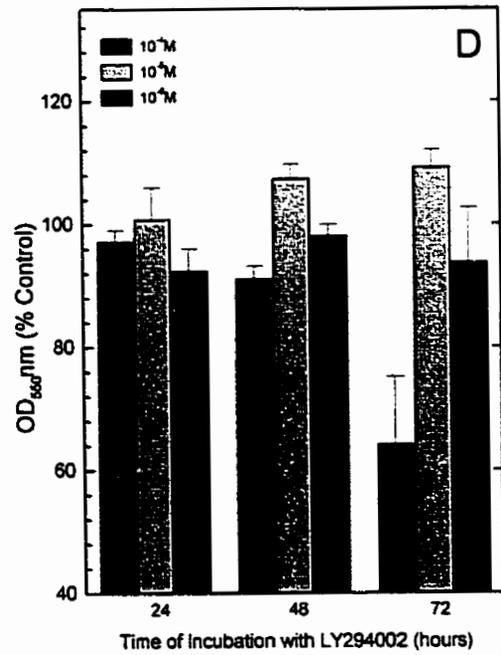
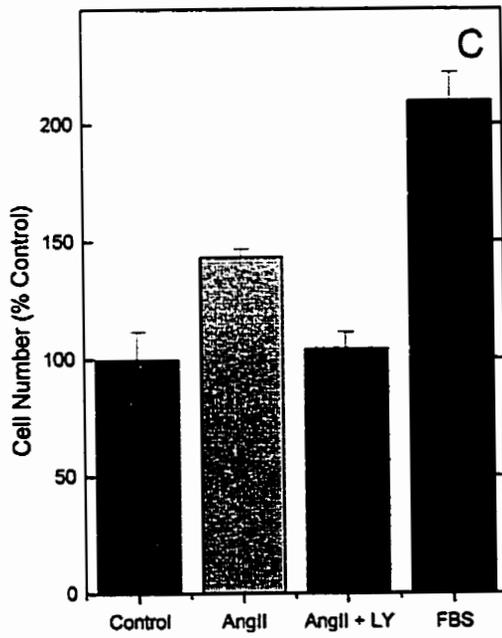
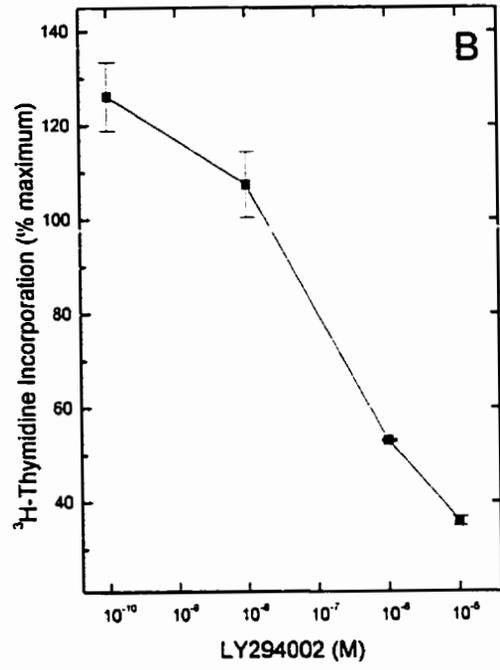
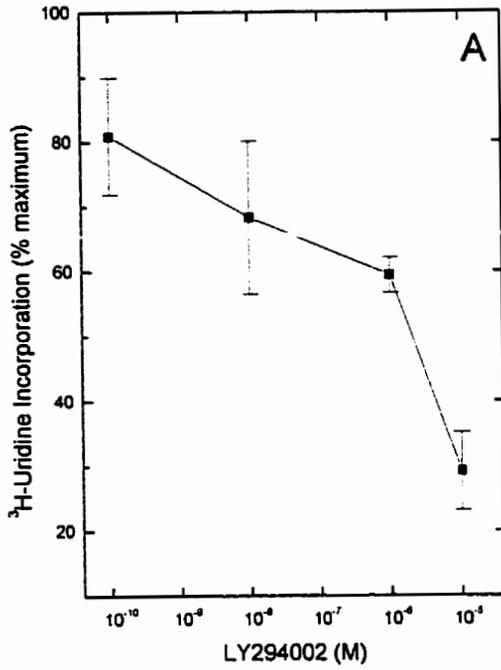
0 5 10 15 30



**FIGURE 28: *Components of p85 signalling complex after AngII stimulation.*** Quiescent SMCs were treated with AngII ( $10^{-6}$  M) for the indicated times (0-30 min) and cell lysates were prepared for immunoprecipitation with anti-p85 antibody as described in “Materials and Methods”. Panel A: Western blot analysis of the immunoprecipitated proteins was performed with anti-AT<sub>1</sub> receptor (Panel A), anti-IRS-1 (Panel B), anti-paxillin (Panel C) and anti-FAK (Panel D) antibodies for a direct comparison of their association with p85 following AngII treatment.

**A IP-p85 / IB-AT1R****B IP-p85 / IB-IRS1****C IP-p85 / IB-Paxillin****D IP-p85 / IB-FAK**

**FIGURE 29: *Effect of PI3K inhibitor LY294002 on AngII-stimulated SMC growth.*** Panel A illustrates the effect of varying concentrations of LY294002 on the stimulation of RNA synthesis by AngII ( $10^{-6}$  M), based on [ $^3$ H]-uridine incorporation over a 6-h period. Panel B shows the efficacy of varying concentration of LY294002 to inhibit AngII-stimulated DNA synthesis, based on [ $^3$ H]-thymidine incorporation over a 48 hour period. Each data point represents the average  $\pm$  S.E. of at least three separate experiments. In both panels, the value of the AngII-stimulated control was set at 100%. These results were confirmed in 5 independent experiments with different SMC isolations. Panel C illustrates the increase in total cell number following AngII ( $10^{-6}$  M) treatment of quiescent SMCs for 96 hours in comparison to 5% FBS treatment and the ability of LY294002 to prevent AngII-dependent hyperplasia. Panel D depicts the cytotoxic effect of various doses of LY294002. Quiescent SMCs were treated with LY294002 ( $10^{-4}$ - $10^{-6}$  M) for 24, 48 and 72 hours and mitochondrial activity was evaluated with the MTT assay as described in "Materials and Methods: Each data point represents the average  $\pm$  S.E. of at least three separate experiments.



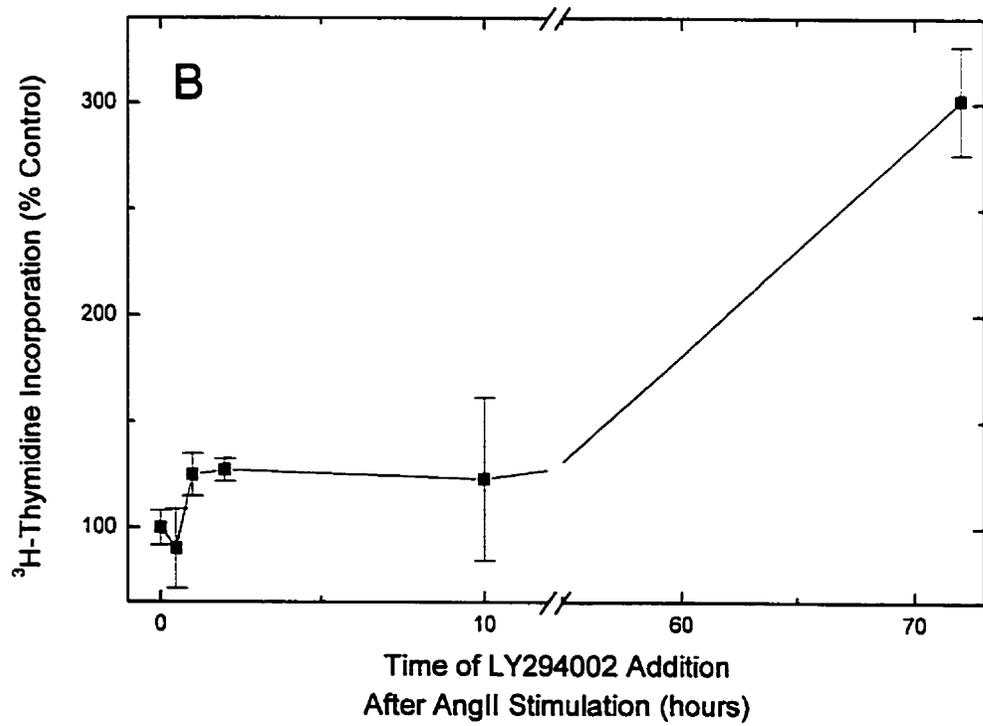
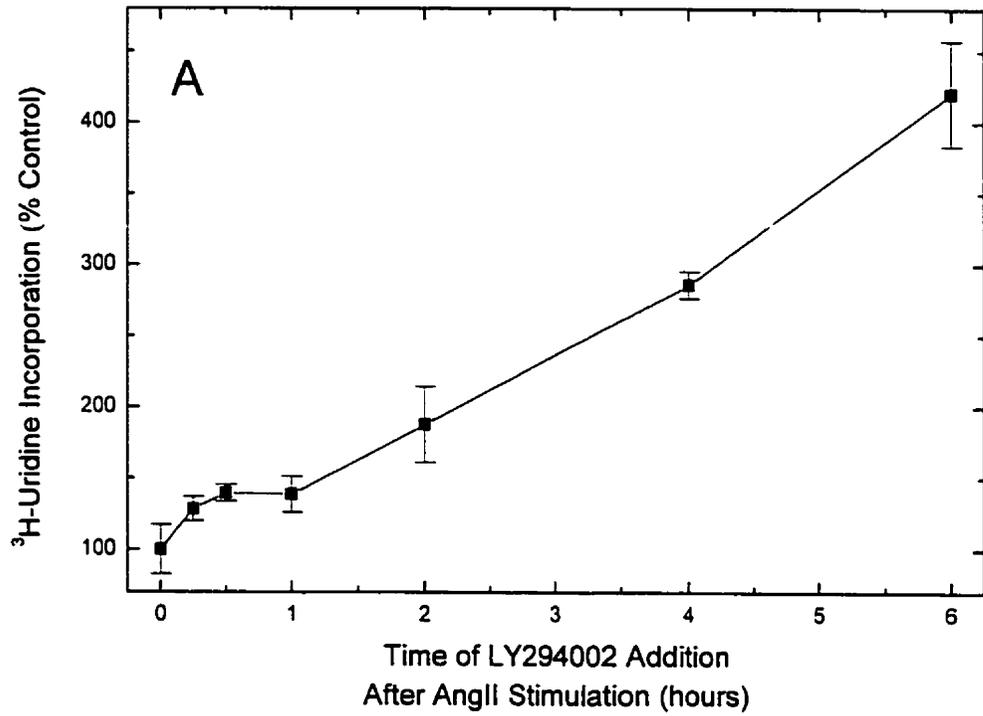
complex in PCA SMCs (data not shown). Only FAK was associated with p85 in the unstimulated control cells which may reflect the involvement of these signalling intermediates in a specific function of PI3K in quiescent SMCs or cells in *in vitro* culture conditions.

#### 6.2.8 Effect of PI3K Inhibitor LY294002 on AngII-stimulated SMC Growth.

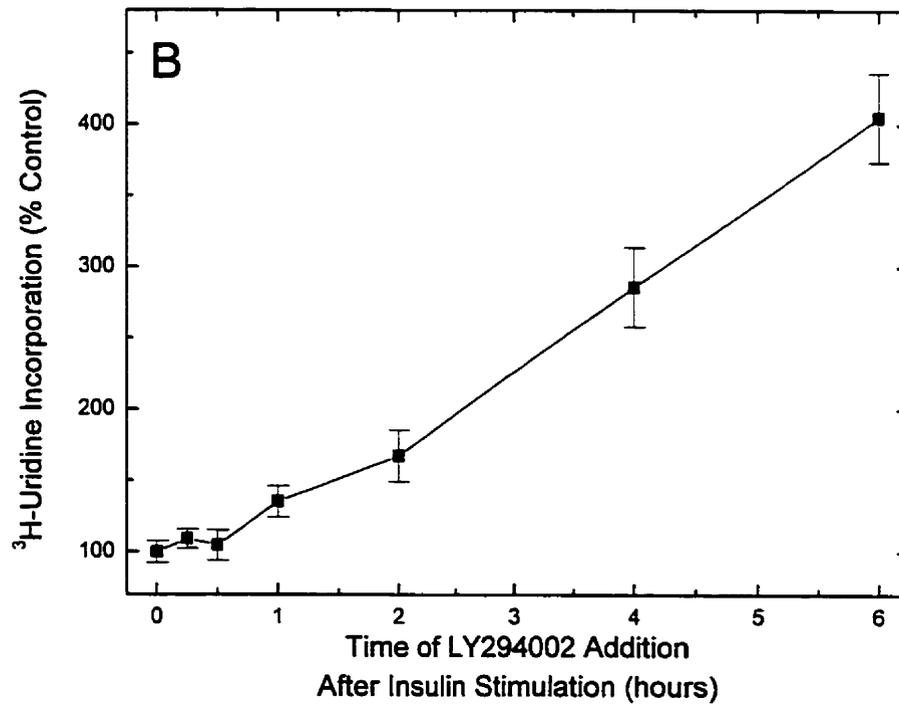
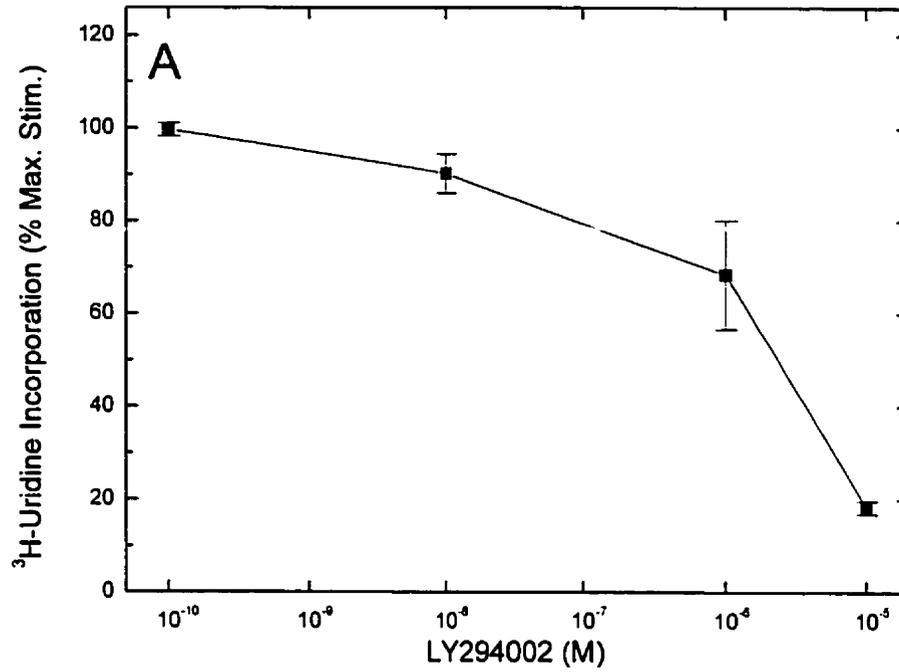
PI3K has been identified as an essential factor for cell proliferation in a number of cell systems following tyrosine kinase receptor activation. However, its potential contribution to G-protein receptor-mediated growth has not been established. The functional role of PI3K in the proliferative response to AngII (Chapter 5) was examined in PCA SMCs using the PI3K inhibitor LY294002. Typically, AngII ( $10^{-6}$  M) stimulates the synthesis of both RNA ( $184\% \pm 13$ ) and DNA ( $172\% \pm 17$ ), as determined by the rate of [ $^3$ H]-uridine and [ $^3$ H]-thymidine incorporation, respectively, but inclusion of LY294002 ( $10^{-5}$ - $10^{-10}$  M) blocked the stimulation in a concentration-dependent manner (Figure 29A & B). In addition, LY294002 ( $10^{-5}$  M) blocked the increase in cell number that is observed 96 hours after stimulation with AngII ( $10^{-6}$  M) (Figure 29C). To eliminate the possibility that the observed effects could be attributed to cellular toxicity of LY294002, the effect of LY294002 on cell survival was monitored with the MTT assay, a quantitative assay for cell viability based on mitochondrial activity (Shi et al., 1993). As demonstrated in Figure 29D, no cell death or cytotoxicity was evident at the concentration of LY294002 ( $10^{-5}$  M) used in this study over an extended incubation of 72 hours. In conclusion, these data support the hypothesis that PI3K is critical for the mitogenic effects of AngII in SMCs.

