

**Angiotensin II-induced Phosphorylation of R-Smad 2 in Cardiac Myofibroblasts is
Mediated by Specific Kinases**

A Thesis Presented to the
University of Manitoba

In partial fulfillment of the requirements
for the degree of
Master of Science in Physiology

by

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Faculty of Medicine

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**THE UNIVERSITY OF MANITOBA
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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University
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MASTER OF SCIENCE

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LIST OF ABBREVIATIONS

AT ₁	angiotensin type I receptor
AT ₂	angiotensin type II receptor
α -SMA	α -smooth muscle actin
BSA	bovine serum albumin
Co-Smad	common-mediator Smad
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxide
ECM	extracellular matrix or the matrix
Erk	extracellular signal-regulated kinase
FBS	fetal bovine serum
IP ₃	1,4,5-inositol triphosphate
I-Smad	inhibitory Smad
JNK	c-jun NH ₂ -terminal kinase
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MH1	Mad homology 1
MH2	Mad homology 2
MI	myocardial infarction
MMP	matrix metalloproteinase
nonRTKs	nonreceptor-induced tyrosine kinases

PICP	procollagen-1-carboxypropeptide
PAGE	polyacrylimide gel electrophoresis
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PI3K	phosphoinositol-3-kinase
PKC	protein kinase C
PLC	phospholipase C
P-Smad	phosphorylated Smad
RAAS	renin angiotensin aldosterone system
RAS	renin angiotensin system
R-Smad	receptor-mediated Smad
RTKs	receptor-induced tyrosine kinases
SARA	Smad anchor for receptor activation
SDS	sodium dodecyl sulfate
TGF- β	transforming growth factor- β
TGF- β_1 NA	anti- transforming growth factor- β_1 neutralizing antibody
T β RI	transforming growth factor- β type I receptor
T β RII	transforming growth factor- β type II receptor
T β RII NA	anti- transforming growth factor- β type II receptor neutralizing antibody

I. ABSTRACT

Cardiac myofibroblasts contribute to remodeling of the cardiac collagen matrix in heart failure of different aetiologies. Transforming growth factor- β_1 (TGF- β_1) and angiotensin II (angiotensin) both influence myofibroblast function. TGF- β_1 signals through Smads and other signaling pathways, including mitogen activated protein kinases (MAPKs). We previously determined that AT₁ activation leads to rapid specific phosphorylation of R-Smad 2, and that this event is linked to enhanced collagen deposition. However, the precise kinase pathways mediating this phenomenon are unresolved. The relationship between angiotensin and TGF- β_1 - signaling was carried out in vitro in primary myofibroblasts using pharmacological inhibitors against p42/p44 Erk MAPKs, p38 MAPK, protein kinase C (PKC), and phosphoinositide 3-kinase (PI3K). We examined i/ phosphorylation of R-Smad 2 ii/ phosphorylation of MAPKs; iii/ the effects of PD 98059 (the upstream inhibitor of p42/p44 Erk MAPKs or MEK 1 inhibitor), SB 203580 (p38 MAPK inhibitor), chelerythrine (PKC inhibitor), and LY 294002 (PI3K inhibitor); iv/ nuclear translocation of R-Smad 2; and v/ the effects of anti-TGF- β_1 and anti-TGF- β type II receptor antibodies on P-Smad 2 induction. We confirmed that angiotensin stimulation specifically phosphorylates R-Smad 2 and facilitates nuclear translocation in myofibroblasts; this was associated with increased collagen type I synthesis. Western analysis revealed that SB 203580 (50 μ M), chelerythrine (1 μ M) or LY294002 (10 μ M) ablated angiotensin-induced R-Smad 2 phosphorylation. Furthermore, immunofluorescent staining of cells pretreated with either chelerythrine or LY 294002 decreased nuclear translocation of R-Smad 2 in TGF- β_1 and angiotensin treatments. Blockade of TGF- β signaling did not alter angiotensin-specific induction of R-Smad 2. We suggest that the p38 MAPK, PKC and PI3K pathways in cardiac myofibroblasts participate in non-

classical (i.e., non-TGF- β_1 induced) phosphorylation of R-Smad 2, and this event leads to enhanced deposition of collagen in myofibroblasts.

Keywords: cardiac myofibroblasts and fibroblasts; R-Smad 2; angiotensin; transforming growth factor β ; mitogen activated protein kinases; phosphoinositide 3-kinase; protein kinase C; collagen type I

II. INTRODUCTION

The basic interplay between trophic factors at the post-receptor level is recognized to play a role in the pathogenesis of heart failure, but many of the precise crosstalk pathways are unknown. Multiple factors, including angiotensin II (angiotensin), transforming growth factor - β_1 (TGF- β_1), endothelins, nitric oxide, prostaglandin F 2α , and norepinephrine, are implicated in this regard and are currently under investigation. However, the focus of this study is on the roles of angiotensin, TGF- β_1 , and the putative Smad nexus they subserve.

Angiotensin is a pleiotropic effector of the renin-angiotensin aldosterone (RAAS) axis that functions as pressor and growth factor. Angiotensin also exerts mitogenic- and inflammatory-like actions that are implicated in the pathogenesis of cardiac hypertrophy present in myocardial infarction (MI), hypertension and cardiomyopathies [1]. For example after MI, the myocardium undergoes progressive gross remodeling that is accompanied by the expansion of interstitial (extracellular matrix, matrix or ECM) collagen mass, transient inflammatory changes, and acute myocytic cell dropout in the infarct and border zones (both of necrotic and apoptotic origin) [2,3]. In the chronic phase, myocytes continue to be lost to these mechanisms. On the other hand matrix remodeling, characterized by the disproportionate accumulation of cardiac matrix proteins, involves both reparative and reactive fibrosis [4,5]. Reparative fibrosis is important for the normal wound healing and scar formation at the site of infarction during the early phase of MI [6,7], whereas reactive fibrosis is defined as the triggering of inappropriate deposition of fibrillar collagens in noninfarcted remnant (remnant) tissue [6,8]. Increased cardiac collagen synthesis and deposition is associated with decreased passive compliance and impaired contractility, which potentiates the progression of overt heart failure [6-8]. The mechanism of myocardial fibrosis has been

extensively investigated and the emerging role of cardiac myofibroblasts in this process is recognized [6-9]. Myofibroblastic cells are hypersynthetic phenotypic variants derived from relatively quiescent fibroblasts [10-12]. TGF- β_1 has been identified as a player in this phenotypic transformation; myofibroblasts are major producers of collagen and other matrix components [13,14]. Myofibroblasts are known to proliferate in the infarct scar as early as 3 days post-MI, and at this time the deposition of matrix protein begins in earnest. However, the pathways by which these cells are regulated by the combination of TGF- β_1 and angiotensin in stimulation of fibrosis are unknown; it is of considerable interest to clinicians and basic scientists to elucidate this mode-signaling crosstalk.