To determine the time frame of the contribution of PI3K to the cellular actions of AngII, the PI3K inhibitor LY294002 was added to cells at various times after stimulation with AngII and the overall growth response was evaluated. In Figure 30A, quiescent SMCs were stimulated with AngII ( $10^{-6}$  M) prior to the addition of LY294002 ( $10^{-5}$  M) at the

**FIGURE 30: *Effect of delayed addition of LY294002 on AngII-stimulated RNA and DNA synthesis.*** Panel A illustrates the effect of addition of LY294002 ( $10^{-5}$  M) at the indicated time points on the total level of RNA synthesis stimulated by AngII ( $10^{-6}$  M), based on [ $^3$ H]-uridine incorporation over the 6 hour period. Panel B represents the effect on DNA synthesis of the addition of LY294002 ( $10^{-5}$  M) at varying time points after AngII ( $10^{-6}$  M) stimulation, based on [ $^3$ H]-thymidine incorporation over the 48 hour period. Each data point represents the average  $\pm$  S.E. of at least three separate experiments. In both panels, value of the AngII-stimulated control was set at 100%.



**FIGURE 31: *Effect of PI3K inhibitor LY294002 on Insulin-stimulated RNA and DNA synthesis.*** Panel A illustrates the effect of varying concentrations of LY294002 on the stimulation of RNA synthesis by Insulin ( $10^{-6}$  M), based on [ $^3$ H]-uridine incorporation over a 6-h period. The value of the Insulin-stimulated control was set at 100%. Panel B illustrates the effect of LY294002 ( $10^{-5}$  M) addition on RNA synthesis at varying time points after Insulin ( $10^{-6}$  M) stimulation, based on [ $^3$ H]-uridine incorporation over a 6 hour period. The value of the untreated control was set at 100%. For both panels, each data point represents the average  $\pm$  S.E. of at least three separate experiments.



indicated time points and the total amount of [ $^3\text{H}$ ]-uridine incorporated over 6 hours was measured. The capacity of LY294002 to inhibit RNA synthesis at any time of addition indicates the involvement of PI3K in mediating AngII-stimulated RNA synthesis over the 6 hour assay time. Thus, as the length of time between addition of AngII and LY294002 was increased, the level of [ $^3\text{H}$ ]-uridine incorporated also increased. The linear relationship between these parameters (coefficient of linearity 0.990), therefore, indicates that PI3K activity is crucial to the early growth response to AngII. In Figure 30B, quiescent SMCs were stimulated with AngII ( $10^{-6}$  M) prior to the addition of LY294002 ( $10^{-5}$  M) at the indicated time points and the total amount of [ $^3\text{H}$ ]-thymidine incorporated over 72 hours was measured. The addition of LY294002 ( $10^{-5}$  M) at any time during the initial 10 hours after AngII stimulation resulted in the inhibition of DNA synthesis, when added up to 10 hours post-AngII addition (Figure 30B). This data suggests the involvement of PI3K in later events essential for AngII-mediated DNA synthesis. In summary, the function of PI3K in the AngII-dependent stimulation of cell growth may be associated with both immediate and late events in the cell cycle.

#### **6.2.9 Efficacy of LY294002 in Insulin-mediated SMC Growth**

Since PI3K has been well-characterized as a key component of the insulin signal transduction mechanism (refer to Section 1.5.1), the efficacy of LY294002 to inhibit insulin-stimulated growth was evaluated in the same SMC system. In PCA SMCs, LY294002 was shown to effectively inhibit insulin-stimulated ( $10^{-6}$  M) RNA synthesis in a dose-dependent manner, similar to AngII (Figure 31A). A parallel analysis of the timecourse of LY294002 efficacy in insulin-stimulated RNA synthesis was also examined (Figure 31B). In agreement with AngII-mediated growth, addition of LY294002 ( $10^{-5}$  M) during the initial 6 hours effectively inhibited insulin-stimulated RNA synthesis. These results further support our conclusion that PI3K is a key signalling component of the early growth response in PCA

SMCs.

### 6.3 DISCUSSION

#### 6.3.1 PI3K is a Component of the AngII Signalling Pathway in PCA SMCs

The most important finding presented in this chapter is the identification of PI3K as an important signalling intermediate for AngII in vascular SMCs. Due to the fact that PI3K has been traditionally associated with receptor tyrosine kinase activation (Kapeller and Cantley, 1994), the contribution of this enzyme to G-protein-coupled receptor signalling pathways remained uncharacterized. In PCA SMCs, AngII stimulated a concentration-dependent increase in PI3K activity that was inhibited by the PI3K inhibitor LY294002. Due to the fact that this assay monitors the activity of the total PI3K pool, it cannot be used to distinguish between the different isoforms of PI3K. Several reports have demonstrated the activation of distinct isoforms of Class I PI3K by G-protein-coupled receptors including a p85-dependent p110 $\beta$  (Kurosu et al., 1997), a p85-independent p110 $\beta$  (Murga et al., 2000) and p85-independent p110 $\gamma$  (Stephens et al., 1994; Tang et al., 1997). The *in vitro* PI3K assay with p85 and p110 immunoprecipitates from SMC extracts indicated that a large portion of the total p110 PI3K activity was associated with p85. Further experiments will be necessary to determine the presence of a pool of p85-independent p110 PI3K in this SMC system. Since the anti-p110 antibody used in this study will not detect p110 $\gamma$ , the contribution of this PI3K isoform in this system remains undefined.

The hyperplastic response of PCA SMC cultures to AngII involves both the AT<sub>1</sub> and AT<sub>2</sub> receptors (Chapter 5). To determine the AngII receptor subtype linked to PI3K activation, the non-peptide receptor antagonists losartan and PD123319 were used to selectively block the AT<sub>1</sub> and AT<sub>2</sub> receptors, respectively. The AT<sub>1</sub> receptor was shown to activate PI3K in these SMCs, since AngII-stimulated PI3K activity could be blocked by

losartan but not PD123319 in both the *in vivo* and *in vitro* PI3K assays. Furthermore, Western blot analysis of anti-p85 immunoprecipitates revealed that the AT<sub>1</sub> receptor may associate directly with the PI3K complex formed after AngII stimulation.

After completion of this study, other laboratories published corroborative results that demonstrate AngII and other G-protein-coupled growth factor receptor systems recruit PI3K as a key component of their signal transduction pathways. AngII, via the AT<sub>1</sub> receptor, has been shown to activate Akt/PKB in SMCs via PI3K- and tyrosine kinase-dependent pathways (Takahashi et al., 1999; Ushio-Fukai et al., 1999b). Dominant-negative Akt/PKB blocked the stimulation of protein synthesis by AngII in SMCs (Ushio-Fukai et al., 1999b). In addition, PI3K was identified as a key signalling pathway in AT<sub>1</sub>-stimulated Raf-1 kinase (Smith et al., 1999), vascular L-type Ca<sup>2+</sup> channels (Viard et al., 1999) and norepinephrine regulation in SHR neurons (Yang and Raizada, 1999).

### **6.3.2 PI3K Signalling Complex Formation by AngII**

The mechanism of PI3K regulation by non-kinase receptors is still undefined. In tyrosine kinase receptor systems, p85 either associates directly with the receptor via its SH2 binding to the motif pYXXM or through an adaptor molecule. Since the AT<sub>1</sub> receptor lacks the pYXXM motif, it is likely that an adaptor molecule mediates this association. The IRS-1 protein is a key docking protein for SH2 containing proteins in a number of receptor signalling systems in addition to its defined role as the substrate for the insulin receptor. Previously, et al. (1995) reported a correlation between AngII stimulation and the association of minute quantities of tyrosine phosphorylated p85 with IRS-1 in cardiac tissue. AngII has also been shown to inhibit PI3K activity through activation of JAK2 and tyrosine phosphorylation of IRS-1 and IRS-2 in rat hearts (Velloso et al., 1996). Alternatively, novel adaptor molecules such as p120<sup>cb1</sup> (Sattler et al., 1996; Soltoff and Cantley, 1996) and p80 (Jucker and Feldman, 1995) have been shown to mediate the binding of p85 to the epidermal

growth factor receptor and the human granulocyte/macrophage colony stimulating factor receptor, respectively. Alternatively, another mechanism to link PI3K to the AT<sub>1</sub> receptors may involve recruitment of Src-family kinases which may activate PI3K through binding to the p85 subunit via SH3 domains, without a direct interaction with the receptor (Pleiman et al., 1994). Thus, due to the lack of the pYXXM motif in the AT<sub>1</sub> receptor, numerous possibilities exist to link the AT<sub>1</sub> receptor with PI3K.

In this study, Western blot analysis was used to examine the proteins that immunoprecipitated with p85 following AngII stimulation of quiescent PCA SMCs. AngII stimulation resulted in a rapid and transient recruitment of IRS-1, paxillin, and FAK to the p85 complex. The AngII-dependent association of IRS-1 with p85 in PCA SMCs is functionally significant since the IRS-1 peptide likely has a key role in the activation of p85/p110 $\beta$  PI3K by G $\beta$  $\gamma$  receptors (Kurosu et al., 1997). In addition, IRS-1 has been identified as a substrate for the serine kinase domain of p110 (Freund et al., 1995). FAK represents an important non-receptor kinase that associates with a number of downstream targets to mediate cellular growth and migration. Previously, PI3K was identified as a substrate for FAK (Chen and Guan, 1994a) and, conversely, PI3K activity was demonstrated to be necessary for PDGF-mediated FAK phosphorylation (Chen and Guan, 1994b). Thus, it is significant that AngII stimulation regulated the association of FAK with the PI3K p85 regulatory unit. The presence of paxillin in the signalling complex induced by AngII treatment is also intriguing. Paxillin contains multiple domains that can interact with FAK, vinculin and other cytoskeletal proteins (Turner and Miller, 1994) and this intermediate has been shown to be negatively regulated by PDGF-induced PI3K activity (Rankin et al., 1996). Previous studies have established a link between activation of FAK and paxillin by tyrosine phosphorylation and reorganization of the actin cytoskeleton (Rankin and Rozengurt, 1994). The association of the p85 signalling complex with the AT<sub>1</sub> receptor was indicated in the

Western blots; however, we failed to detect PI3K activity in AT<sub>1</sub> immunoprecipitates. This lack of PI3K activity does not necessarily contradict the association observed between the AT<sub>1</sub> receptor and PI3K since a dissociation of p110 from p85 may have occurred at this time after AngII treatment. Alternatively, the conditions used during the immunoprecipitation may destabilize an indirect association between p85 and the AT<sub>1</sub> receptor. Similar to the insulin receptor system where p85 does not bind directly to the receptor but instead, associates with IRS-1, little PI3K activity was observed in anti-insulin receptor immunoprecipitates; however, anti-IRS-1 immunoprecipitates contained significant PI3K activity (Ruderman et al., 1990). Future studies with this SMC system should investigate the ability of Src kinases to link the AT<sub>1</sub> receptor to PI3K and possibly activate the intermediates identified in the signalling complex since preliminary results did indicate the association of Src with p85.

### **6.3.3 Mechanism of PI3K Regulation by AngII**

Two potential mechanisms for regulating the recruitment of p85 by AngII and the downstream effects of PI3K were investigated in this study: changes in subcellular localization and modification of p85 by tyrosine phosphorylation. Tyrosine phosphorylation of p85 may represent a key activation event in PI3K activity and/or association with other signalling intermediates. In addition, the subcellular localization of PI3K likely plays a key role in targeting the active enzyme to the site of its substrates. In PCA SMCs, the kinetics of both PI3K activation and tyrosine phosphorylation of the regulatory subunit p85 in response to AngII suggest these events are coupled. In quiescent SMCs, staining for p85 exhibited a diffuse pattern concentrated in the nuclear/perinuclear region. Our results are in agreement with previous reports which indicate that there is a large pool of available PI3K in resting cells (Šuša et al., 1992). Stimulation with Ang II resulted in the translocation of p85 from the nuclear region to the cytoplasm where it exhibited a punctate pattern of distribution. Our double immunofluorescent staining for p85 and actin fibers demonstrated

that a large proportion of p85 did not associate with actin stress fibers. These data were further supported by Western blot analysis of subcellular fractions of AngII-treated SMCs that demonstrated an increase in p85 in the membrane fraction and a parallel decrease in the cytoskeletal and nuclear fractions within a time period consistent with p85 tyrosine phosphorylation and translocation. A significant increase in the amount of p85 within the nucleus was demonstrated 30 minutes after AngII treatment by immunofluorescence microscopy and Western blot analysis.

Although there is no report in the literature on the dynamic distribution pattern of p85 in SMCs in response to a growth factor such as AngII, the patterns and kinetics observed are very similar to those previously described by Kapellar et al. (1993) in PDGF-stimulated 3T3-L1 fibroblast cells. Their study describes an association of p85 with the microtubule network which is consistent with a role for PI3K in microtubule-based motility. Since it has been postulated that PI3K mediates the reorganization of the cytoskeletal apparatus in cell growth, AngII-dependent tyrosine phosphorylation of adhesion factors such as paxillin (Leduc and Meloche, 1995) and FAK (Turner et al., 1995; Polte et al., 1994) may be important for defining the subcellular localization of PI3K. In addition, a recent study supports our data of p85 subcellular localization with the conclusions that p85 does not associate with actin stress fibers but it is confined to Golgi-like distribution and a smaller portion in actin-associated membrane sites (Johanson et al., 1999). This pattern of p85 subcellular distribution would be consistent with a role for p85 PI3K in vesicle trafficking. Another established role for PI3K is the control of endosome trafficking and receptor endocytosis (Shepherd et al., 1996; Li G et al., 1995). The punctate staining observed in our SMC system and the increase in the p85 content of the membrane fraction with AngII stimulation, may reflect an increase in PI3K association with endosomes. A similar pattern of immunofluorescence has been observed for Src kinase, a protein also associated with

endosomes (Kaplan et al., 1992; David-Pfeuty and Nouvian-Dooghe, 1990). The association of PI3K with endosomes would provide an opportunity for p85 to colocalize and potentially directly interact with the AT<sub>1</sub> receptor following receptor internalization into endosomes or caveolae. In fact, immunofluorescent analysis of AT<sub>1</sub> receptor distribution did reveal an intracellular, endosomal pattern following AngII stimulation of PCA SMCs (Saward et al., submitted).