Both TGF- β_1 and angiotensin stimulate structural remodeling of the myocardium, contributing to the pathologic myocardial fibrosis associated with the progression of heart failure [4,6,7,15-17]. The turnover of ECM in diseased hearts has been linked to the elevated systemic as well as locally generated angiotensin, leading to the increased type I and III collagen mRNA expression and increased rates of collagen synthesis at the remnant myocardium and scar by myofibroblasts [7,8,18,19]. Angiotensin's effects on cardiac fibroblasts are largely attenuated by angiotensin II type I (AT₁) receptor antagonism [6,20,21]. AT₁ receptor stimulation is associated with trimeric G-protein mediated stimulation of phospholipase C- β (PLC- β) [1,22], tyrosine phosphorylation and activation of mitogen-activated protein kinase (MAPK) cascade [1,23]. As well, this pathway is associated with non-receptor tyrosine kinase activation, such as Src, Pyk2, p130Cas, FAK, JAK/STAT, and phosphatidylinositol 3-kinase (PI3K) [1,5,16,24]. PLC-dependent hydrolysis of phosphatidylinositol -4,5-biphosphate (PIP₂) leads to intracellular 1,4,5-inositol triphosphate (IP₃) and diacylglycerol (DAG) production, which is associated with sarcoplasmic reticular

(SR) Ca^{2+} release and activation of protein kinase C (PKC), respectively [1,16,25]. Other factors contribute to cardiac fibrosis [26] and TGF- β_1 is a very potent agonist for fibroblast and myofibroblast-mediated collagen synthesis and deposition [8,14,27]. The main mode of postreceptor signaling of TGF- β_1 in cardiac fibroblasts and myofibroblasts is via activation of Smad proteins (R-Smad 2, R-Smad 3, Co-Smad 4 and I-Smad 7). In heart, R-Smad 2 is the most studied protein of the Smad superfamily. Upon activation of TGF- β type I receptor (T β RI) after TGF- β type II receptor (T β RII)-ligand binding, downstream phosphorylation of R-Smad 2 (P-Smad 2) and subsequent complex formation with Co-Smad 4 occurs in these cells [6,19]. Following the receptor-induced activation, the P-Smad 2/Co-Smad 4 complex translocates to the nucleus where it acts as a transcription factor to initiate specific gene transcription [6,28-31]. P-Smad 2 is implicated in collagen synthesis in response to TGF- β_1 [32], however it is known that angiotensin may also contribute to non-classical phosphorylation of Smads. These precise pathways for angiotensin's contribution to this phenomenon are not clear, and this is the aim of the current study.

We hypothesize that angiotensin stimulates the very rapid direct phosphorylation of R-Smad 2 i.e., without secondary release of TGF- β_1 , via the induction of a specific kinase mediator. Second, we believe that this mechanism is important for modulation of collagen synthesis and deposition by cardiac myofibroblasts. Our approach was to characterize and compare angiotensin and TGF- β_1 - signaling systems in our *in vitro* myofibroblast system using pharmacological inhibitors of specific downstream kinases, and examine the data for relationships between the two modes. In both pathways we examined i/ the accumulation of P-Smad 2 protein and its nuclear translocation following the stimulation and/or inhibition; ii/ the phosphorylation of MAPKs; and iii/ the effect of LY 294002 (PI3K inhibitor),

chelerythrine chloride (PKC inhibitor), SB 203580 (p38 MAPK inhibitor), and PD 98059 (the upstream inhibitor of p42/p44 Erk MAPKs or MEK 1 inhibitor) on R-Smad 2 phosphorylation. In the final section of the study iv/ we verified that angiotensin played a role in R-Smad 2 expression vis-a-vis TGF- β_1 in myofibroblasts in contributing to collagen synthesis *per se*.

III. LITERATURE REVIEW

1. Extracellular Matrix

The extracellular matrix (ECM or matrix) is a complex gel-like structural entity that fills the extracellular space i.e., between parenchymal cells, in mammalian tissues. Its role is to surround, support and hold the cells within a tissue together as well as to provide a pathway for the diffusion of nutrients and oxygen to individual cells. In addition to its structural role, the ECM also plays a crucial part in directing cell migration, proliferation, and tissue development [33]. In normal adult heart, 75 % of the myocardial tissue volume is taken up by cardiac myocytes, whereas 25 % is attributed to the cardiac interstitium containing non-myocytes. In normal healthy heart, these cells include cardiac fibroblasts, vascular smooth muscle cells, endothelial cells, and macrophages [34]. Embedded in the ECM, nonmyocytes account for the two-thirds of all cells found in the heart [35]. The progressive decline of cardiac function that accompanies several prominent etiologies of heart failure is attended by structural abnormalities within the cardiac interstitium, which are accompanied by the loss of normal physical tethering between cardiac myocytes and non-myocytes [35,36].

1.1. Components of Extracellular Matrix

The ECM is composed of an interlocking meshwork of glycosaminoglycans, fibrous proteins, and different types of matrix metalloproteinases [37, 38]. Glycosaminoglycans are associated with extracellular proteins, forming high molecular weight ECM components referred to as proteoglycans [39]. Interwoven with the proteoglycans are adhesion proteins and fibrous proteins, including collagen and elastin [37, 40]. Adhesion proteins i.e., laminin and fibronectin, contain binding sites within their sequence for proteoglycans and the

extracellular domains of integral membrane proteins (integrins), and thereby mediate the association between the ECM and cells [41,42]. Collagen and elastin proteins cross-link with various molecules, where the crosslinking increases tensile strength and integrity of the matrix [7,43-45]. The integrity of the matrix is modulated by metalloproteinase enzymes, which have the ability to degrade the matrix in both physiological and pathological states [46,47].

1.1.1. Laminin

Laminins are a family of heterotrimeric adhesion glycoproteins composed of α , β and γ chains [41]. They represent the most abundant noncollagenous matrix macromolecule component and serve as structural components of basement membranes [48]. Although laminins provide structural support, they are also essential during tissue development and in the process of morphogenesis [49]. Laminins form multiple interactions with other components of basal lamina (e.g., collagen type IV and proteoglycans), and their cell surface receptors such as integrins (e.g., integrin $\alpha 6\beta 1$) [41]. The association with integrins plays an important role in mediating the signaling events that regulate cellular organization and differentiation, which are triggered due to structural changes in the ECM [48].

1.1.2. Fibronectin

Fibronectin is a glycoprotein found in the ECM of healing and embryonic tissues as an insoluble form, and in plasma as a soluble glycoprotein [50]. Fibronectin is composed of two subunits, which contain three types of repeating modular units, known as FN type I, FN type II, and FN type III repeats [51]. These modules carry multiple binding sites, including those for certain glycosaminoglycans and integrins [51]. The insoluble form of fibronectin forms a

direct interaction with integrins (e.g., integrin $\alpha 5\beta 3$) [42]. This coupling is important in mediating the signal-transduction pathways from the ECM to cells, affecting cell differentiation, migration, adhesion, and the cellular cytoskeleton organization [52,53]. The soluble plasma fibronectin is primarily involved in blood clotting and wound healing [3,54,55].

1.1.3. Elastin

Elastin is the insoluble, elastic protein composed of tropoelastin monomers [44]. These monomers are secreted from cells in a soluble form, which become modified and cross-linked in the extracellular space [44]. The resultant polymerized protein meshwork of tropoelastins is insoluble with a high degree of reversible distensibility and deformability [56]. The assembly of elastins into the ECM depends on microfibrils, which are also structural components of elastic fibers. Microfibrils act as a scaffold for the proper orientation, deposition, and polymerization of tropoelastins [56]. The elastin assembly is a process associated only throughout the elastogenic period in developing tissues with little or no synthesis in adult life [56]. These fibers are involved in controlling cell proliferation and reorganization, which stabilizes the tissue structure during the period of development. Elastins are remarkable for their extremely long biological half-life, and their function in providing passive distensibility to tissues are largely related to the degree of fenestration present in the elastin sheet [45].

1.1.4. Collagen

Collagens as a group, are the most abundant fibrous protein in the ECM [7, 37]. The polypeptide chain that composes collagen is a unique left-handed α helix, which contains the repeating tri-peptide sequence of Gly-X-Pro or Gly-X-Hyp [57]. Three of such helices twist around each other, forming tropocollagen superhelix, which is right-handed and opposite from the twisting of individual polypeptides [57]. The twisting allows the helices to pack as close as possible, providing collagen's tensile strength with no capacity to stretch [58]. To date, there are at least twenty different collagen isoforms identified [37].

1.1.4.1. Types of Collagens

Type I and III collagens are the most abundant fibrillar collagens in the ECM [7]. Type I collagen is a heterotrimer of two α_1 - (I) and one α_2 - (II) subunit polypeptides, and represents a major matrix protein in the heart (Table 1) [8, 37]. Type II collagen is a homotrimer composed of three α_1 - (II) subunit polypeptides, and is commonly found in cartilage and vitreous humor [8,59,59]. Type III collagen is another homotrimer, which typically occurs together with the type I collagen. It consists of three α_1 - (III) subunit polypeptides, and is also present in the heart [8]. Type I collagen accounts for about 80% of the total cardiac collagen content, whereas the type III only for 10% [60, 61]. The other 10% is composed of type IV, V, and VI collagens [61, 62]. Type IV collagen is nonfibrillar collagen, and a major constitute of the basal lamina [8, 63]. Type V and VI collagens are fibrillar proteins that are most often associated with the type I collagen in the interstitial tissue [8,64,65]. Due to different structural compositions, various collagen types provide different tensile strength, which decreases in the following order: type I > type III > type VI collagen

[8]. As the collagen type I comprises most of the cardiac ECM, it represents a major determinant of myocardial stiffness [66]. While we recognize that the intracellular cytoskeletal proteins and intercalated discs are components of the myocytic syncytium that contribute to global passive myocardial stiffness, a discussion of their protein components is beyond the scope of the current literature review, and have been previously reviewed [67].

TABLE 1

Collagen Sub-types in the Myocardium [8]

Type	Chain Composition	Procollagen MW (kDa)	Collagen MW (kDa)
I	[2 α_1 (I):1 α_2 (I)] heterotrimer	α_1 -140	95
		α_2 -125	95
III	[3 α_1 (I)] homotrimer	α_1 -140	95
IV	[1 α_1 (IV):1 α_2 (IV)] heterotrimer	α_1 -185	185
		α_2 -170	170
V	[1 α_1 (V):1 α_2 (V):1 α_3 (V)] heterotrimer	-	-
VI	[1 α_1 (VI):1 α_2 (VI):1 α_3 (VI)] heterotrimer	-	α_1 -150
		-	α_2 -160-280
		-	α_3 -140

1.1.4.2. Type I Collagen Synthesis

Type I collagen is synthesized in fibroblasts, myofibroblasts (i.e. phenotypic variants of fibroblasts), osteoblasts and odontoblasts [8,10,37]. In the heart, this fibrillar protein is produced by cardiac fibroblast and myofibroblasts, which are the non-myocyte cells that play a key role in regulating the cardiac ECM formation and distribution in normal physiological and pathological processes, respectively [8,15,68,69]. Collagen I is a heterotrimer of two α_1 - (I) and one α_2 - (II) subunit polypeptides, which are the products of two genes [8, 70]. The first polypeptide is synthesized in the form of pro-COL1A1 polypeptide chain, and the latter in the form of pro-COL1A2 [37]. The pro-polypeptide chains are modified in the endoplasmatic reticulum. The modification involves the hydroxylation of specific proline and lysine residues, forming hydroxyproline and hydroxylysine, respectively [71,72]. The hydroxylation of prolines is undertaken by prolyl-4-hydroxylase and prolyl-3-hydroxylase, while the hydroxylation of lysine residues is catalyzed by lysyl-hydroxylase. The hydroxylated residues are important for hydrogen bonding of pro-polypeptide chains, which subsequently combine to form a triple helix of procollagen [37]. Procollagen I differs from the mature collagen I by having an additional 150-long amino acid propeptide at the amino-terminal site, and 250-long amino acid propeptide at the carboxy-terminal site [8]. These terminal propeptides are cleaved in the Golgi apparatus by the procollagen-specific terminal proteinases to produce a mature collagen I [8]. The removed propeptide terminals can be used as markers for collagen synthesis. The measurement of the procollagen type I carboxy-terminal propeptide (PICP) removal is a commonly employed technique for the indication of collagen type I synthesis [8,73]. The mature collagen I is secreted into the extracellular space,

where it becomes integrated into the growing fibrils that compose the ECM. As the type I collagen accounts for about 80% of the total cardiac collagen content, its normal structure, synthesis, and degradation are important processes from the physiological point of view [8]. Any abnormalities in the processing of this collagen type contribute to defective formation of the ECM, and lead to development of various cardiac diseases, including fibrosis following myocardial infarction [6-8].

1.1.4.3. Regulatory Elements and Protein Factors Involved in Type I Collagen Expression

The structural and regulatory regions of human pro-COL1A1 and pro-COL1A2 genes exhibit high homology among various species, indicating that the collagen I genes are evolutionarily conserved [37]. The regulation of transcription of both genes is highly controlled, resulting in two pro-mRNAs of COL1A1 and one pro-mRNA of COL1A2 [70]. The regulation of transcription is achieved by modulating the repressor and enhancer activities within the promoters of the genes, and transcription factors, which control the basal, cell type-specific, and cytokine-modulated expression of the pro-collagen polypeptides [37]. The major cytokine-responsive elements are TGF- β , TNF- α (tumor necrosis factor- α), and IFN- γ (interferon- γ), whereas the group of transcription factors consists of CBF (the CCAAT binding factor), NF-1 (nuclear factor 1; required for the TGF- β -mediated stimulation of COL1A2 transcription), IF-1 and IF-2 (the negative regulatory factors), C/EBP (CCAAT/enhancer binding proteins), Sp-1 (GC-binding protein), AP-1 (activator protein 1; required for the TGF- β -mediated stimulation of COL1A1 transcription), NF- κ B (transcription factor responsive to TNF- α), Smads (TGF- β -activated proteins), CBP/p300 (transcriptional

coactivators), Myc (the negative transcription factor), Erk ½ (mitogen activated protein kinase; involved in the TGF- β -mediated synthesis of collagen), and p53 (tumor suppressor protein; inhibitor of TGF- β -induced collagen production) [37].

1.1.4.4. Degradation of Type I Collagen

The degradation of type I collagen is catalyzed by collagenases, which are a group of enzymes that belong to the family of matrix metalloproteinases. The products of collagen degradation include telopeptides of different sizes. The serum-derived small telopeptide (12 kDa) serves as an indicator of the intensity of collagen degradation [74]. The big telopeptide (36 kDa) is usually denaturated and completely degraded into inactive fragments by gelatinases, which are also the enzymes that belong to a group of matrix metalloproteinases [8,46].

1.2. Matrix Metalloproteinases (MMPs)

MMPs compose a family of Zn²⁺ - and Ca²⁺ -dependant enzymes that represent a driving force behind the modulation and degradation of the ECM molecules in both normal physiological processes and pathological states [46,47]. There are nine important MMPs in cardiac tissue, and depending on the substrate specificity, these proteins generally divide into the following three groups:

- 1) collagenases, which degrade collagens types I and III
- 2) type IV collagenases/gelatinases, which specifically degrade components of the basement membrane, and partially degraded collagen

3) stromelysins, stromelysins-like MMPs and membrane-anchored MMPs, which degrade collagens, proteoglycans, laminin, fibronectin, and gelatin [46,75]. The activation of MMPs is tightly controlled as these enzymes have the ability to completely degrade the ECM. The regulatory control is achieved by IL-6 (interleukin-6), PDGF (platelet-derived growth factor), TNF- α (tumor necrosis factor α), TGF- β , the radicals derived from superoxide (O₂⁻), TIMPs (tissue MMP-inhibitors), and PAIs (plasminogen activator inhibitors) [46,76,77].

In the period of post-myocardial infarction (MI), the heart undergoes remodeling that is accompanied with changes in MMP and TIMP levels. The enhanced expression of different MMPs is region-specific with no TIMP detectable at the MI site [78]. In addition to the loss of TIMPs, the net increase in proteolytic bioactivity of MMPs is also associated with contractile protein degradation [79]. Degradation of contractile proteins by MMPs is a significant contributing factor that results in the misalignment between myocytes and the ECM, which eventually leads to the loss of structural and functional integrity of the heart. [47,76,77,80]. The structural changes of the heart muscle that are induced by an enhanced activity of MMPs involve degradation of collagen ultrastructure, and molecules that function as cytokine reservoirs. For example, MMP-3 (stromelysin) and MMP-7 (stromelysins-like MMP) cleave proteoglycan decorin, releasing TGF- β ₁ cytokine from the decorin- TGF- β ₁ complex into the ECM [75]. The freed TGF- β ₁ can subsequently control the collagen expression from cardiac fibroblasts and myofibroblasts, which in turn further remodels the ECM [7, 14]. The remodeling of the ECM ultimately results in the thinning of the ventricle wall, ventricular dilation, myocardial dysfunction, and the progression of heart failure [46,80,81].