A parallel analysis of the cellular distribution of the p110 catalytic subunit by immunofluorescent microscopy revealed a similar pattern of staining to p85 in quiescent PCA SMCs. In agreement with the p85 analysis, AngII stimulation resulted in a rapid translocation of p110 from the nuclear region to foci throughout the cytoplasm. Previous reports of p110 subcellular localization revealed different patterns of regulation for different isoforms of p110 within the same cell type. The p110 $\alpha$  was shown to associate with actin filaments whereas the p110 $\gamma$  colocalized with tubulin in microtubule network (Johanson et al., 1999). Different subcellular locations were also noted in sympathetic and sensory neurons of mice with p110 $\alpha$  found predominantly in the plasma membrane while p110 $\beta$  and p110 $\gamma$  localized in the perinuclear region (Barlett et al., 1999). The possibility that the antibody to p110 used in this study may be detecting multiple isoforms ( $\alpha,\beta,\delta$ ) should be considered. In addition, the Ang II-mediated translocation of p110 to the putative nucleoli presents another interesting avenue for future research. The potential role for p110 kinase activity in the direct regulation of transcription by Ang II must be investigated. Interestingly, recent studies have demonstrated AngII-induced nuclear targeting of the AT<sub>1</sub> receptor (Lu et al., 1998b) and AT<sub>1</sub>-mediated translocation of Raf-1 kinase (Lu et al., 1998a) to the nucleolus, possibly through MAPK-dependent mechanism (Lu et al., 1998b). Although the function of these molecules localized in the nucleolus is not known, previously a direct role for nuclear AngII has been postulated based on the ability of Ang II to increase RNA synthesis and alter

chromatin conformation in isolated nuclei (Re and Parab, 1984; Re et al., 1983). Future studies should address the intriguing possibility that the direct translocation of signal effectors such as PI3K and Raf-1 to the nucleolus may play a direct role in the regulation of rRNA

#### **6.3.4 Association of PI3K p85 and p110 Subunits**

Various isoforms of p85 ( $\alpha$  and  $\beta$ ) and p110 ( $\alpha$  and  $\beta$ ) associate to form functional PI3K; however, the role of each p110 and p85 isoform in assembly of functionally-active PI3K remains to be defined. A generally accepted concept is that the catalytic p110 subunit, without a regulatory subunit, does not exhibit any PI3K activity (Hiles et al., 1992); however, membrane-targeted p110 $\alpha$  subunit (p85-free) has been shown to activate signal transduction (Klippel et al., 1996). Our study revealed significant differences in the subcellular localization of the p85 and p110 subunits. After 30 min of AngII treatment, the pattern of p110 distribution did not return to the nuclear/ perinuclear pattern observed in quiescent SMCs. In addition, the localization of p110, but not p85, within the nucleoli following AngII treatment was noted. These visual observations were supported by a subcellular fractionation study of p110 from different cellular compartments of AngII-treated SMCs that demonstrated an increase in p110 in the cytoskeletal and nuclear fractions within the time period examined microscopically. To directly address the question of kinetics of p85 and p110 association, immunoprecipitation experiments with anti-p85 and anti-p110 antibodies were performed. These data clearly demonstrated that AngII-stimulation results in a pool of p110, detected with the p110 antibody used in this study (p110 $\alpha$  or  $\beta$ , not  $\gamma$ ), that is independent of p85. Whether the p85-independent p110 is catalytically active and/or is coupled to a unique regulatory unit such as p55 (Inukai et al., 1996; Pons et al., 1995) remains to be determined.

Although the biological significance of the differences noted in p85 and p110 localization is not addressed in this study, these data strongly support the concept that p85-

independent p110-associated PI3K activity may be involved in AngII-mediated signal transduction. In agreement with these observations, a recent study has identified a pool of p110 $\alpha$  that is p85-independent, since depletion of p85 from cell lysates resulted in only partial removal of p110 $\alpha$ -associated PI3K activity (Johanson et al., 1999). A specific role for this PI3K activity was indicated for actin stress fiber formation in a study utilizing thrombin, another G-protein-coupled receptor system. It was observed that thrombin increased the association of p85-independent p110 $\alpha$  with p70 S6 kinase and the actin network. The possibility that another regulatory subunit is coupled to p110 $\alpha$  merits further investigation. Thus, our concept of the biological function of PI3K must broaden to integrate a role for differentially localized pools of PI3K that are subject to distinct regulation and unique functions.

### **6.3.5 Contribution of PI3K to AngII-mediated Events**

In addition to establishing a direct link between PI3K activity and AngII, the functional contribution of PI3K to AngII-dependent SMC growth was addressed with the specific PI3K inhibitor LY294002. This study clearly demonstrates that inhibition of PI3K with LY294002 prevents the stimulation of SMC growth by AngII, as determined with both RNA and DNA synthesis, as well as cellular hyperplasia. The delayed addition experiments with LY294002 suggest that PI3K may be involved in both the early and late events in AngII-stimulated growth. Since PI3K participates in the early tyrosine phosphorylation cascades leading to activation of MAP kinase and S6 kinase (Romanelli et al., 1999; Hawes et al., 1996), as well as membrane traffic processes and cytoskeletal organization (Shepherd et al., 1996; Li G et al., 1995; Kapeller and Cantley, 1994), it is plausible that activation of PI3K by AngII is necessary for both intracellular signalling and structural reorganization. Activation of pp60<sup>src</sup> by AngII (Ishida et al. 1995) provides a plausible mechanism by which PI3K integrates these events. It has become evident that *c-src* is a critical mediator of cell

proliferative processes, influencing a variety of intracellular events. Karnitz *et al.* (1994) reports that members of the Src-kinase family control PI3K activation by IL-2. Thus, the binding and phosphorylation of proteins such as FAK by *c-src* may influence both the intracellular location and activity of PI3K.

It is clear that treatment of SMCs with AngII results in the immediate stimulation of protein phosphorylation and gene expression, eventually leading to DNA synthesis and cell division. These metabolic changes are often accompanied by a reorganization of the cytoskeletal apparatus, an event that alters cell shape and provides the spatial cues needed for mitosis. With these criteria, we have established that both the catalytic activity and the subcellular localization of PI3K respond to AngII and these events correlate with tyrosine phosphorylation of the p85 regulatory subunit. Furthermore, AngII stimulation resulted in a rapid association of the p85 subunit with IRS-1, paxillin and possibly the AT<sub>1</sub> receptor as well as altered the association with FAK, within a 15 minute time period, consistent with the observed increase in PI3K activity.

### 6.3.6 Specificity of PI3K Inhibitors

One of the keys to unravelling the complex array of functions associated with PI3K has been the availability of specific PI3K inhibitors. The most popular PI3K inhibitor, LY294002 {2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one} was synthesized by modification of the broad spectrum tyrosine kinase inhibitor quercetin (Vlahos *et al.*, 1994). This compound was identified as a highly specific inhibitor of PI3K with an IC<sub>50</sub> of 1.4  $\mu$ M that exhibited no effects on other ATP-requiring enzymes such as PI 4-kinase, insulin receptor, PDGF receptor, MAP kinase, PKA, PKC and ATPase (Vlahos *et al.*, 1994). Although LY294002 has been shown to specifically inhibit the formation of 3'-phosphorylated phosphoinositides (Vlahos *et al.*, 1994) and the downstream effects of PI3K activation in a number of cell types (Yano *et al.*, 1995; Sanchez-Margalet *et al.*, 1994),

another target of this inhibitor is the kinase termed mammalian target of rapamycin (mTOR) (Brunn et al., 1996). mTOR, a key enzyme for the G<sub>1</sub> to S phase transition in cell cycle progression, was identified as an enzyme amenable to inhibition by LY294002 and wortmannin due to the similarity between the catalytic domain of mTOR and PI3K. Wortmannin is another commonly used PI3K inhibitor that is structurally unrelated to LY294002 (Powis et al., 1994), but has been reported to inhibit several unrelated enzymes, such as myosin light chain kinase (Arcaro et al., 1993), phospholipases C and D (Bonser et al., 1991), and PLA<sub>2</sub> (Cross et al., 1995), as well as mTOR (Brunn et al., 1996). Although we have confirmed that both wortmannin and LY294002 block AngII- and insulin-mediated SMC growth (data not shown), the questionable specificity of wortmannin led to the selection of LY294002 as the inhibitor of choice for our experiments. The efficacy of LY294002 in PCA SMCs demonstrates that the rapid activation of PI3K by AngII may be an essential requirement for cell proliferation. Although it is possible that PI3K inhibitors are affecting mTOR directly in this SMC system, the direct stimulation of PI3K by AngII and physical association of p85 with the AT<sub>1</sub> receptor suggest this is unlikely. Subsequent work in this laboratory has reconfirmed that LY294002 is selective for PI3K in PCA SMCs, as demonstrated by the inhibition of AT<sub>1</sub>-mediated regulation of AT<sub>2</sub> mRNA expression with LY294002 but not the mTOR inhibitor, rapamycin (Zahradka et al., 1998).

#### **6.4 CONCLUSIONS**

The changes in PI3K activity and translocation of the p85 and p110 subunits clearly indicate that PI3K is an important signal transduction intermediate for AngII. Furthermore, the inhibition of AngII-dependent RNA and DNA synthesis and cellular hyperplasia by

LY294002 support the hypothesis that early activation of PI3K may be necessary for AngII-mediated cell cycle progression. Given that AngII is a growth factor for vascular tissue and potentially contributes to the pathophysiology of several cardiovascular diseases, the identification of PI3K as an important mediator of SMC growth *in vitro* warrants further investigation with *in vivo* models of vascular growth.

## **7. INSULIN INTERACTION WITH RENIN-ANGIOTENSIN SYSTEM IN SMC**

### **7.1 INTRODUCTION**

Another independent risk factor for the development of cardiovascular disease has been identified as insulin resistance, and consequently hyperinsulinemia, however, the mechanism by which insulin affects the cardiovascular system remains undefined (Sowers, 1992; Tuck et al., 1992; Ferrannini *et al.*, 1991). The renin-angiotensin system (RAS), which plays a key role in the pathophysiology of the cardiovascular system, has also been implicated in the hyperinsulinemic state based on the decrease in insulin resistance observed clinically in humans (Galletti et al., 1999; Morris *et al.*, 1994; Torlone et al., 1993) and in animal models of insulin-resistant hypertension (Kost et al., 2000; Nawano et al., 1999; Ura et al., 1999) obtained with ACE inhibitor treatment. Recently, ACE polymorphisms have been associated with hyperinsulinemia and Type 2 diabetes (Bengtsson et al., 1999). This interaction between two apparently independent physiological systems, the RAS and the insulin-glucose transport system, has initiated a new area of research to define the level of cross-talk.

The effect of long-term exposure to insulin on AngII-mediated growth was examined in the A10 SMC line: (i) The effect of pretreatment with insulin and the length of incubation required to induce an AngII-responsive state were defined. (ii) The growth response to AngII and receptor subtype contribution was evaluated at the level of ribosomal RNA synthesis, cell size and proto-oncogene *c-fos* expression. (iii) The ability of A10 SMCs to respond to insulin, IGF-1 and synergism with AngII was examined. (iv) The regulation of AngII receptor levels by insulin was examined as a possible mechanism for insulin regulation of the renin-angiotensin system at the cellular level. These data provide a better understanding of how insulin facilitates the growth promoting activities of AngII at the cellular level.

## 7.2 RESULTS

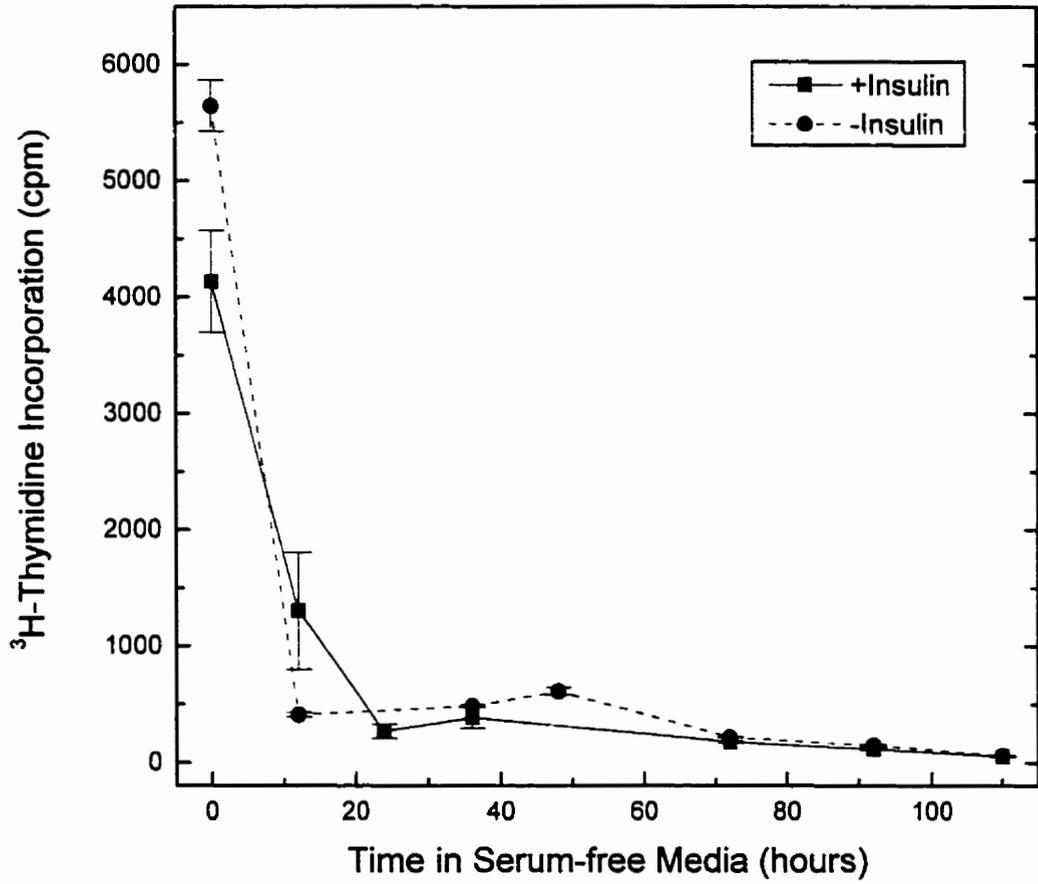
### 7.2.1 Effect of Long-term Insulin Exposure on A10 SMC Differentiation:

Insulin is an important agent for both the differentiation and growth of smooth muscle cells (Stout, 1991). The effect of pathophysiological concentrations of insulin on the SMC phenotype was characterized in the A10 SMC cell line. During the establishment of quiescence, insulin ( $10^{-8}$  M) did not alter the time required for proliferating A10 cells to reach quiescence nor the basal rate of DNA synthesis (Figure 32). In these experiments, a "differentiated" state was induced in A10 cells after a 96 hour incubation in serum-free medium containing insulin ( $10^{-8}$  M) to ensure that the cells were quiescent according to their rate of DNA synthesis and that sufficient time had expired to allow expression of the smooth muscle phenotype. Immunohistochemical analysis demonstrated that under the defined conditions, A10 cells exhibit definitive markers for differentiated smooth muscle with fibrillar staining pattern for smooth muscle isoforms of  $\alpha$ -actin, myosin heavy chain (204, 200 kDa) and h-caldesmon (150 kDa)(Figure 33). Additionally, the inclusion of insulin during the differentiation period did not influence the expression or organization of the muscle-specific markers  $\alpha$ -actin and myosin.

### 7.2.2 Characterization of AngII Receptor Population in A10 cells:

To define the angiotensin receptor subtypes expressed in the different growth states, RT-PCR analysis was employed. In growing A10 SMCs, mRNA for the  $AT_2$  as well as the  $AT_{1A}$  and  $AT_{1B}$  receptors was present; however, only the  $AT_2$  receptor was expressed in quiescent A10 cells (Figure 34A). To avoid variations in loading, each reaction for growing or quiescent conditions was aliquoted from a common mix and the specific primers were added. Negative and positive control lanes confirmed the primers used were specific to the respective receptor sequences and produced single bands of the expected size (data not shown).