1.3. Extracellular Matrix Receptors: the Integrins

Integrins are the group of heterodimeric transmembrane receptor proteins that modulate cell-cell and cell-ECM interactions [82]. The heterodimer is composed of α and β membrane-spanning proteins, both of which contribute to matrix binding [82]. Divalent cations (Ca^{2+} or Mg^{2+} , depending on the integrin) are found mainly in the α chain structure, and play an important role in the cell-ECM interactions [83]. When activated by matrix binding, integrins function as signal transducers by converting the mechanical tension into a biochemical signal [84]. Such signals can subsequently trigger various intracellular signaling pathways, including pathways that regulate alterations in cell morphology, migration, proliferation, differentiation, gene expression, and death [82,84]. The interaction between ECM and the cytoskeleton is bidirectional, where the cytoskeletal molecule (e.g., actin) reorganization can reciprocally signal integrins for the orientation of secreted matrix proteins [84]. The reciprocal signaling is important as it assures the proper organization of the matrix between cells, which in turn influences the cell-cell interaction. Binding of the cytoskeleton to the intracellular portion of integrins contributes to the formation of focal adhesions, which are complexes of intracellular signaling and structural proteins that involve kinases (FAK, Akt, Raf, Erk, PI3K), cytoskeletal organizers (eg, paxillin), and small GTP-ases (eg, Rho, Rac, Ras) [82]. Although some integrins can bind only one ligand, others can bind more than one. For example, integrin $\alpha_3\beta_1$ recognizes ligands such as type I collagen, fibronectin and laminin, whereas integrin $\alpha_5\beta_1$ can bind only fibronectin [82]. The specificity toward ligand binding arises from the pairing of α and β subunits, forming a unique heterodimeric structure. Although each subunit differentiates into 18 different types, not all heterodimers can form [82].

In the heart, integrins play an important role as mechanotransducers. However, the remodeling of the ECM structure that occurs during the development of cardiac hypertrophy, dilated cardiomyopathy, and MI can significantly influence the expression and function of integrins [2,3,82]. The factors that appear to modulate the expression of integrins in diseased heart include PDGF (platelet-derived growth factor), IGF (insulin-like growth factor), angiotensin, and TGF- β_1 [82]. During cardiac disease, integrins change the position on the cell surface as cardiac myocytes undergo changes in cell shape and size. In cardiac hypertrophy, the expression of the myocyte integrins (e.g., $\alpha_5\beta_1$) is increased, and is initially coordinated with the amount of the ECM proteins synthesized [82]. However, in later stages of hypertrophic induction characterized by myocyte branching, the coordinated expression of ECM and integrins is disrupted due to release or shedding of integrins into the extracellular space [85]. The release of integrins into the ECM space is enhanced by the activation of various ECM proteinases, including matrix metalloproteinases [85-87]. The loss of integrins changes cell-signaling events, resulting in myocytic death, and thus altered generation of the myocardial mechanical forces, which is detrimental for the heart survival [88].

2. Development of Heart Failure: Cardiac Hypertrophy and Fibrosis

Cardiovascular diseases are, collectively, the leading health problem in North America [7]. The mechanisms that lead to irreversible pathological remodeling of heart tissue structure contribute to an enhanced risk of adverse cardiovascular events, including myocardial infarction, mechanical dysfunction, symptomatic heart failure and arrhythmias [35]. Due to

these changes, the heart becomes unable to pump enough blood to meet the needs of the body's vital systems, which subsequently break down.

2.1. Causes of Heart Failure

Although the heart loses some of its ability to effectively pump blood as a consequence of aging, a sudden life-threatening loss of cardiac performance may be induced by various factors. Loss of heart pumping activity can occur directly from the development of pathological conditions caused by certain diseases, or it can occur over time as the activation of compensatory mechanisms may become maladaptive, and thereby cause adverse effects on cardiac function.

2.1.1. Myocardial Infarction, Coronary Artery Disease, Hypertension, and Hypertrophy

A major cause of heart failure may result from the imbalance between the oxygen supply and demand of myocardium [9, 89]. Deprivation or lack of oxygen via impeded blood delivery may damage the heart muscle due to impaired energy metabolism of cardiac cells [90]. Such damage often results from coronary artery occlusion, which critically reduces the blood flow that supplies the heart with oxygen and nutrients [90]. The occlusion of coronary arteries may develop from either an excessive collagen deposition, or from the buildup of cholesterol on the walls of the arteries, which is termed atherosclerosis [9,91]. Over time, the injuries that develop from this lack of oxygen cause irreversible physical damage to the heart, and can result in myocardial infarction [89]. Following infarction, the heart undergoes structural changes to compensate for the loss of its tissue. The remodeling of cardiac structure

constitutes an important long-term adaptive mechanism, and involves development of hypertrophy, growth of capillary network, and an increase in interstitial collagen [9]. However, the structural changes are not limited only to the infarcted myocardium, but are extended into the non-infarcted area. Cardiac function eventually decreases due to transition of adaptive compensatory into the maladaptive mechanisms [9]. This transition depends on the nature of load imposed on the heart, where the most prominent heart failure risks are hypertension and volume overload [9,92]. Both risks are linked to the development of pathological hypertrophy and fibrosis, which ultimately result in heart failure [9]. However, the pathological hypertrophy has to be distinguished from the physiological hypertrophy as the latter is considered as benign, and contributes to an enhanced cardiac performance without development of heart failure [9,35].

2.1.2. Diabetes

Diabetes contributes to the development of heart failure even in the absence of coronary artery disease, supporting the concept of primary diabetic cardiomyopathy [93]. Although this disease is often associated with obesity, hypertension and high total cholesterol levels, the mechanisms that underlie the process of diabetes may also damage the heart muscle. These mechanisms primarily involve the activation of the cardiac renin-angiotensin system, which is linked to hypertension that eventually leads to heart damage [9,93].

2.1.3. Valvular Heart Diseases

Valvular heart diseases develop from stenosis (i.e. narrowing) or improper closing of cardiac valves, which control the flow of blood leaving the heart, and thus the normal routing

of blood flow is disrupted. Most common valvular diseases include mitral and aortic stenotic lesions that result in a backup of blood in left atrium during diastole (the filling phase of the heart), and in left ventricle during systole (the emptying phase of the heart), respectively. The increase in blood volume ultimately leads to increase in left-diastolic pressure, which over time induces development of hypertrophy and heart failure [94].

2.1.4. Cardiomyopathies

Genetic factors may play a role in development of certain cardiomyopathies, which damage heart muscle. The most common cardiomyopathies are hypertrophic cardiomyopathy and dilated cardiomyopathy. Hypertrophic cardiomyopathy is an idiopathic heart disease, affecting the interventricular septum and the left ventricle [95, 96]. In dilated cardiomyopathy, the heart ventricular wall undergoes progressive thinning that leads to cavity enlargement [95]. Both types of cardiomyopathies are associated with hemodynamic abnormalities, arrhythmias, and premature sudden cardiac death [97, 98]. Apoptosis of cardiac myocytes is observed in toxic cardiomyopathies, which was in the past commonly induced by the drug called doxorubicin (Adriamycin) [89].

2.1.5. Heart Failure and the Neurohormonal Milieu

Heart muscle damage is often associated with altered neurohumoral equilibrium, which in turn affects the vascular function and blood volume. Modifications in circulatory homeostasis serve as compensatory mechanisms for maintaining the cardiac output, and are commonly observed in the post-MI period [35,99,100]. Mechanisms triggered after tissue injury involve the overproduction of stimulatory and inhibitory signals. Stimulatory signals

are evoked by the increased production of angiotensin, antidiuretic hormone (vasopressin), endothelins and catecholamines, whereas the inhibitory signals are triggered by ligands including bradykinin, nitric oxide, prostaglandins, and natriuretic peptides [35,101]. Over time, the reciprocal regulation between the two groups of signals is lost due to excessive stimulation of the former, leading ultimately to the development of fibrosis, pathological hypertrophy, and thereby heart failure [35].

2.1.6. Other Causes of Heart Failure

Alcoholism is a heart failure risk as it is associated with chronic hypertension that leads to progressive cardiac damage [102]. Other factors that contribute to the development of heart failure involve excessive salt consumption, hyperthyroidism, thiamine deficiency, pneumonia, severe emphysema, high fever, and failure of liver or kidneys. Acute myocarditis is a viral infection of the heart muscle, and can also produce heart failure [103]. While similar mechanisms operate in the induction of heart failure in men and women, gender-related differences in the anatomy and physiology of myocardium should not be neglected as the presence of sex hormones could impact the onset of heart failure and modify the course of the disease between the two genders [104].

2.2. Heart Failure and the post-Myocardial Infarction (MI)

MI is irreversible damage to the heart muscle, where some of the cardiac muscle cells die as a result of the imbalance between the oxygen supply and demand of myocardium [89]. Inadequate oxygenation occurs due to ischemia or insufficient blood flow, which subsequently causes tissue hypoxia or anoxia often accompanied by angina or chest pain.

After MI, the heart undergoes structural changes that involve both infarcted and non-infarcted myocardium.

2.2.1. Wound Healing of Infarcted Myocardium

The injured ischemic heart tissue is characterized by the presence of the necrotic core composed of dead cardiomyocytes, and a border zone that surrounds it [9]. The border zone plays an important role during the scar formation at the site of infarction [105]. Infarct healing is an active process, which can be divided into four phases; the first phase involves cardiomyocyte cell death or myocyte drop out, which occurs by either apoptosis or overt necrosis (6-8 h after infarction) [9]. Myocyte necrosis evokes the second phase of the infarct healing that is characterized by the activation of inflammatory cells, cytokines, and neutrophilic granulocytes, which help to remove the dead myocytes (12 h till 4 days after infarction) [9]. The third phase is marked by onset of structural remodeling of the infarct site, and formation of granulation tissue. Granulation tissue itself is characterized by the appearance of myofibroblasts, enhanced activation of proteolytic matrix metalloproteinases, and neovascularization (2-3 days after infarction) [9]. Myofibroblasts are phenotypic variants of quiescent fibroblasts, and are involved in the synthesis and deposition of the extracellular matrix (ECM) proteins [10]. The importance of the matrix protein synthesis includes alterations in the tensile strength of the infarct, and may abrogate cardiac rupture [8,9]. Enhanced synthesis of fibrillar collagens *de novo* promotes development of cardiac fibrosis, whereas enhanced activities of interstitial collagenase (i.e. MMP-1) and gelatinases (MMP-2 and MMP-9) lead to degradation of the matrix in acute MI - the net action of these proteases contributes to infarct expansion and myocardial rupture [35,106]. The process of

neovascularization involves the formation of new blood vessels, which role is to increase the blood supply into the infarct area [9]. In the fourth phase of cardiac wound healing, granulation tissue becomes replaced by the scar formation. This phase is characterized by the complete cross-linkage of matrix proteins, and the relative abundance of myofibroblasts which do not undergo apoptosis at the completion of the wound healing phase (2-3 weeks after infarction) [9]. The role of myofibroblasts in the scar is important as they govern both acute and chronic matrix remodeling in the post MI-period [8].

2.2.2. Changes in Non-infarcted Myocardium

Changes that occur within the infarcted myocardium are attended by those in the non-infarcted tissue. As the infarct scar is unequipped and unable to generate rhythmic contractions as does the remnant heart, the non-ischemic myocardium adapts to the increased workload by changing the structural morphology of the heart muscle [88]. Changes in mechanical loading directly affect the function of cardiomyocytes, endothelial cells and fibroblasts [9,107]. Cardiomyocyte hypertrophy and apoptosis are prominent features of remnant post-MI heart; these changes are accompanied by the endothelial cell proliferation lining the capillaries [9]. As the proliferation of endothelial cells cannot compensate for the amount of cardiomyocyte hypertrophy, the myocytic phenotype shifts into the fetal state [9]. Fetal cardiomyocytes subsequently function with lower energy consumption associated with decreased myocardial function [9]. Concomitantly with the process of cardiomyocyte hypertrophy, mechanical feedback input affects fibroblast function in the remodeling heart; these cells transduce the mechanical stimuli from matrix to specific intracellular signaling responses that induce an altered pattern of protein expression. This sequence of events then

leads to remodeling of the ECM as fibroblasts increase their synthetic output [108,109]. Over time, cardiac fibrosis develops as a result of the increased production of the ECM proteins, which in most cases/etiologies seems to overwhelm the altered bioactivity of collagenase matrix metalloproteinases [107].

2.3. Cardiac Fibrosis

Cardiac fibrosis, or expansion of the cardiac interstitial space, is characterized by the elevated level of fibrillar collagen turnover, which occurs due to increased rate of collagen gene transcription and protein secretion in fibroblasts and myofibroblasts, as well as the enhanced bioactivity of proteolytic matrix metalloproteinases [15,37]. The fibrogenic process involves both reactive and reparative responses [4]. The reactive response is important for the normal wound healing at the early phase of post-MI tissue injury [6, 7]. It is characterized by the thickening of preexisting collagen fibers, and an increased number of fibers that appear between viable parenchymal cells [8]. On the other hand, the reparative fibrosis or scarring is an adaptative process to the loss of parenchyma, and involves the increased interstitial collagen deposition in remnant tissue, which prevents tissue destruction [8]. Fibrous tissue development is independent of the presence of parenchymal cells, which are terminally differentiated cells and thus cannot proliferate or regenerate [8]. Continuous parenchymal loss and abnormal collagen accumulation result in decreased passive compliance and impaired cardiac contractility, which potentiates the progression of overt heart failure [6,6-8].

2.4. Myofibroblasts

In the fibrogenic process, myofibroblasts play an important role in matrix remodeling by synthesizing and depositing collagen proteins [8, 13]. Myofibroblasts resemble fibroblast cells by having an extensive rough endoplasmic reticulum and Golgi apparatus, but in contrast to fibroblasts, myofibroblasts contain the *de novo* expression of α -smooth muscle actin (α -SMA) in more extensively developed stress fibers, supermature focal adhesions, and in several cases, desmin and embryonal isoform of myosin heavy chain isoform (SMemb) [8, 13, 90,110]. The expression of α -SMA by myofibroblasts is similar to the contractile myofibrils found in smooth muscle cells [8,13], and is thus considered as smooth muscle differentiation marker along with desmin and smooth muscle myosine heavy chain [110]. Although fibroblasts *per se* might contain actin, the distinguishing feature of these cells is that they do not exhibit mature stress fibers, and they do not form adhesion complexes with the matrix. This is held to be related to graded or incremental cellular expression of α -SMA [110]. The precursor candidates of myofibroblasts are fibroblasts, which under normal physiological conditions represent a heterogeneous population of cells [11,111]. In fibrosis, a specific fibroblast subpopulation of cells may become predominant, or alternatively fibroblasts undergo phenotypic modulation [13]. Among the factors that are involved in the phenotypic modulation of fibroblasts to myofibroblasts is TGF- β , a cytokine which is also responsible for the induction of mechanisms implicated in the enhanced collagen synthesis in the post-MI myocardium [13,15]. Collagen deposition by myofibroblasts prevents the rupture of the infarct tissue, and contributes toward the scar formation in the post-MI [8,9]. These cells can persist in myocardial scars for many years, and it has been suggested that α -SMA contributes to the infarct scar tissue contraction as well as maintenance of scar tonus, serving as an

important adaptation to the loss of the myocytes [8]. Myosin is also a required protein for cellular contraction and in the case of myofibroblasts, tissue contraction during cardiac healing may be also mediated via SMemb that are expressed by myofibroblasts [90].

3. Molecular Signaling Mechanisms Involved in Cardiac Diseases

The molecular signals involved in the induction of collagen synthesis, cardiac myocyte hypertrophy, and cell proliferation are mediated by various signaling pathways, including TGF- β and angiotensin signaling pathways [8,15]. It is now becoming clear that crosstalk between the signaling pathways may be of considerable importance in determining the net response of the cell in both normal physiological and pathological conditions.