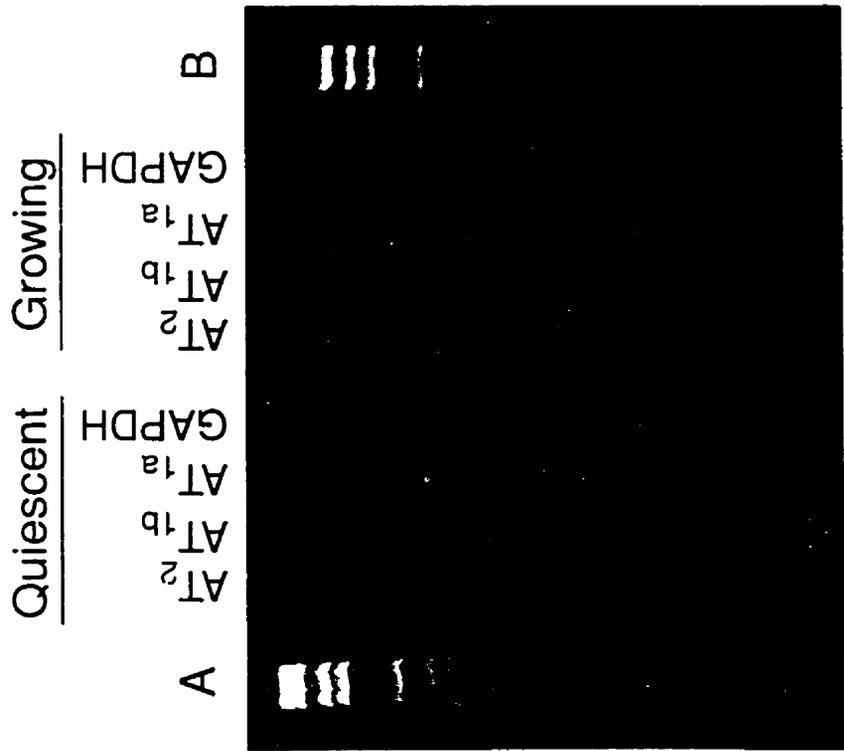
**FIGURE 32: *Effect of Long-term Insulin Exposure on Cell Cycle Withdrawal in A10 SMCs.*** The growth medium for A10 SMCs was replaced with defined serum-free medium with (■) or without (●) insulin ( $10^{-8}$  M) supplementation. At specific time points over a 5 day period,  $^3\text{H}$ -thymidine was added to triplicate sets of cells for 30 min to measure the rate of DNA synthesis as described in “Materials and Methods”. Each value represents the mean  $\pm$  S.E. for three separate experiments.

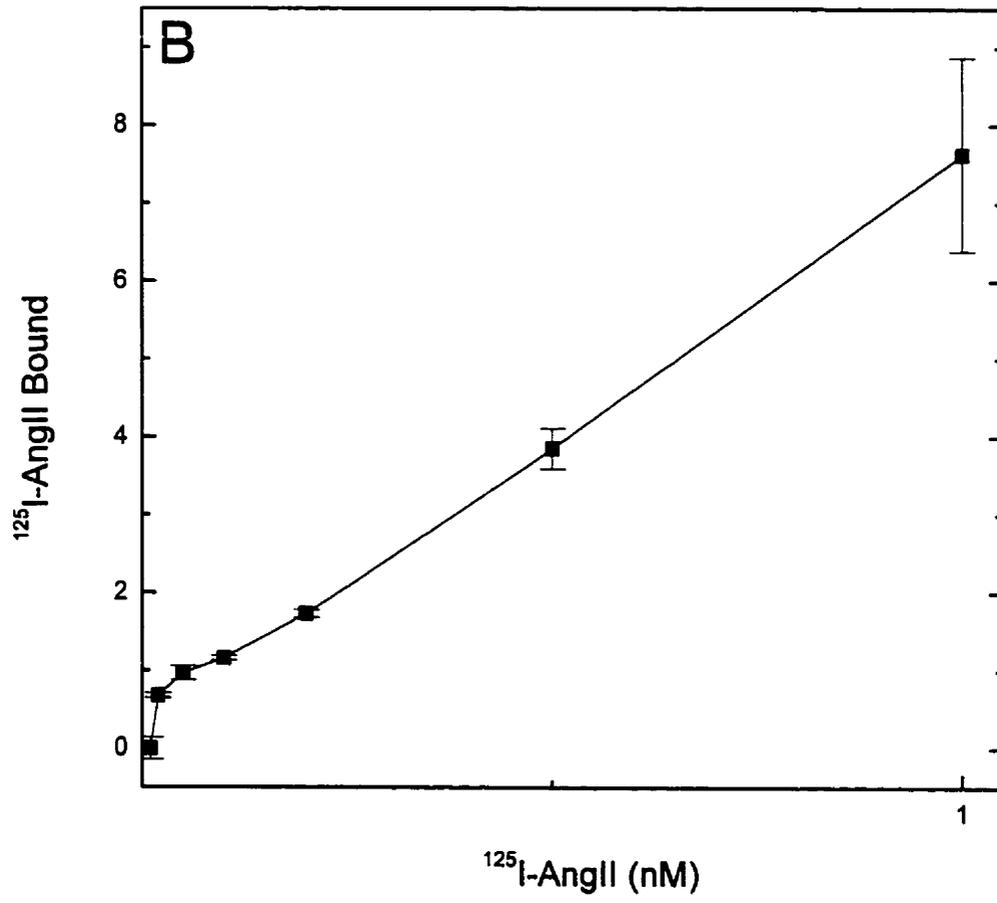


**FIGURE 33: *Smooth Muscle Cell Phenotype of Quiescent A10 cells.*** Quiescent A10 cells were stained with antibodies to smooth muscle-specific isoforms of  $\alpha$ -actin (Panel A), myosin heavy chain (Panel B) and caldesmon (Panel C) and visualized by indirect immunofluorescent microscopy. Each panel represents the staining observed in >90% of the cells. Bar=100  $\mu$ m.



**FIGURE 34: Characterization of AngII receptors in A10 SMCs.** Panel A: The expression of AngII receptor subtypes AT<sub>1A</sub>, AT<sub>1B</sub> and AT<sub>2</sub> with respect to GAPDH was examined using RT-PCR. The receptor subtype expression pattern is illustrated for both quiescent and growing A10 populations. DNA size markers are provided in lanes marked A (mixture of *Hinf*I and *Bgl*II digested pBR328) and B (*Hae*III digested  $\phi$ X174). Panel B: The presence of AngII receptors on quiescent A10 SMCs was examined by [<sup>125</sup>I]-SarIle AngII binding studies as described in "Materials and Methods. Specific binding was measured in the presence of increasing concentrations of [<sup>125</sup>I]-SarIle AngII (0.01-1 nM). Non-specific binding was determined in the presence of unlabelled AngII (10<sup>-6</sup> M). Each value represents the mean  $\pm$  SE of at least three separate experiments.





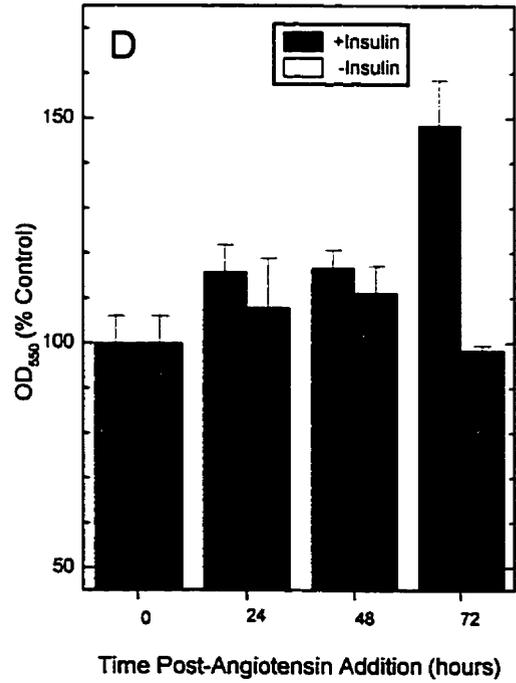
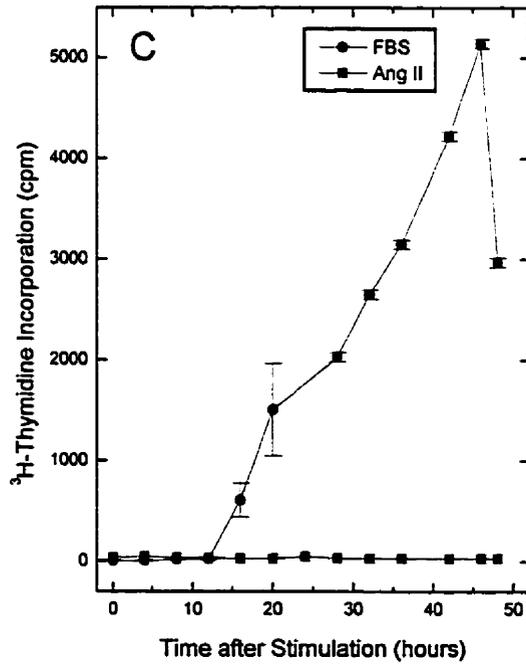
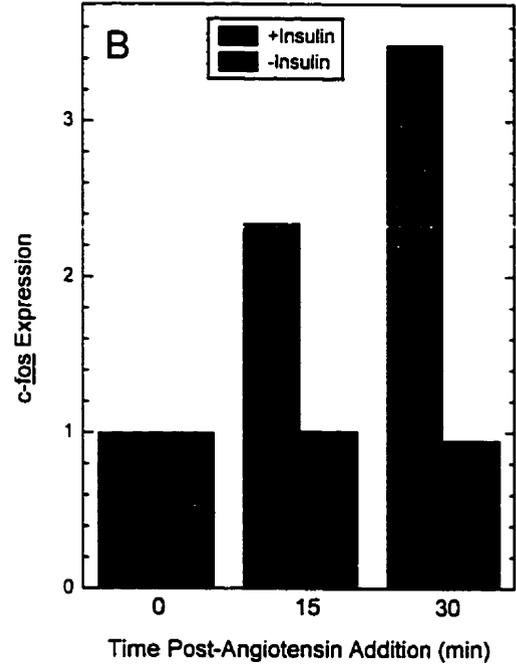
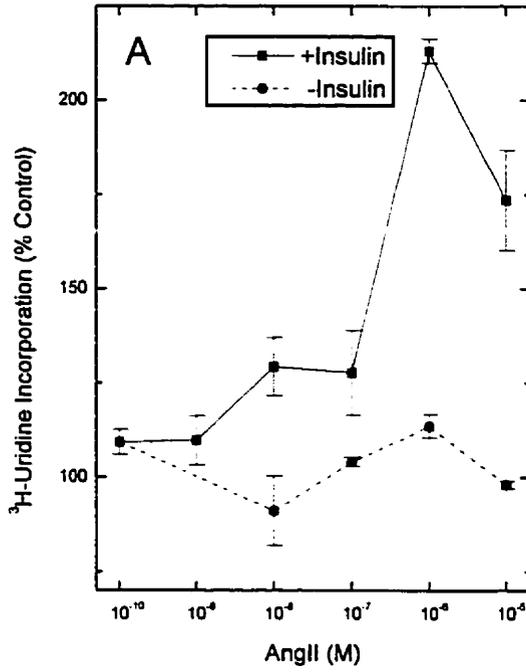
To confirm the presence of an AngII receptor population in differentiated A10 SMCs at the protein level, the binding profile of varying concentrations of [<sup>125</sup>I]-SarIle AngII (0.01-1 nM) was examined on monolayers of quiescent A10 SMCs differentiated in the presence insulin (10<sup>-8</sup> M). The non-specific binding was based on the levels of [<sup>125</sup>I]-SarIle AngII in the presence of excess unlabelled AngII (10<sup>-6</sup> M). As shown in Figure 34B, a population of AngII receptors was detected with a high level of specific binding observed with 0.125-1 nM [<sup>125</sup>I]-SarIle AngII.

### 7.2.3 Long-term Insulin Exposure Promotes AngII-mediated SMC Growth:

An early indicator of cell growth is ribosomal RNA synthesis which can be monitored by the rate of [<sup>3</sup>H]-uridine incorporation due to the high proportion of rRNA to mRNA (Zahradka and Yau, 1994). The ability of AngII (10<sup>-10</sup>-10<sup>-5</sup> M) to stimulate RNA synthesis was evaluated in A10 cells differentiated in the presence or absence of insulin (10<sup>-8</sup> M)(Figure 35A). No increase in RNA synthesis was observed upon addition of AngII when the differentiation media lacked insulin. In contrast, cells incubated in differentiation media containing insulin (10<sup>-8</sup> M) responded to AngII (10<sup>-6</sup> M) treatment with a reproducible increase in RNA synthesis (max 214%±3 of unstimulated control). It is important to note that inclusion of insulin (10<sup>-8</sup> M) in the differentiation media did not alter the level of basal RNA synthesis in quiescent SMCs (data not shown), confirming the previous supposition that insulin is not affecting the quiescent SMC phenotype or acting as a direct mitogen under these conditions.

Another frequently used marker of the early growth response is the expression of the proto-oncogene *c-fos*. Treatment with AngII (10<sup>-6</sup> M) induced a rapid increase in *c-fos* mRNA, relative to ribosomal protein mRNA, only when the A10 cells were differentiated in the presence of insulin (10<sup>-8</sup> M) (Figure 35B). The results shown represent the densitometric analysis of *c-fos* mRNA levels relative to the ribosomal protein L32 mRNA

**FIGURE 35: *Insulin-dependent Promotion of SMC Growth by AngII.*** Panel A: RNA synthesis was measured by the rate of [<sup>3</sup>H]-uridine incorporation over a 6 hour period following addition of AngII to quiescent A10 cells that had been incubated for 96 hours in defined medium with (■) or without (●) insulin (10<sup>-8</sup> M). Panel B: Expression of *c-fos* mRNA at varying times after AngII treatment (0-30 min) was monitored by slot blot hybridization of total RNA from A10 cells differentiated in the presence (shaded) or absence (unshaded) of insulin (10<sup>-8</sup> M). Values are expressed in densitometric units relative to the level of ribosomal protein L32 mRNA. These results represent the typical pattern of *c-fos* expression observed in three independent experiments. Panel C: The time course required for quiescent A10 SMCs to traverse G1 and enter S phase after stimulation with 20% FBS (●) in comparison to 10<sup>-6</sup> M AngII (■) is shown. At the indicated time points, the rate of [<sup>3</sup>H]-thymidine incorporation into DNA was measured over a 30 minute period as described in "Materials and Methods". Panel D: Hypertrophic growth of A10 cells was determined by monitoring cellular mitochondrial activity via reduction of MTT to a product detectable at 550 nm. Cell growth was measured at 24, 48 and 72 hours after the stimulation with AngII (10<sup>-6</sup> M) of triplicate sets of cells previously incubated for 96 hours in the presence (shaded) or absence (unshaded) of insulin (10<sup>-8</sup>). In all panels, the value of unstimulated controls were set at 100% and each data point represents the mean ± SE of at least three separate experiments.