3.1. Transforming Growth Factor β (TGF- β) Signaling Pathway

The TGF- β superfamily consists of a variety of structurally related multifunctional cytokines that include TGF- β s, activins/inhibins, glial cell-derived neurotrophic factor (GDNF), growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs), and anti-Mullerian hormone/Mullerian inhibiting substance (AMH/MIS) [29,112]. Signaling induced by TGF- β s plays an important role in cell proliferation, differentiation, migration, apoptosis, the extracellular matrix (ECM) production, and angiogenesis [31,113]. TGF- β isoforms are critical for embryonic development, and in maintaining biological processes that regulate tissue homeostasis during adult life [30, 31]. Perturbations of the TGF- β pathway result in a loss of cell growth regulation, which then triggers the progression of various clinical diseases, including auto-immune diseases, vascular disorders, tissue fibrosis, and cancer [31,112,114]. In the heart, perturbations of TGF- β signaling contribute toward

initiation and progression of cardiac hypertrophy, ventricular remodeling, the early response to MI, and heart failure [8,19].

3.1.1. TGF- β Isoforms

More than thirty proteins have structures similar to TGF- β s; therefore, they are collectively referred to as TGF- β related group of proteins (Table 2). In mammalian heart tissues, three isoforms of TGF- β s are identified, i.e. TGF- β_1 , TGF- β_2 , and TGF- β_3 [115]. These ligands have homodimeric structures, which helps TGF- β s to bind and bring together two different types of signaling TGF- β receptors named as type I and type II receptors [28,112]. Heterodimeric ligands of TGF- β s also exist, and include TGF- $\beta_{1,2}$ and TGF- $\beta_{2,3}$ [28].

TABLE 2

Representative Homologues of TGF- β Superfamily [29]

Name	% Homology	Representative Activities
BMP 2 subfamily		
BMP 2	100	Gastrulation, neurogenesis, chondrogenesis, apoptosis; in frog: mesoderm patterning; in fly: dorsalization, eyes, wings.
BMP 4	92	
BMP 5 subfamily		
BMP 5	61	Development of nearly all organs, neurogenesis.
BMP 6	61	
BMP 7	60	
BMP 8	55	
BMP 3 subfamily		
BMP 3	48	Osteogenic differentiation, endochondral bone formation, monocyte chemotaxis.
GDF 10	46	
Activin subfamily		
Activin β A	42	Pituitary follicle-stimulating hormone (FSH) production, erythroid cell differentiation; in frog: mesoderm induction.
Activin β B	42	
Activin β C	37	
Activin β E	40	
TGF-β subfamily		
TGF- β 1	35	Cell cycle arrest in epithelial and hematopoietic cells, control of mesenchymal cell proliferation and wound healing, extracellular matrix production, immunosuppression.
TGF- β 2	34	
TGF- β 3	36	
Distant members		
MIS/AMH	27	Mullerian duct regression.
Inhibin α	22	
GDNF	23	Dopaminergic neuron survival, kidney development.

3.1.2. Controlling TGF- β Ligand Activation

TGF- β is secreted as a latent complex, which maintains the ligand in an inactive state until its conversion to an active form. The latent complex is composed of TGF- β , the latent TGF- β associated propeptide (LAP), and the latent TGF- β binding protein (LTBP) [116]. The activity of TGF- β ligands is modulated by various ligand-binding proteins. One of the most important regulatory modes is achieved by LAP, which remains noncovalently coupled to the secreted TGF- β , making the ligand unfamiliar to its receptor. The TGF- β -LAP complex activation is also controlled by LTBPs. LTBPs govern proper folding and secretion of TGF- β into the ECM via binding to LAPs [30]. Among other activators of the latent TGF- β are plasminogen, thrombospondin-1 (TSP-1), and certain types of integrins [30,117]. The latent forms of activins and BMPs are likewise regulated. Follistatin is the Activin and BMP antagonist, whereas Noggin, Chordin, and the DAN family of proteins are antagonists of BMPs [30].

3.1.3. TGF- β Receptors

The members of TGF- β superfamily exert their effects through the ligand-induced heterodimerization of specific type I and type II serine/threonine kinase receptors [29,31,112]. Most mammalian cells express different members of this receptor family. Five type II receptors and seven type I receptors, which are also referred to as activin receptor-like kinases (ALKs), are identified [29,31,112]. The type II receptors differentiate into activin type II and type IIB receptors (ActR-II and ActR-IIB), TGF- β type II receptor (T β R-II), BMP type II receptor (BMPR-II), and AMH type II receptor (AMHR-II) [112]. Type I receptors include ALK-4 and ALK-5 that represent activin and TGF- β type I receptors (T β R-I), respectively;

ALK-2, ALK-3 and ALK-6 that are recognized as type I receptors for BMP signal transduction; ALK-1, which is known as an endothelial specific TGF- β type I receptor; and ALK-7 that still remains to be identified [31]. Both type II and type I receptors contain intracellular domains of serine and threonine residues, whereas their extracellular portions exist in various oligomeric forms such as type II homomers, type I homomers, and type II-type I heteromers [112]. The type II receptor, which is a constitutively active kinase, forms a complex with the type I receptor in a ligand dependent manner [29,112]. Theoretically, more than thirty different combinations of type II and type I receptor complexes are possible. However, under the physiological conditions the outcome in the signaling transduction becomes limited as the certain type II receptors tend to interact with certain type I receptors, and because some of the receptors are shared by different TGF- β ligands [29,112].

In TGF- β signaling, the formation of T β R-II/ T β R-I receptor complex triggers phosphorylation of particular serine and threonine residues at the TTSGSGSG sequence in the T β R-I [28,29,31]. This sequence is termed as the juxtamembrane domain or GS region [28]. The phosphorylation of GS by T β R-II activates T β R-I [28].

3.1.4. Controlling TGF- β Receptor Activation

The activity of TGF- β receptors is regulated by accessory receptors and the factors that inhibit the type I receptor activation. The accessory receptors are betaglycan and endoglin [30]. Betaglycan is a membrane-bound proteoglycan that binds to the isoforms of TGF- β (e.g., TGF- β_1 , TGF- β_2 , and TGF- β_3) to facilitate the ligand-induced receptor activation [30]. However, betaglycan cannot participate as a true signaling receptor as it lacks the signaling domain important for the signal transduction [30]. Endoglin is primarily involved in the

promotion of the ALK-1 activation [30]. The factors that inhibit the type I receptor activation involve inhibitory Smad proteins (I-Smad 6 and I-Smad 7), FKBP 12 (FK 506 binding protein of 12 kDa), FKBP 12.6 (FK 506 binding protein of 12.6 kDa), BAMBI (BMP and Activin membrane-bound inhibitor), and less known TRIP-1 (TGF- β -receptor interacting protein-1), STRAP (T β RI-interacting protein) and TRAP-1 (T β RI-associated protein-1) [29,30]. Ligand-induced receptor activation transduces signal to a family of intracellular signal mediators known as Smad proteins.

3.1.5. TGF- β signaling effectors: Smad Proteins

The Smad family of the TGF- β signaling effectors was discovered through genetic studies in *Drosophila melanogaster* and *Caenorhabditis elegans* [29, 31]. The “Smad” moniker originates from the fusion of two gene names: *Drosophila mothers against decapentaplegic* (Mad) and *Caenorhabditis elegans Small body size* (Sma) [29,118,119]. In vertebrates, Smads were identified as homologs to *Mad* and *Sma* genes [120]. Depending on the mechanism of their action, Smad proteins generally divide into three major groups: the receptor-mediated proteins or R-Smads, the common-mediator proteins or Co-Smads, and the inhibitory proteins or I-Smads (Figure 18). R-Smad group includes Smad 1, Smad 2, Smad 3, Smad 5, and Smad 8. These Smads are directly phosphorylated by TGF- β receptors. Co-Smads differentiate between Smad 4 in mammals, and Smad 4 α and Smad 4 β (i.e.Smads-10) in *Xenopus*. Co-Smads promote TGF- β signaling by forming heteromeric complexes with R-Smads. I-Smads include Smad 6 and Smad 7, which interfere with TGF- β downstream signaling, and thereby inhibit the signaling function of the other two Smad groups [28, 31].

3.1.5.1. Structure of Smads

The N- and C-termini of R-Smads and Co-Smads have two domains of high sequence similarity referred to as Mad homology (MH)1 and MH2, respectively [31]. The MH2 domain contains the L3 loop, which structure in part determines the specificity toward the R-Smad isoform phosphorylation by the activated receptor [29,31]. Accordingly, R-Smad 1, R-Smad 5, and R-Smad 8 are activated by the type I receptor for BMPs, GDFs and AMH/MIS, whereas R-Smad 2 and R-Smad 3 are activated by the type I receptor for TGF- β and activins [29]. The phosphorylation of R-Smads by traditional TGF- β mediated mechanisms occurs due to a SSXS (Ser-Ser-X-Ser) motif that is found on the L3 loop at the MH2 region [29]. As this motif is not found on the MH2 domain in Co-Smads and I-Smads, these two groups of Smads cannot be phosphorylated by the activated receptor [121]. It is known that angiotensin may also contribute to non-classical phosphorylation of R-Smads; however, little information is available regarding the angiotensin-induced intracellular signaling, which may regulate the induction of R-Smad phosphorylation [6]. The MH2 domain has several other functions in addition to providing the phosphorylation sites in R-Smads. These processes include binding to SARA (Smad anchor for receptor activation), regulation of transcriptional activity, induction of nuclear translocation, formation of homo- and heterooligomer complexes, and repression of the MH1 domain activity [31,121]. Although separated by a proline-rich linker region, the MH1 and MH2 domains show intrinsic affinity to each other, which when associated induce reciprocal inhibition [31,121]. The inhibitory association between the domains is relieved with the activation of the receptor [31,121]. The MH1 is found in R-Smads and Co-Smads, but not in I-Smads [121]. The primary function of this domain is to drive DNA-binding activity of Smads, which is however absent in R-Smad 2 [29,31,121].

However, the MH1 domain is recently observed to be also involved in non-traditional phosphorylation of R-Smads (i.e. R-Smad 2 and R-Smad 3) [111]. This type of Smad protein phosphorylation is mediated by protein kinase C (PKC), suggesting that this kinase may be involved in modulating the signaling responsiveness of R-Smads to TGF- β [111]. On the other hand, the function of the proline-rich linker region is to associate Smads with various proteins, including the ubiquitination regulatory factor Smurf-1 (R-Smad 1 and R-Smad 5), the transcriptional repressor Hoxc-8 (R-Smad 1), transcriptional factors c-jun, Jun B, and Jun D (R-Smad 3 and R-Smad 4), p42/p44 Erk MAP kinases (R-Smad 1, R-Smad 2 and R-Smad 3) and calcium/calmodulin-dependent protein kinase (R-Smad 2 and R-Smad 3) [30,31]. The proline linker can also be phosphorylated by MAP kinases, including p42/p44 Erk MAPKs [28].

3.1.5.2. Activation of Smads

In the basal state, the MH1 domain inhibits the biological activities of the MH2, which forces R-Smads to form an association with the membrane-associated FYVE-domain-containing protein SARA (Smad anchor for receptor activation) [29]. This association retains R-Smads in the cytoplasm when not activated by the receptor. However, SARA can in addition to R-Smads interact with T β R-I through its C-terminal region, or else it can bind phosphatidylinositol-3-phosphate (PI3P) via its FYVE domain [31]. Upon the phosphorylation of R-Smads by the activated receptor, the complex between R-Smad, SARA and T β R-I dissociates into individual components, which in turn triggers SARA to recruit other Smads for their phosphorylation [31]. The phosphorylated R-Smads form a new assembly, which can be either a heteromer if it binds Co-Smads, or homomer if composed of

R-Smads alone [31]. Once the complex is formed, R-Smads enter the nucleus where they regulate the gene expression. Independently of the receptor-induced R-Smad phosphorylation, the regulation of R-Smad activation may likewise be achieved by p42/p44 Erk mitogen activated protein (MAP) kinases, which can phosphorylate R-Smads at their linker domain [28]. In the basal state, Co-Smad is also retained in the cytoplasm. This is due to a leucine-rich nuclear export sequence (NES) found in the Co-Smad structure [31]. Upon R-Smad phosphorylation followed by the receptor activation, NES becomes inactivated by R-Smad binding to Co-Smad, which ensures that R-Smad/Co-Smad complex translocates into the nucleus [31]. I-Smads, including I-Smad 6 and I-Smad 7, are located in the nucleus in non-stimulated cells [31]. However, in the presence of TGF- β ligand and activation of the receptor, I-Smads are exported into the cytoplasm where they interact with the activated T β R-I, preventing R-Smads to become phosphorylated [14,31]. Molecular mechanisms that drive I-Smads out of the nucleus into the cytoplasm still remain unknown.

3.1.5.3. Nuclear Import of Smads

In the absence of ligand, T β R-I cannot be activated, and R-Smads are retained in the cytoplasm due to their association with SARA (Smad anchor for receptor activation) [29]. The ligand-induced receptor activation triggers R-Smad phosphorylation followed by their translocation into the nucleus. The nuclear accumulation of activated R-Smads does not depend on the presence of Co-Smads, although the complex between the phosphorylated R-Smads and Co-Smads is favorable [31]. The nuclear translocation of R-Smads depends on a specific sequence located at the N-terminal region referred to as the nuclear localization (NLS)-like sequence [31]. The NLS-like sequence is present in all R-Smads except R-Smad 2

[31]. The primary structure of R-Smad 3 features an NLS-like region, which serves for the importin β binding necessary for the process of nuclear translocation [31]. The mechanism of R-Smad 2 nuclear import is still not known. However, it is possible that this process is regulated at the specific site in the MH2 region, which is masked by SARA in the basal state [122]. Upon the ligand-induced phosphorylation of R-Smad-2, SARA dissociates from the MH-2 domain, triggering the nuclear accumulation of R-Smad-2 [31]. In contrast to R-Smads, Co-Smads require the phosphorylated R-Smads to translocate into the nucleus. The nuclear translocation of R-Smad/Co-Smad complex occurs due to inactivation of the nuclear export sequence (NES) in Co-Smads [31]. The inactivation of NES is triggered by the R-Smad binding to Co-Smads [31]. In contrast to R-Smads and Co-Smads, I-Smads are located in the nucleus in the basal state until stimulation when they become exported into the cytoplasm [31].

3.1.5.4. DNA -binding Function of Smads

Once in the nucleus, the activated R-Smad/Co-Smad complexes may undergo selective interactions with DNA. Although the R-Smad-2/Co-Smad-4 complex binds DNA only indirectly through the association with DNA-transcriptional adaptors, the R-Smad 3/Co-Smad 4 complex can directly interact with DNA binding sites [28,29]. The latter complexes have the ability to bind DNA at specific sequences referred to as Smad-binding elements (SBEs; 5'-CAGAC-3'), whereas the first bind degenerate SBEs (5'-GTCT-3') [121]. SBEs are found in many TGF- β responsive promoter regions, including PAI-1, type VII collagen, and $\alpha 2$ (I) procollagen. Although the full length of Co-Smad 4 can bind DNA at SBEs, R-Smad 3 binds SBEs via the MH1 domain, which possesses a β hairpin loop necessary for an

indirect DNA contact [31]. In contrast to R-Smad 3, R-Smad 2 lacks the intrinsic ability to bind DNA. The MH1 domain in R-Smad 2 has an extra exon upstream of the β hairpin, which produces an interference with the binding to SBEs [31].