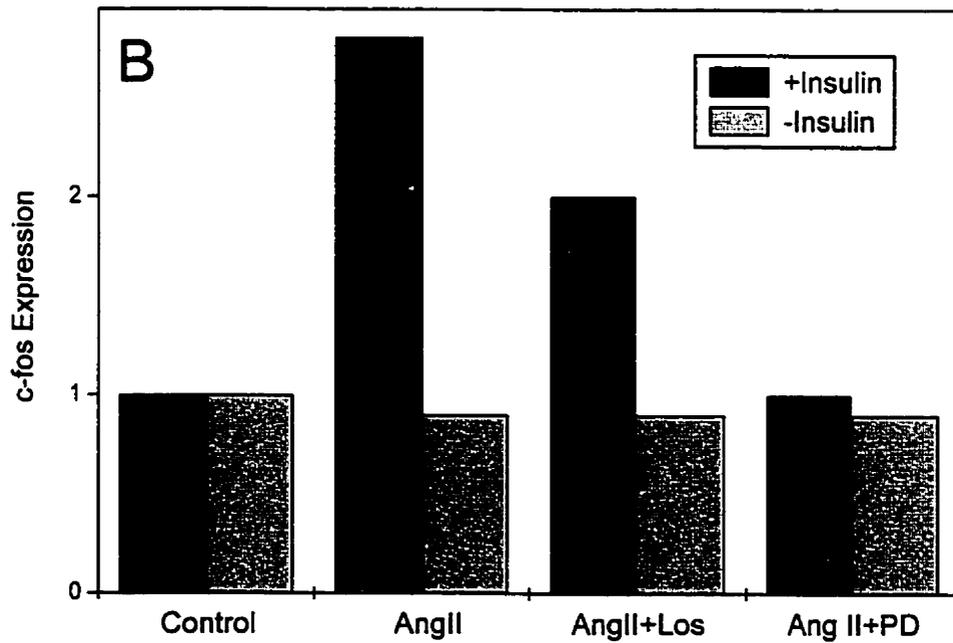
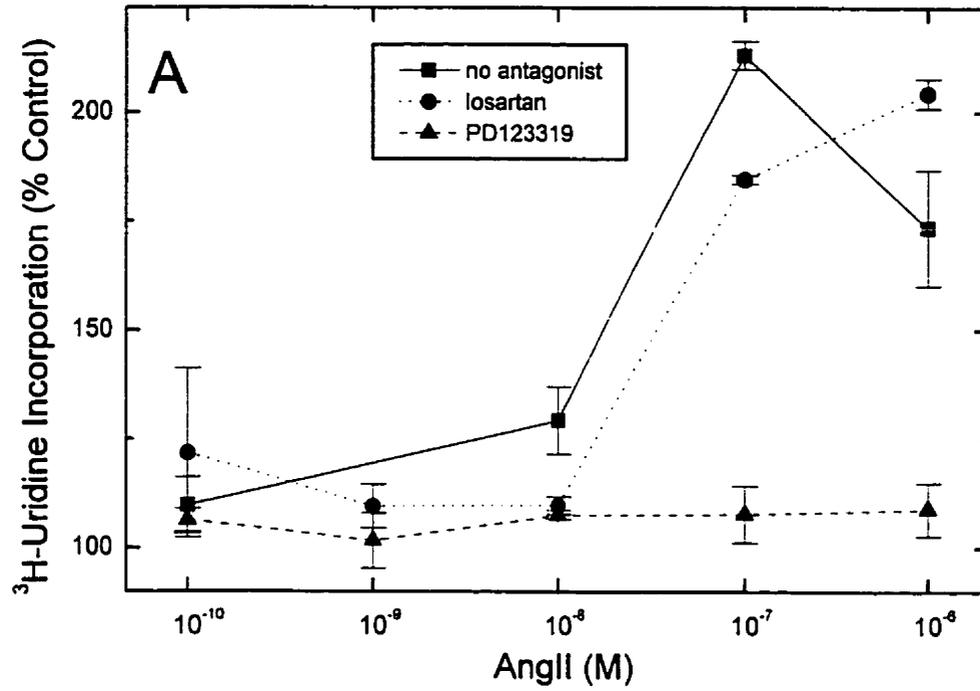


from a representative experiment of the pattern observed in three separate experiments. This result supports the conclusion that insulin pretreatment mediates the induction of A10 SMC growth by AngII.

DNA synthesis represents another key parameter of cellular growth that is involved in both the hypertrophic and hyperplastic growth response. In contrast to the other growth assays, short-term analysis of the level of DNA synthesis (48 hours) revealed that AngII did not stimulate DNA synthesis in A10 SMCs differentiated in the absence or presence of insulin pretreatment (data not shown). As a result, the time-course required for the differentiated A10 SMCs to traverse G1 and enter S phase in response to AngII or FBS stimulation was monitored by the rate of [<sup>3</sup>H]-thymidine incorporation into DNA during 30 minute pulses over an extended period of time (4 days)(Figure 35C). Treatment with FBS (20%) increased the rate of DNA synthesis within 18 hours with maximum rates of DNA synthesis within 40 hours, indicative of a synchronous re-entry into the cell cycle and S phase. In comparison, AngII ( $10^{-6}$  M) did not stimulate DNA synthesis over a 4 day period. Based on these results, it can be concluded that AngII activates a set of early growth processes in A10 smooth muscle cells that are characteristic of a hypertrophic response that does not involve DNA synthesis.

To further investigate the insulin-dependence of the overall growth response to AngII, the colorimetric MTT assay was utilized (Shi et al., 1993). This assay, which measures the metabolism by active mitochondria of MTT to a coloured product that can be quantified by absorbance at 550 nm, provides a convenient method for evaluating the overall cellular response to a growth factor in the absence of DNA synthesis. In agreement with the other measurements of growth, the ability of AngII ( $10^{-6}$  M) to increase absorbance at 550 nm ( $149\% \pm 10$ ) over a 72 hour period was dependent on the presence of insulin ( $10^{-8}$  M) in the differentiation media (Figure 35D). Thus, all three assays for early and late cell growth

**FIGURE 36: *AngII* Receptor Subtype Contribution to A10 SMC Growth.** In Panel A, total RNA synthesis was measured by the rate of [<sup>3</sup>H]-uridine incorporation over a 6 hour time period following stimulation of quiescent SMCs by AngII ( $10^{-5}$ - $10^{-12}$  M) alone (■) or in the presence of the AT<sub>1</sub> receptor antagonist losartan ( $10^{-6}$  M)(●) and the AT<sub>2</sub> receptor antagonist PD123319 ( $10^{-5}$  M)(▲). Each data point represents the mean  $\pm$  SE of at least three separate experiments. The value of unstimulated controls was set at 100%. In Panel B, the inhibition of AngII-stimulated *c-fos* expression by losartan and PD123319 was monitored with slot blot hybridization. The relative level of *c-fos* mRNA in comparison to ribosomal protein L32 mRNA is shown in arbitrary densitometric units.



events indicate that long-term exposure to insulin was required to make A10 SMCs permissive to the growth stimulatory effects of AngII.

#### **7.2.4 AT<sub>2</sub> Receptor Mediates AngII-stimulated Growth of A10 SMCs:**

To define the receptor through which AngII stimulates rRNA synthesis, treatment with either losartan, a non-peptide antagonist of the AT<sub>1</sub> receptor, or PD123319, a non-peptide antagonist of the AT<sub>2</sub> receptor, was used to selectively block each receptor subtype (Figure 36A). Typically, losartan is an effective inhibitor of AngII-dependent cell growth (Chiu et al., 1991); however, losartan ( $10^{-6}$  M) was unable to inhibit the increase in the rate of RNA synthesis by AngII in quiescent A10 cells. This observation supports the RT-PCR data indicating an absence of AT<sub>1</sub> receptors in quiescent A10 SMCs. In contrast, the AT<sub>2</sub> antagonist, PD123319 ( $10^{-6}$  M), under identical conditions, blocked the stimulation of RNA synthesis by AngII. Thus, the AT<sub>2</sub> receptor mediates AngII-dependent stimulation of RNA synthesis in A10 cells which suggests that this receptor is associated with an early growth response, independent of the AT<sub>1</sub> receptor.

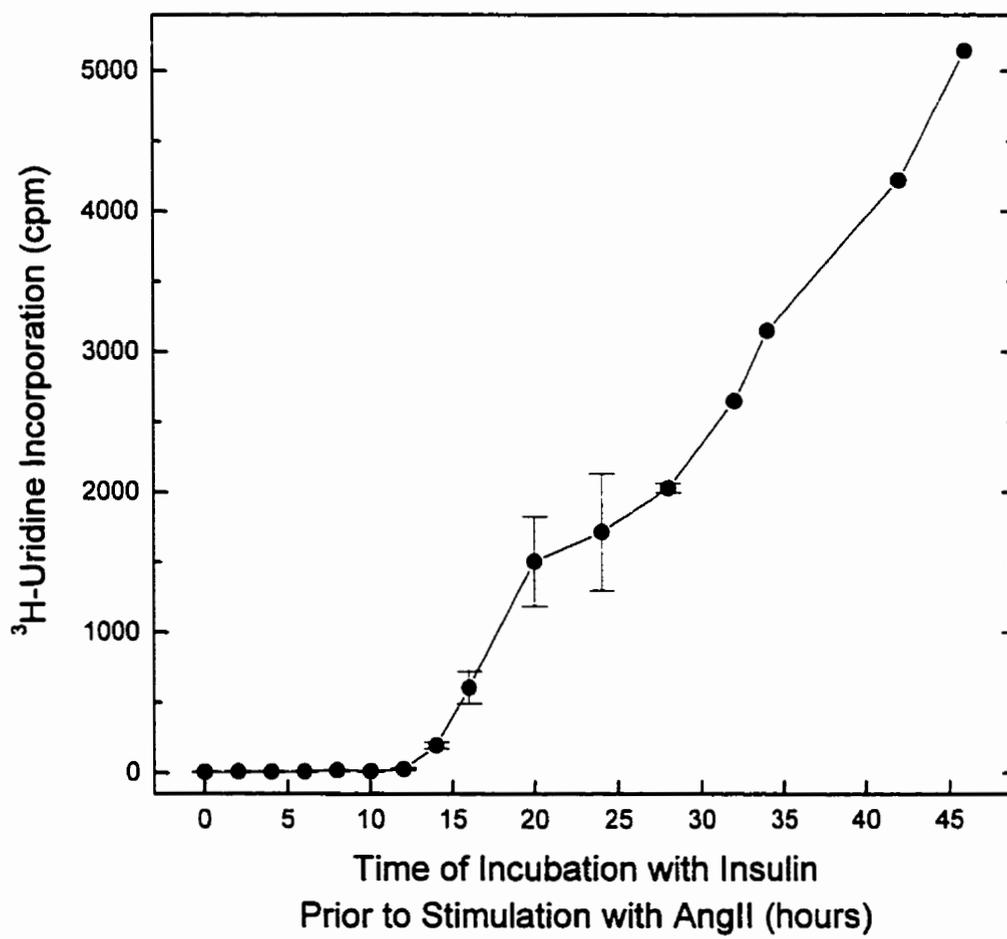
The contribution of the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes to the induction of *c-fos* mRNA by AngII was also examined. As shown in Figure 36B, AngII stimulated a visible increase in the levels of *c-fos* mRNA in comparison to the stable levels of ribosomal protein mRNA in A10 SMCs differentiated in the presence of insulin ( $10^{-8}$  M). The relative contribution of the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes was assessed by the efficacy of receptor antagonists losartan and PD123319 ( $10^{-6}$  M), respectively. Only PD123319 blocked the increase in *c-fos* mRNA stimulated by AngII. The data shown represents the relative densitometric units of *c-fos* mRNA relative to ribosomal protein L32 mRNA in representative results from three separate experiments.

#### **7.2.5 Length of Pretreatment with Insulin Required Prior to AngII Treatment:**

To define the temporal limits required for the induction of an AngII-responsive state

**FIGURE 37: *Time-course of Insulin Pretreatment for AngII-dependent RNA Synthesis.***

Growing A10 cells were incubated in serum-free defined media and insulin ( $10^{-8}$  M) was added at various time points during the differentiation period (total of 96 hours). Quiescent A10 SMCs were stimulated with AngII ( $10^{-6}$  M) and the level of RNA synthesis was measured by [ $^3$ H]-uridine incorporation over 6 hours. Each value represents the mean  $\pm$  SE of three separate experiments.



in A10 cells by insulin, insulin ( $10^{-8}$  M) was added to the differentiation media at various times prior to measuring AngII-dependent RNA synthesis. It was observed that a minimum incubation period of 18-24 hours with insulin was necessary before AngII could stimulate RNA synthesis significantly above control values (Figure 37). These data suggest that insulin's effects on the A10 cell phenotype requires an extended period of incubation that is sufficient for the synthesis of new proteins. Also, the level of AngII-mediated RNA synthesis correlated with the length of insulin incubation.

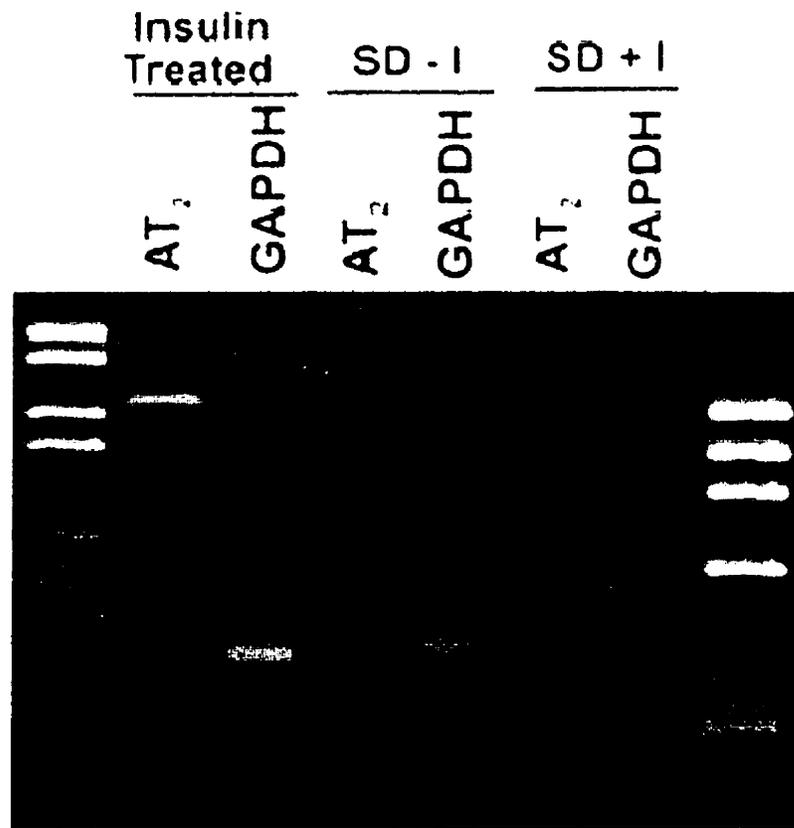
#### **7.2.6 Regulation of AT<sub>2</sub> Receptor Expression by Insulin:**

A potential target for insulin regulation of AngII-responsiveness in A10 SMCs is the AngII receptors. Due to the AT<sub>2</sub> receptor-dependent growth in A10 SMC, the ability of insulin to regulate AT<sub>2</sub> receptor mRNA in A10 SMCs was examined by RT-PCR (Figure 38). To obtain semi-quantitative data, all experiments were conducted simultaneously with an equal amount of total RNA added to each RT-PCR reaction and the results were examined relative to the level of the housekeeping protein, GAPDH. Quiescent A10 SMCs differentiated in the presence of insulin ( $10^{-8}$  M) exhibited an approximately 4-fold higher level of AT<sub>2</sub> receptor mRNA relative to GAPDH. The direct stimulation of quiescent A10 SMCs by insulin ( $10^{-6}$  M) increased the AT<sub>2</sub> receptor mRNA content approximately 12-fold higher than in cells not exposed to insulin. These data indicate that insulin directly regulates expression of the AT<sub>2</sub> receptor in A10 SMCs and thus has the potential to influence the cellular response to AngII.

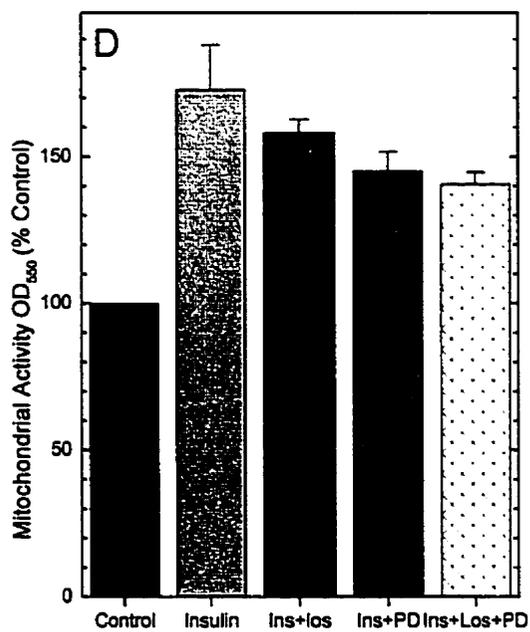
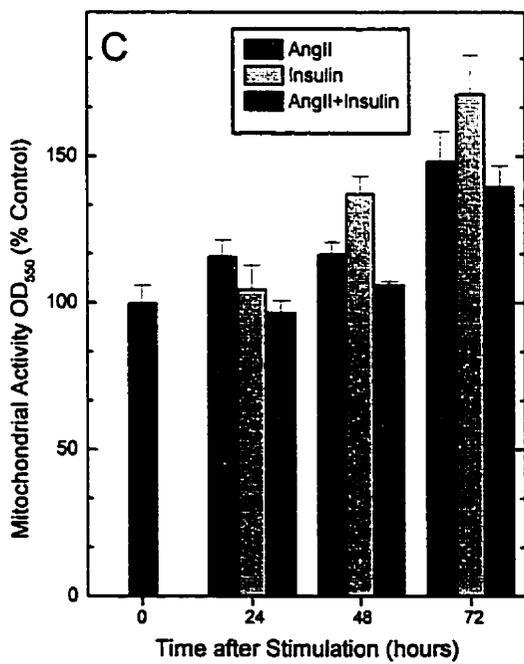
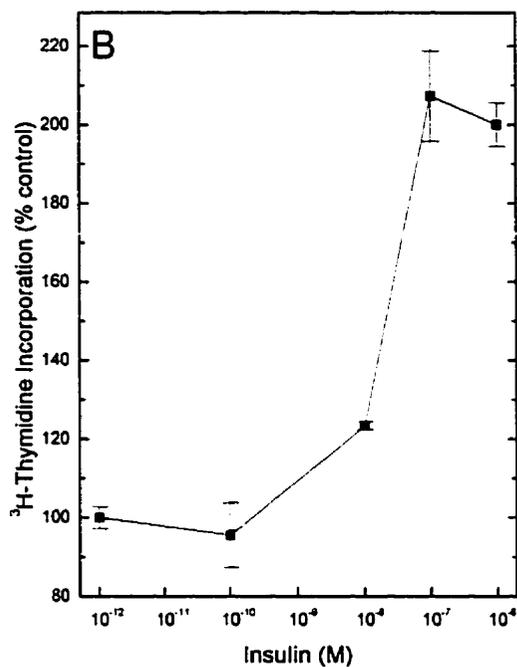
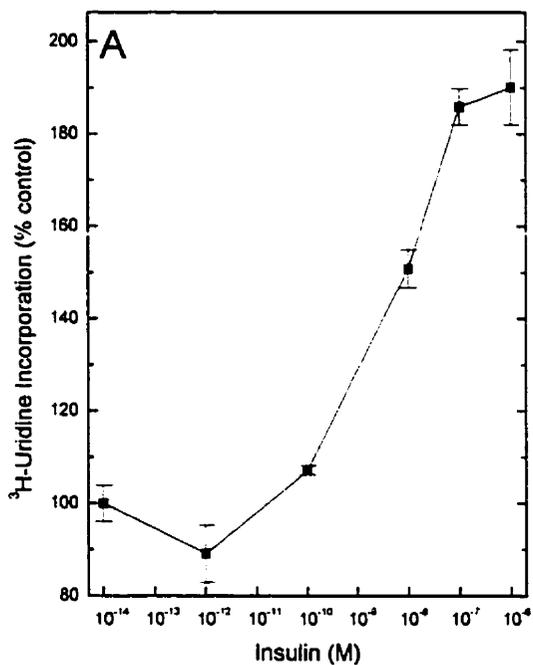
#### **7.2.7 The Effect of Insulin is not due to Direct Synergism with AngII:**

Since long-term exposure to pathophysiological concentrations of insulin has been associated with the development of insulin resistance, we examined the ability of insulin to mediate a growth response in A10 SMC differentiated in the presence of insulin ( $10^{-8}$  M). Treatment of quiescent A10 SMCs with insulin ( $10^{-12}$ - $10^{-6}$  M) resulted in a dose-dependent

**FIGURE 38: Regulation of AT<sub>2</sub> Receptor mRNA Levels by Insulin.** AT<sub>2</sub> receptor mRNA content was evaluated by RT-PCR in quiescent A10 SMC that had been incubated in differentiation media for 96 hours in the presence or absence of insulin (10<sup>-8</sup> M). In addition, the effect of insulin (10<sup>-6</sup> M) stimulation of quiescent A10 SMCs after 24 hours is shown. Total RNA was isolated and 1 µg was amplified as described in “Materials and Methods”. The AT<sub>2</sub> receptor mRNA levels were compared to the relative amount of GAPDH mRNA.



**FIGURE 39: *Effect of Insulin as a Growth Factor for A10 SMCs.*** RNA (panel A) and DNA (panel B) synthesis by A10 SMCs were measured following stimulation by insulin ( $10^{-14}$ - $10^{-6}$  M). Panel C: The growth of A10 SMCs in response to a 72 hour treatment with AngII ( $10^{-9}$  M; unshaded), insulin ( $10^{-6}$  M; shaded) or both (hatched) was monitored with the MTT assay (at 550 nm). Panel D: The effect of the AngII receptor antagonists losartan and PD123319 ( $10^{-6}$  M; hatched) on insulin stimulated ( $10^{-6}$  M; shaded) A10 SMC growth over for 72 hours. In all panels, each value represents the mean  $\pm$  SE of at least 3 separate experiments, and the value of unstimulated control cells were set to 100%.



increase in the rate of both RNA and DNA synthesis (Figure 39A,B). Thus, exposure of A10 SMCs to insulin during the differentiation period did not alter their capacity to respond to insulin.

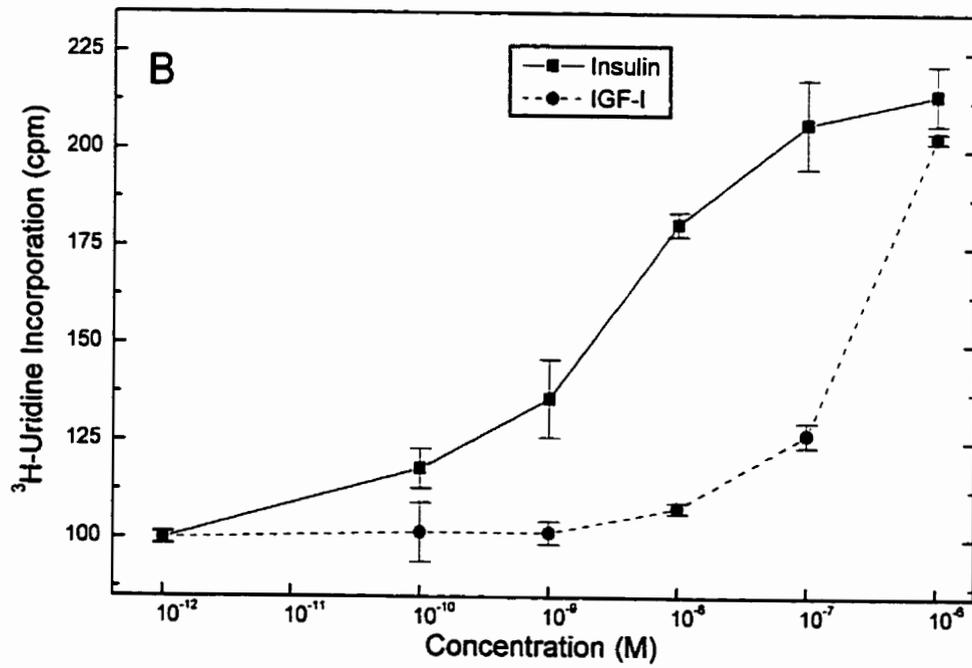
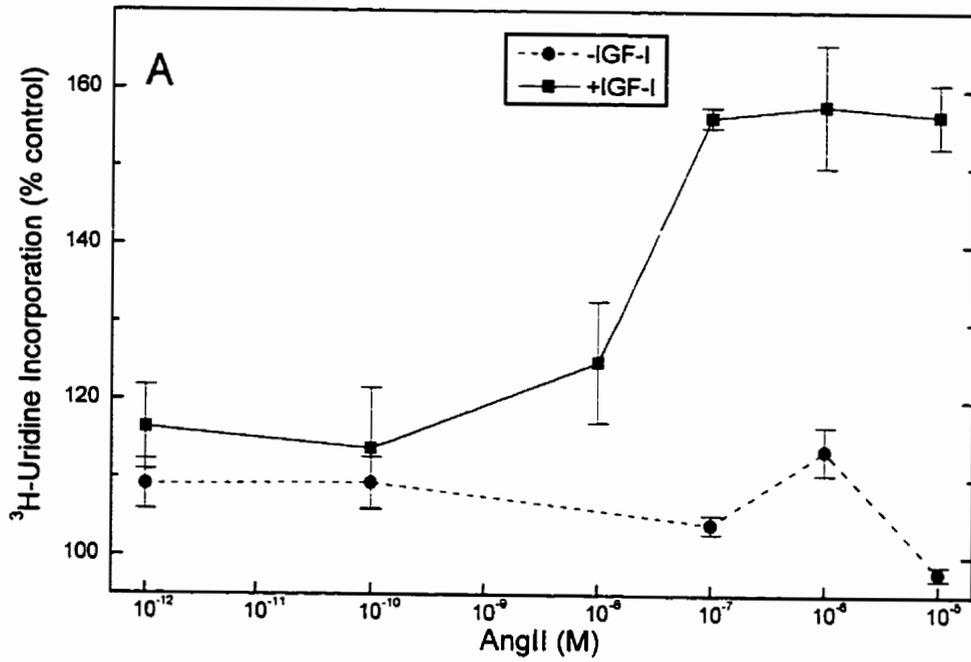
Since insulin can itself act as a growth factor for A10 SMCs at high concentrations, it was necessary to ascertain whether the observed AngII-responsiveness could be attributed to a direct synergy between insulin and AngII, both acting as growth factors. To determine whether insulin could influence the growth stimulatory actions of AngII directly, the ability of these agents, alone and in combination, to stimulate hypertrophy of quiescent A10 SMCs was assessed using the MTT assay. As previously established, the addition of either AngII ( $10^{-6}$  M) or insulin ( $10^{-6}$  M) stimulated an increase in cell size and/or number (Figure 39C). Simultaneous treatment with both AngII and insulin, however, did not enhance the hypertrophic response above that observed with either agent alone. It is interesting to note that the hypertrophic response was noticeably lower when A10 cells were stimulated with both insulin and AngII. These results support the premise that there is no direct synergism between these two growth factors and suggests that pretreatment with insulin is necessary to alter a specific cell component required for the response to AngII.

The level of interplay between insulin and RAS has not been defined at the cellular level. Recently, Kamide et al. (1998) reported that insulin-stimulated growth of aortic SMC could be attributed to activation of AngII receptors. Thus, the contribution of the signal transduction mechanisms of the AngII receptors to insulin-mediated growth was examined with the MTT assay. Insulin-stimulated hypertrophy of A10 SMCs ( $173\% \pm 15$ ) was partially inhibited by the inclusion of the  $AT_2$  receptor antagonist, PD123319 ( $140\% \pm 4$ ), and to a lesser extent by the  $AT_1$  receptor antagonist, losartan ( $158\% \pm 6.5$ ) (Figure 39D).

### **7.2.8 Comparison of Insulin and IGF-1 in A10 SMCs**

Due to the cross-reactivity of insulin with IGF-1 receptor system, the ability of IGF-1

**FIGURE 40: Comparison of Insulin and IGF-1 Treatment of A10 SMCs.** In Panel A, the effect of long-term exposure to IGF-1 ( $10^{-6}$  M) was examined. The ability of AngII ( $10^{-5}$ - $10^{-12}$  M) to stimulate RNA synthesis in A10 SMCs, differentiated in the presence (■) or absence (●) of IGF-1 ( $10^{-6}$  M) was monitored by [ $^3$ H]-uridine incorporation over a 6 hour period. In Panel B, the ability of IGF-1 to stimulate RNA synthesis in a dose-dependent manner ( $10^{-6}$ - $10^{-12}$  M) is shown in comparison to insulin, based on the incorporation of [ $^3$ H]-uridine over a 6 hour period. In all panels, the value of unstimulated control cells was set at 100%. Each value represents the mean  $\pm$  SE of at least three independent experiments.



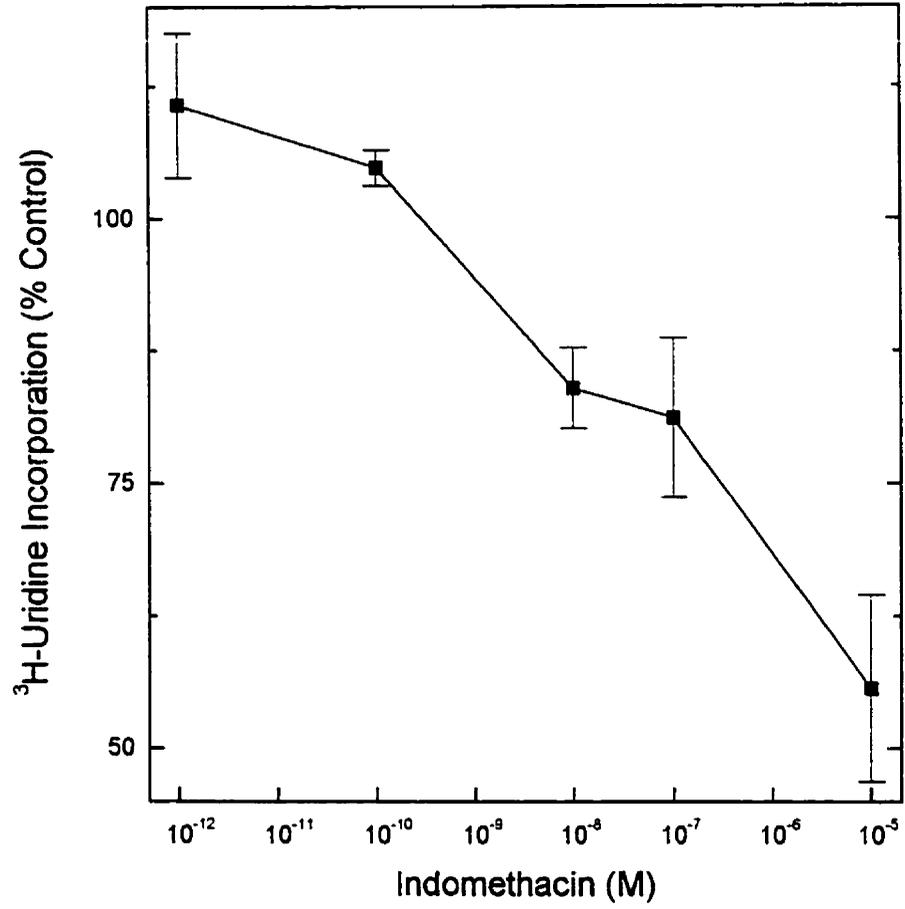
to mediate similar effects in A10 SMCs was investigated. The effect of IGF-1 pretreatment on AngII-mediated RNA synthesis was examined by the level of [<sup>3</sup>H]-uridine incorporation. A10 cells were incubated in serum-free defined media with IGF-1 ( $10^{-6}$  M) for 96 hours to induce quiescence. Similar to insulin-pretreated cells, AngII ( $10^{-12}$ - $10^{-5}$  M) stimulated a dose-dependent increase in the rate of RNA synthesis (Figure 40A). Cells in differentiation media lacking IGF-1 did not respond to AngII and maintained a basal level of RNA synthesis.

Ideally, to determine the specific receptor activated by both insulin and IGF-1 to mediate this change in SMC phenotype, specific antibodies to both receptors would be necessary. Due to the lack of availability of these antibodies, preliminary information on the active receptor in these cells was obtained based on the differences in the concentration-dependent stimulation of growth by insulin and IGF-1. Stimulation by insulin or IGF-1 resulted in an increase in total RNA synthesis (Figure 40B) in a dose-dependent manner; however, the rightward shift of the dose-dependency curve for IGF-1 indicates these growth factors act through the insulin receptor system (Yau et al., 1999). Although a direct correlation between the insulin receptor-mediated growth response and the insulin-mediated differentiated phenotype cannot be assumed, this observation suggests that the insulin receptor system is a likely candidate.

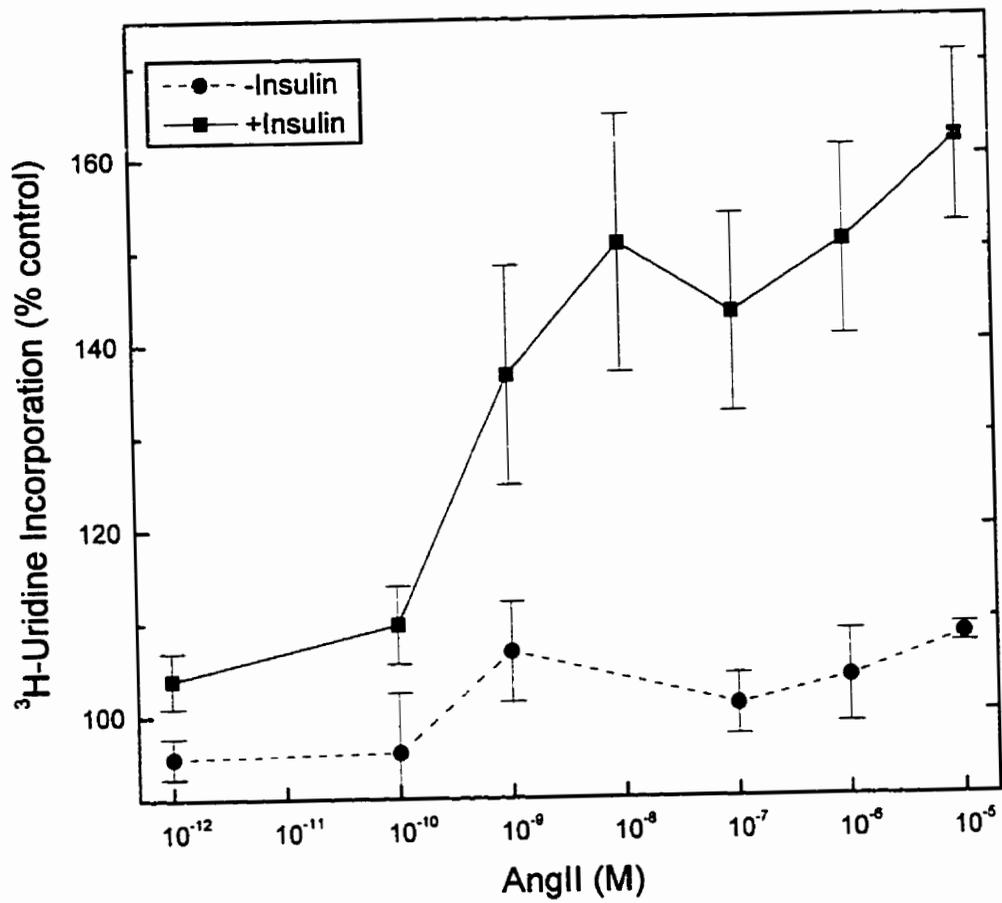
#### **7.2.9 AT<sub>2</sub> Receptor-mediated Prostanoid Synthesis in A10 SMC Growth:**

The involvement of prostaglandin synthesis in AT<sub>2</sub>-mediated growth was demonstrated in PCA SMCs (Chapter 5). The A10 SMC system characterized in this chapter provides an ideal system to corroborate this data due to the complete dependence of AngII-stimulated growth on AT<sub>2</sub> receptor activation in this system. The effect of indomethacin, a general cyclooxygenase inhibitor, on AngII-dependent RNA synthesis was examined. Indomethacin ( $10^{-10}$ - $10^{-5}$  M) blocked the stimulation of RNA synthesis by AngII in a concentration-dependent manner (Figure 41) which suggests prostaglandins are also

**FIGURE 41: *Effect of Indomethacin on AngII-dependent RNA Synthesis.*** The contribution of prostaglandin synthesis to AngII-mediated growth was examined with the general cyclo-oxygenase inhibitor indomethacin. Quiescent A10 SMCs were treated with AngII ( $10^{-6}$  M) and varying concentrations of indomethacin ( $10^{-10}$  -  $10^{-5}$  M), and [ $^3$ H]-uridine incorporation over a 6 hour period was measured. RNA synthesis in AngII-stimulated cells (in the presence of ethanol vehicle) was set 100%. Each data point represents the mean  $\pm$  SE of three separate experiments.



**FIGURE 42: *Effect of Long-term Insulin Exposure on AngII-stimulated Growth of PCA SMCs.*** Porcine coronary artery SMCs were incubated in serum-free defined medium with (■) or without (●) insulin ( $10^{-8}$  M) for 7 days as described in “Materials and Methods”. [ $^3$ H]-Uridine incorporation over a 6 hour period was measured following addition of AngII ( $10^{-12}$ - $10^{-6}$  M).



important mediators for AT<sub>2</sub>-dependent cell growth in this SMC system.

#### **7.2.10 Insulin Pretreatment Facilitates AngII-stimulated Growth of PCA SMCs:**

To assess the universality of the requirement for insulin in AngII-stimulated SMC growth, we examined PCA SMCs. An initial analysis of the AngII-stimulated RNA synthesis in PCA SMC cultures revealed that a significant increase in the levels of uridine incorporated occurred only in the PCA SMCs pre-incubated with 10<sup>-8</sup> M insulin (Figure 42). As a result, all experiments in PCA SMCs summarized in Chapter 5 & 6 were conducted with insulin in the differentiation media. The requirement for insulin in generating an AngII-responsive state in two SMC cultures that differ in species (rodent, porcine), vessel type (thoracic artery, coronary artery) and length of culture (cell line, primary culture) suggests this phenomenon may be universal in SMCs.

### **7.3 DISCUSSION**

The causal link between hyperinsulinemia and renin-angiotensin system activation as it pertains to vascular disease has been clearly established *in vivo*, but the direct effect of insulin on the renin-angiotensin system remains poorly understood. Originally, the sympathoadrenal system was considered to be the most likely link between the renin-angiotensin system and circulating insulin (Gans et al., 1991). However, based on the ability of isolated adipocytes from fructose-fed rats to exhibit insulin resistance (Reaven et al., 1989) and the improvement provided by an AT<sub>1</sub> receptor antagonist (Shimamoto et al., 1994), a connection at the cellular level must exist between the renin-angiotensin system and insulin resistant state.

Smooth muscle cells express the AT<sub>2</sub> receptor abundantly in fetal cells and its expression rapidly decreases or disappears in adult tissue, leaving the AT<sub>1</sub> receptor predominant (Viswanathan et al., 1991). Adult SMCs, however, retain the ability to revert

to a "fetal" phenotype in response to vascular injury, contributing significantly to the pathogenesis of vascular diseases. Therefore, due to the fetal origins of the A10 SMCs and the elevated levels of AT<sub>2</sub> receptors, this cell line provides a unique model for studying the effects of AngII which can complement studies of the role of angiotensin receptors in adult vascular smooth muscle.

### 7.3.1 Requirement for Insulin in AngII-Responsive Phenotype

The hypothesis that insulin regulates the AngII-dependent growth processes at the cellular level was investigated in the A10 SMC line. Characterization of A10 cells demonstrated that reduced-serum conditions induce a quiescent phenotype with markers of a differentiated smooth muscle phenotype (Figure 33) and the ability to re-enter the cell cycle in response to growth factor stimulation (Figure 35 & 39) (Saward and Zahradka, 1996a,b). The presence of AngII receptors in A10 SMCs was established by <sup>125</sup>I-AngII binding studies and RT-PCR (Figure 34 & 38). The pattern of expression of the AngII receptor subtypes was shown to be growth-state dependent, since growing A10 SMCs expressed AT<sub>1A</sub>, AT<sub>1B</sub> and AT<sub>2</sub> mRNA but quiescent A10 SMCs expressed only AT<sub>2</sub> mRNA (Figure 34). AngII stimulated a hypertrophic growth response in A10 SMCs via the AT<sub>2</sub> receptor based on the induction of RNA synthesis and *c-fos* proto-oncogene mRNA expression as well as an examination of the long-term growth effects of AngII with the MTT assay (Figures 35 & 36). Although quiescent A10 SMCs initiated DNA synthesis in response to FBS treatment, no response to AngII was evident (Figure 35), in agreement with a number of studies of other rodent SMC cultures (Gibbons et al., 1992; Geisterfer et al., 1988). The novel finding presented in this data is that insulin pretreatment was necessary to achieve a SMC phenotype that is permissive to growth stimulation by AngII (Figure 35). The addition of insulin during the differentiation period did not alter establishment of the quiescent state, since no changes in either the time course required to reach quiescence or the basal rate of DNA and RNA

synthesis were observed (Figure 32 & 35).

Prostaglandin synthesis was identified as a key signalling intermediate in AngII-mediated growth of PCA SMCs and causally linked to the AT<sub>2</sub> receptor (Chapter 5). In agreement with these results, AngII-stimulated growth of A10 SMCs was inhibited by the cyclo-oxygenase inhibitor, indomethacin. Due to the complete dependence of AngII-stimulated growth on AT<sub>2</sub> receptor activation in this system, these data further support the essential role of prostaglandins in AngII-mediated SMC growth.

### 7.3.2 Mechanism for Insulin Regulation of AngII-mediated Growth

To elucidate the mechanism by which insulin mediates the growth effects of AngII, both the time required for insulin to induce the change in SMC phenotype and the possibility of a direct synergism between insulin and AngII were investigated. Our results clearly indicate that an extended pretreatment with insulin, at a concentration comparable to the hyperinsulinemic state *in vivo*, for at least 24 hours was necessary to induce the AngII-responsive state; however, this altered phenotype does not influence the quiescent state. In agreement with our findings, a slow onset of the insulin-dependent alterations in RAS was observed in primary rat aortic SMCs (Kambayashi et al., 1996). A similar requirement for insulin has been noted in a human neuroblastoma cell line, SHSY54, where the induction of DNA synthesis by AngII was insulin-dependent (Chen et al., 1993). It is difficult to speculate about the universality of the requirement for insulin since most studies of AngII-stimulated SMC growth have used a medium that contained either low concentrations of FBS or a supplement containing insulin to establish quiescence. In our investigation of primary cultures of PCA SMCs (Chapter 5 & 6), all experiments were conducted with insulin ( $10^{-8}$  M) in the differentiation media since preliminary studies indicated that the presence of insulin was also crucial for acquiring an AngII-responsive state (Figure 42). Kambayashi et al. (1996) have suggested that the requirement for insulin regulation of AngII receptors may

be specific to VSMC, since this effect was not evident in PC12W cells or adrenal glands.

Although both insulin and IGF-1 act as growth factors in A10 SMCs (Figure 39 & 40), the possibility that the observed effects could be attributed to the additive effects of costimulation with insulin and AngII is unlikely due to the length of incubation required and the absence of a direct additive effect on cell growth. In A10 SMCs, costimulation with insulin and AngII resulted in a slower growth response than treatment with insulin alone (Figure 39C). Direct cross-talk between AngII and insulin signalling pathways has been reported at the level of IRS-1 and phosphatidylinositol 3-kinase (Solow et al., 1999; Folli et al., 1997; Vellosso et al., 1996), which mediate the reduced growth response to insulin in the presence of AngII. Previously, long-term treatment (1-14 weeks) of VSMC with insulin (100 ng/mL) was also shown to attenuate the synergistic effect of insulin and AngII on growth through down-regulation of IGF-1 receptors (Ko et al., 1993). Although the participation of AngII receptors and their associated signalling pathways in the SMC response to insulin remains undefined, the ability of PD123319 to reduce insulin-stimulated growth suggests a potential role for the endogenous renin-angiotensin system in A10 SMCs (Figure 39D). This concept is supported by recent evidence that AT<sub>1</sub> receptor blockade and ACE inhibition can reduce the growth response to insulin in rat VSMCs (Kamide et al., 1998). Thus, these data support the hypothesis that SMCs exhibit a complex interaction between the insulin and angiotensin systems at the cellular level.

### **7.3.3 Insulin-dependent Regulation of the AT<sub>2</sub> Receptor**

Further investigation will be required to elucidate the mechanism by which insulin influences the actions of AngII. One potential site of regulation involves an interaction between insulin and AngII at the receptor level. In this study, the length of insulin pretreatment that is required suggests *de novo* protein synthesis may be necessary to facilitate A10 SMCs' response to AngII, possibly through synthesis and transport of new AngII

**FIGURE 43:** *Alignment of a putative Insulin-Responsive Element (IRE) in the AT<sub>2</sub> receptor promoter with the IRE of phosphoenolpyruvate carboxykinase (PEPCK) gene.*

**Gene**                      **Sequence (5'-3')**

**PEPCK**

**TGGTGTTTG**

**AT<sub>2</sub>-R**

**TAAATGTTTG**

receptors. The results of this study provide direct evidence that insulin regulates AT<sub>2</sub> receptor expression (Figure 38) and directly influences the response to AngII at the cellular level. We have identified a potential insulin-responsive element (IRE) that is located in the mouse AT<sub>2</sub> receptor promoter (Genebank U10928) between -126 and -117 TAATGTTTG (Figure 43). This putative IRE exhibits 80% homology to the well-characterized canonical IRE present in the phosphoenolpyruvate carboxykinase (PEPCK) gene (Lemaigre and Rousseau, 1994), with only two conservative substitutions of adenine for guanine, and 90% sequence homology to the IRE in malic enzyme (Morioka et al., 1988). In agreement with our findings, the same IRE was identified by Kambayashi et al. (1996) in rat AT<sub>2</sub> genomic sequence. Prolonged pretreatment with insulin was also shown to be necessary for the selective upregulation of AT<sub>2</sub> receptor transcription, but not the mRNA half-life, in these rat VSMCs (Kambayashi et al., 1996; Ichiki et al., 1996). Another mechanism for synergy has been reported through the direct induction of AT<sub>2</sub> receptor transcription by IGF-1 in conjunction with enhanced translation of this receptor by AngII (Li JY et al., 1999).

Currently, the physiological significance of the AT<sub>2</sub> receptor is unclear; however, enhanced levels of expression or activation are associated with several cardiovascular disease states such as diabetes (Sechi et al., 1994), hyperinsulinemia (Kambayashi et al., 1996), post-myocardial infarction (Nio et al., 1995; Kuizinga et al., 1998), ischemia (Wiemer et al., 1993), hypertension (Wu et al., 1994), heart failure (Rogg et al., 1996) and following vascular injury (Hutchinson et al., 1999; Kambayashi et al., 1996; Panek et al., 1992; Pratt et al., 1992). Therefore, the direct actions of insulin on AT<sub>2</sub> receptor abundance may indicate that hyperinsulinemia is a contributing factor to the pathophysiological role of AT<sub>2</sub> receptor and provide a possible target for the treatment of the vascular complications of diabetes. The effect of long-term insulin exposure on the other components of the renin-angiotensin system will need to be addressed. For example, insulin or IGF-1 has been shown to upregulate

angiotensinogen mRNA (Kamide et al., 1998) and renin release (Jost-Vu et al., 1992) but not the AT<sub>1</sub> receptor (Kambayashi et al., 1996). Further understanding of how insulin facilitates the growth promoting activities of AngII at the cellular level should provide a novel perspective on the association of cardiovascular disease and hyperinsulinemia.

#### **7.3.4 Comparison of IGF-1 with Insulin in AngII-responsiveness**

Due to the structural homology of insulin and IGF-1, heterologous receptor activation can occur. The ability of IGF-1 to mediate similar effects to those described for insulin in the A10 SMCs was therefore investigated. Pretreatment with a higher concentration of IGF-1 ( $10^{-6}$  M) was required before an AngII-responsive state could be detected (Figure 40A). An evaluation of the relative potency of these two growth factors to stimulate RNA synthesis in A10 SMCs revealed a rightward shift of the IGF-1 dose-response curve in comparison to insulin (Figure 40B), which implicates the insulin receptor in this process. Although a direct correlation between the insulin receptor-mediated growth response and the insulin-mediated phenotype that is AngII responsive cannot be assumed, this observation suggests that the insulin receptor system is a likely candidate.

#### **7.4 CONCLUSIONS**

This study has identified a link between AngII and insulin at the cellular level. In A10 SMCs, long-term exposure to insulin altered the SMC phenotype to an AngII-responsive state. One of the targets of insulin was identified as the AT<sub>2</sub> receptor gene and a putative IRE was identified that may permit direct transcriptional regulation by insulin. These data provide a definitive link between AT<sub>2</sub> receptor activation and cell growth due to the downregulation of the AT<sub>1</sub> receptor and the lack of any inhibitory effect with the AT<sub>1</sub> receptor antagonist losartan. Thus, the A10 SMC line is a unique model for studying AT<sub>2</sub> receptor-associated signalling pathways that can complement studies of the role of angiotensin receptors in adult

vascular smooth muscle. Further understanding of how insulin facilitates the growth promoting activities of AngII at the cellular level should provide a novel perspective on the association of cardiovascular disease and hyperinsulinemia.

## 8. CONCLUSIONS AND FUTURE DIRECTIONS:

The time-span covered by my research has represented one of the most exciting and innovative times to be involved in the RAS field. Although AngII was discovered more than 100 years ago, the details of its relative importance and complexity have unfolded slowly. Many of the discoveries relating to the RAS over the last decade have redefined the traditional concepts with far-reaching implications in our understanding of the physiology of local hormone systems, G-protein-coupled receptor signal transduction and the interaction between multiple receptor subtypes. The extensive body of research on the RAS has established this hormone system as a pivotal endocrine, paracrine and autocrine modulator of cardiovascular development and pathology. Although initially viewed as a mechanism for controlling blood pressure, the existence of local tissue-based RASs that differ in their biological function, target tissues and regulation has been established. This advance in our understanding of the local RAS has changed our view of the cell types that contribute to vascular disease, shifting the focus to the local tissues for changes associated with the initiation and progression of disease. The RAS has remained a major focus of research due to extensive interest in devising modalities for pharmacological intervention in this system. ACE inhibitors offered a significant advance in the treatment of some cardiovascular disorders with the potential to stop the progression and possibly even reverse certain diseases, rather than treating the resultant symptoms. As research unfolds the complexity of the regulation of this system, including the multiple receptor subtypes and signalling pathways that are involved, more precise targets for selectively blocking AngII will be identified. Currently, several AT<sub>1</sub> receptor antagonists are being investigated as alternatives to ACE inhibitors. The resultant clinical data should provide new insight into the complex role of the AT<sub>1</sub> receptor and provide clues as to the role of other subtypes such as the AT<sub>2</sub> receptor. Ideally, future treatment regimes for cardiovascular disease will involve a

combination of inhibitors and receptor antagonists that are more specific to the disease processes involved.

At the initiation of my research, the AT<sub>2</sub> receptor was not considered to be a relevant receptor within the RAS. Although the AT<sub>2</sub> receptor is highly regulated in developmental and injury- and disease-related processes, no biological function was identified until the last few years. The powerful techniques of molecular biology facilitated the cloning and functional analysis of the AT<sub>2</sub> receptor with the development of transgenic and receptor knockout models. The realization that more than one receptor was responsible for mediating AngII's downstream effects caused another dramatic expansion of the view of RAS. It seems incredulous now to imagine that a receptor that is highly regulated and broadly expressed would be assumed to have no significant function. It is now evident that the initial difficulties encountered in deciphering its contribution likely reflect the complexity of the AT<sub>2</sub> receptor's role. Indeed, even the recent reports of mice with the AT<sub>1</sub> or AT<sub>2</sub> receptors ablated did not produce definitive answers regarding their function but, instead, highlighted the functional redundancy and interplay within the RAS. The data presented in this thesis and new research from other laboratories have established that a unique interplay and division of function exists between the AT<sub>1</sub> and AT<sub>2</sub> receptor subtypes. The AngII receptors system is complex due to the cross-talk between multiple receptor subtypes to produce an integrated response at the cellular level and, therefore, a novel model of AngII receptor function is required.

The current perception of the RAS has defined the functional roles of the receptors: i) the AT<sub>1</sub> receptor mediates all the growth effects of AngII, and ii) the AT<sub>2</sub> receptor exerts an anti-growth effect. This view has adhered to rigidly defined roles for the AT<sub>1</sub> and AT<sub>2</sub> receptors with reluctance to acknowledge reports that do not fit this model. This should cause us to question whether this model is comprehensive or valid in only one particular environment or cell-type. If we are willing to broaden our view of the RAS to include the

many studies that have demonstrated regulation and functions that do not fit with this narrow concept of the RAS, we may arrive at a more accurate, albeit more complex, picture. Thus, a more comprehensive model is required to encompass the many studies that have demonstrated a role for both the AT<sub>1</sub> and AT<sub>2</sub> receptors in proliferation, differentiation and apoptotic processes. Further research will be necessary to provide a greater understanding of the multiplicity of AngII receptor functions, the key environmental and intracellular factors that modulate their expression and downstream signalling pathways as well as the mechanism of crosstalk between different receptor subtypes. Research focussed on the AT<sub>2</sub> receptor, in particular, has been plagued by conflicting reports on regulation, signal transduction and function that have been attributed to variations in cell types, culture conditions, and differentiation status. Defining the factors that affect AT<sub>2</sub> receptor coupling to downstream events may provide a key to understanding the environmental influences on the cellular RAS. From this perspective, the findings presented in this thesis that indicate both AT<sub>1</sub> and AT<sub>2</sub> receptors mediated SMC growth should not be considered a contradiction to the current model where the AT<sub>2</sub> receptor antagonizes the growth effects of the AT<sub>1</sub> receptor. Instead, these results lend credence to the view of the AT<sub>2</sub> receptor as a modulatory receptor that can exert both synergistic and antagonistic effects on AT<sub>1</sub> receptor-mediated pathways. We have proposed a dual receptor model (Figure 44) for the cellular response to AngII that involves parallel and independent signalling pathways mediated by both the AT<sub>1</sub> and AT<sub>2</sub> receptors. The convergence of these pathways leads to an integration of the separate signals and contributes to a common cellular response. As a result, the net biological response to AngII would depend on the cellular ratio of the AngII receptor subtypes. Thus, interference with activation of either receptor could disrupt the balance and alter the resultant phenotypic end-point of growth, apoptosis, or anti-growth/differentiation. From this perspective, the system of multiple receptor subtypes

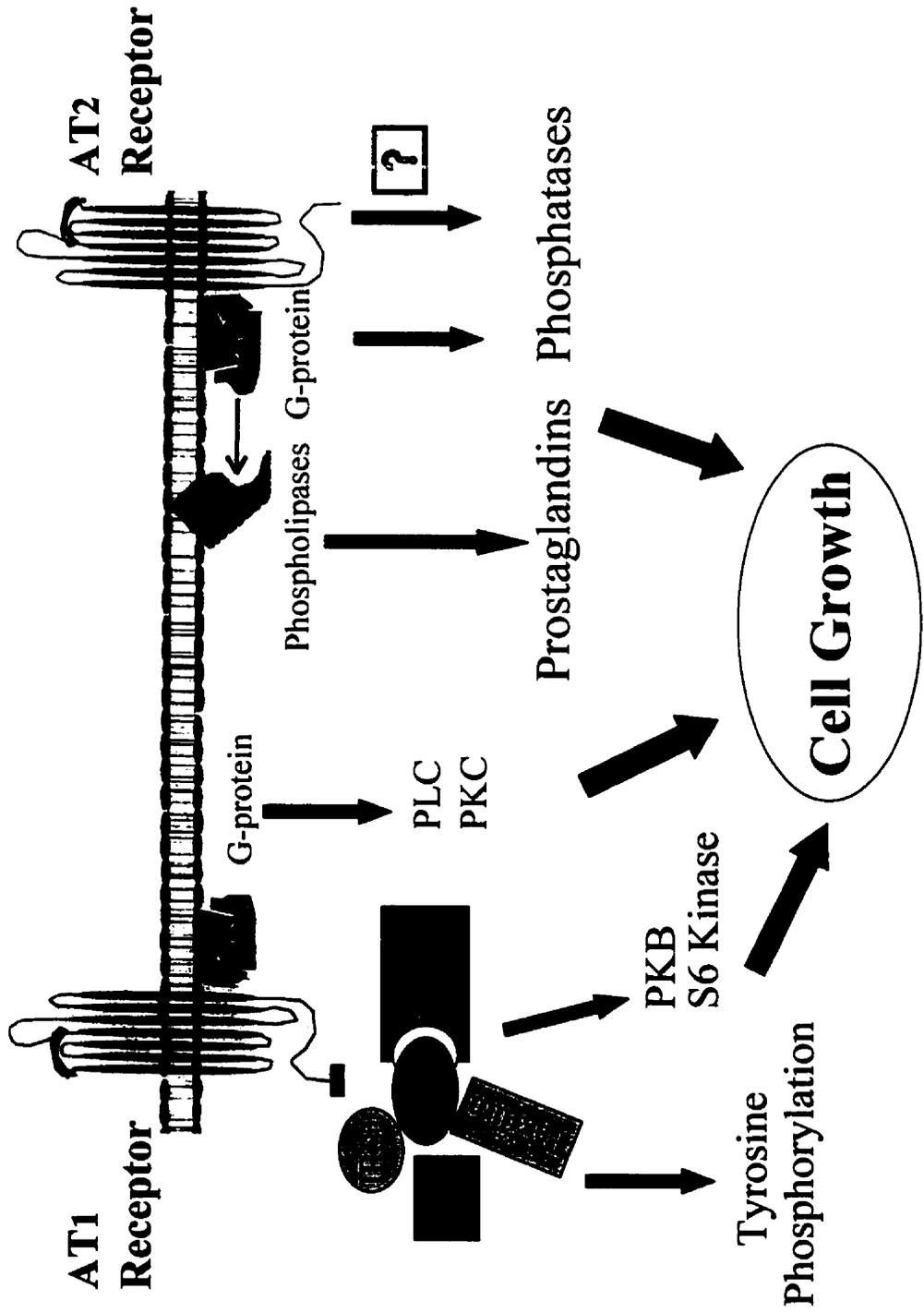
would provide a sensitive mechanism for controlling the cellular response to this multifaceted growth factor.

Attempts to elucidate the signalling pathways activated by AngII have resulted in the discovery of crosstalk between G-protein-coupled receptor and tyrosine kinase receptor signal transduction. The traditional view of compartmentalized signalling pathways has been discarded with the realization that SH2-binding effectors and adapters could directly interact with G-protein receptors and play a key role in their downstream effects. Mounting evidence indicates that the AT<sub>1</sub> receptor signal transduction mechanism involves many traditional tyrosine kinase receptor effectors such as Src, Ras, and MAPK. This thesis has demonstrated that the lipid kinase PI3K plays a key role in AngII-mediated SMC growth. The involvement of various kinase in the proliferative pathway for G protein-coupled receptors has provided new targets for intervention in AngII's biological effects. Recently, a novel mechanism for the recruitment of the tyrosine kinase-associated signalling pathways has been described that involves AT<sub>1</sub>-mediated transactivation of the epidermal growth factor receptor which may serve as a scaffold for the effectors such as Src and Shc/Grb (Eguchi et al., 1999). In addition, we have characterized prostaglandins as key mediators for the AT<sub>2</sub> receptor and future studies need to define the specific prostanoids and receptors involved. Another novel area of research into the contribution of inflammatory signalling pathways to the pathophysiology of the AT<sub>2</sub> receptor became relevant with the discovery of AT<sub>2</sub>-dependent induction of ceramides (Lehtonen et al., 1999). Future research in the RAS field may modify the traditional concept of cell-surface receptors mediating the response to growth factors. Internalization of AT<sub>1</sub> receptor complexes and translocation to the nucleus (Lu et al., 1998) has initiated studies into the intracrine effects of AngII and the function of nuclear AngII receptors.

Extensive research over the last decade has expanded the narrow original view of the

RAS as a linear enzyme cascade involved in systemic blood pressure regulation to a multifaceted local hormone system involved in tissue development, function and disease. Reflective of the myriad biological functions connected with the RAS, this hormonal system has proven very complex. Oversimplification of our understanding of its regulation will not further our success in therapeutic intervention. Instead we must carefully interpret and extrapolate the results and conclusions from both *in vitro* and *in vivo* systems. In hindsight, the reliance on experimental systems with limited expression of AngII receptor subtypes may have hampered our comprehension of the RAS. The research summarized in this thesis has just begun to touch on the interplay between different AngII receptor subtypes, Over the next decade, new research should clarify the relative contribution of these receptors, as well as identify the systems directing crosstalk between their respective signalling pathways, to produce a more complete picture of the fine-tuned regulation of the cellular response to AngII. In addition, many aspects of the RAS require further definition, such as the role of nuclear AngII receptors, the contribution of the degradation products of AngII and their receptors, as well as the impact of matrix interactions on AngII receptor function and signal transduction. In the end, a broader perspective of the interwoven web of complexities in the RAS should provide considerable insight into the regulation and pathophysiology of this pivotal hormone system and identify more precise targets for intervention.

**FIGURE 44: *Dual receptor model for involvement of AT<sub>1</sub> and AT<sub>2</sub> receptors in SMC growth.***



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