3.1.5.5. Smad Transcriptional Regulators

Although the activated Smads can recognize and bind the specific DNA sequences at 5'-CAGAC-3' and 5'-GTCT-3', their affinity toward these sequences is low under physiological conditions, and thus they require assistance by other factors for establishing a selective DNA interaction [29]. Partner DNA-binding cofactors are a group of proteins that have a high affinity for the activated R-Smads, while they can simultaneously associate with DNA specific sequences [29]. One group of Smad cofactors function as DNA-binding adaptors, whereas others play a role of transcriptional partners. DNA-binding adaptors lack intrinsic transcriptional activity, and R-Smads are responsible for recruiting their own coactivators [29]. The adaptors include OAZ (Olf-associated zing finger) for R-Smad 1, and FAST (Forkhead Activin signal transducer) and Mixer for R-Smad 2 [29]. Transcriptional partners also facilitate the Smad-DNA binding, but they differ from adaptors by having the ability to function independently of R-Smads. Transcriptional partners for R-Smads are Jun B, TFE-3 (transcription factor binding to immunoglobulin heavy constant mu enhancer-3), CBFA/AML (core-binding factor A/acute myeloneous leukemia proteins), and LEF-1/TCF (lymphoid enhancer-binding factor-1/T-cell-specific factor) [29]. Depending on whether the activated R-Smads need to activate or repress gene expression, they bind either coactivators or corepressors, respectively. The R-Smad coactivators are p300 and CBP (CREB binding protein) and FOXH1, whereas corepressors include TGIF (TG 3-interacting factor), Ski

(Sloan-Kettering Institute proto-oncogene), and Sno N (Ski-related novel gene N) [29]. The level of corepressors activation versus coactivators determines the intensity in TGF- β /R-Smad responses [123].

3.2. Angiotensin II (Angiotensin) Signaling Pathway

Angiotensin is a multifunctional hormone of the renin-angiotensin aldosterone (RAAS) system. It is produced systemically via the classical circulating or renal RAAS, and locally via tissue RAAS present in the heart, brain, blood vessels, adrenals, the kidneys, liver and reproductive organs [100,124,125]. In the classical RAAS, hepatic-derived angiotensinogen is cleaved by renal-derived renin, which forms a decapeptide angiotensin I. This decapeptide is subsequently converted into octapeptide angiotensin II (angiotensin) via angiotensin-converting enzymes (ACEs) [100]. In tissues, angiotensin I may also form the heptapeptide angiotensin (1-7) through the activation of tissue endopeptidases [125]. As a pleiotropic peptide, angiotensin acts as a pressor agent, growth factor and cytokine [1]. It influences cell growth, apoptosis and differentiation, cell migration, deposition of the matrix proteins, and activates various intracellular signaling cascades, including MAP kinases, tyrosine kinases, and transcription factors [92]. Angiotensin is proinflammatory as it stimulates the production of certain growth factors, vasoactive hormones and cytokines [92]. It is also transactivator of several growth factor receptors, including PDGFR (platelet-derived growth factor receptor), EGFR (epidermal growth factor receptor), and IGF1R (insulin-like growth factor receptor) [1]. The actions of angiotensin are mediated via interrelated intracellular signaling pathways that transmit information from specific membrane receptors to the molecular cascades of proteins responsible for regulation of various cell activities. The

activated angiotensin signaling plays an important role in the etiology of hypertension and pathophysiology of cardiac hypertrophy, myocardial infarction (MI), cardiac ECM remodeling and heart failure [92]. Angiotensin type I (AT₁) receptors are highly expressed in cardiac myofibroblasts at the site of infarct in post-MI hearts [6,126,127]. Although these receptors participate in the induction of matrix protein synthesis and stimulate hypertrophy in infarct myofibroblasts [7,18,19,128], they do not appear to mediate proliferation of these cells [128]. The observed proliferative effect of angiotensin on fibroblasts may possibly occur via mechanisms that differentiate fibroblasts into growth-secreting phenotype, which by secreting growth or inflammatory substances can alter the cell number [129].

3.2.1. Angiotensin Receptors

In mammalian cells, angiotensin mediates its effects via angiotensin type I (AT₁) and type II (AT₂) plasma membrane receptors. The receptor subtypes can be distinguished by a pharmacological approach using specific antagonists. AT₁ receptors are antagonized by biphenylimidazoles including losartan, whereas AT₂ receptors are specifically inhibited by tetrahydroimidazopyridines such as PD 123319 [1]. AT₃ and AT₄ receptors also exist, but these are not included in the classification of mammalian angiotensin receptors because they are still not pharmacologically characterized [125].

3.2.1.1. Angiotensin Type I (AT₁) Receptor

The AT₁ is the major isoform of the angiotensin receptor that mediates most of the angiotensin physiological actions [125]. In rodents, AT₁ receptor has functionally two different subtypes (i.e. AT_{1A} and AT_{1B}) that have 94% amino sequence identity, and are

mapped to chromosomes 17 and 2, respectively [92]. In humans, AT₁ receptor has no subtypes, and it is mapped to chromosome 3 [92,125]. AT₁ is a member of the seven membrane- spanning G protein-coupled receptor family, which does not possess an intrinsic kinase activity [125]. The receptor activation is followed by the ligand binding, and involves the phosphorylation at serine and threonine residues by G protein receptor kinases (GRKs), or at tyrosine residues by various tyrosine kinases, including Janus kinases (JAK and TYK), Src kinases, and focal adhesion kinase (FAK) [1,125]. AT₁ typically activates G_{qαβγ} proteins that regulate PLC signaling pathways [1]. Other G proteins may also be activated (e.g., G_i, G_{q11}, G_{α12}, G_{α13}, and G_s), where the activation of each G isoform couples to specific signaling cascade [1]. AT₁ is abundantly distributed throughout the cardiovascular, renal, endocrine and nervous system [100]. In the heart, this receptor is present in both cardiomyocytes and fibroblasts, where the cellular actions of angiotensin may directly participate in the pathogenesis of various cardiovascular diseases [1,92].

3.2.1.2. Angiotensin Type II (AT₂) Receptor

The AT₂ is the second major isoform of the angiotensin receptors mapped as a single copy to the X chromosome [100]. It is a seven membrane- spanning G protein-coupled receptor, which acts as an antagonist of the AT₁ – mediated effects under physiological conditions [1,100]. The activation of AT₂ induces vasodilatory, antigrowth and apoptotic actions [1]. This receptor is highly expressed in developing fetal tissue, which amount declines rapidly after birth [100]. The re-expression of the AT₂ in adults is observed in vascular or cardiac injury, suggesting that this receptor may also play an important role in

tissue remodeling, growth and development [1]. In the heart, AT₂ receptor is present in both cardiomyocytes and fibroblasts, and appears in healthy and failing human hearts tissues [100].

3.2.2. Angiotensin Type I (AT₁) Receptor-mediated Signaling

The AT₁ receptor couples most of angiotensin physiological actions. It induces the signaling processes that are multiphasic with distinct temporal characteristics [1]. The immediate and early signaling events are triggered within seconds and minutes, respectively, whereas the late events require hours for their activation [125].

3.2.2.1. Phospholipid Activation

Following the ligand binding, the activated AT₁ induces rapid activation of phospholipase C (PLC), phospholipase D (PLD) and phospholipase A₂ (PLA₂). The phosphorylation of PLC results in the hydrolysis of phosphatidylinositol-4, 5-bisphosphate with subsequent generation of water soluble inositol triphosphate (IP₃) and membrane bound diacylglycerol accumulation (DAG) [1,125]. IP₃ is involved in Ca²⁺ sarcoplasmic mobilization, whereas DAG triggers the activation of PKC [1]. PLC isoforms exist, and these include PLC-β, PLC-γ and PLC-δ. These phospholipases are regulated by α and βγ subunits of G proteins, tyrosine phosphorylation and Ca²⁺, respectively [1]. The G_{qα}/PLC-β pathway plays an important role in cardiac hypertrophy, fibrosis, and in the remodeling of scar tissue in the post MI-heart [22]. The angiotensin-induced activation of PLD leads to the PLD-dependent hydrolysis of phosphatidylcholine, which in turn triggers DAG accumulation and PKC activation [125]. The PLD-mediated actions generate prolonged activation of secondary messengers in cardiomyocytes, suggesting its important role in the angiotensin-induced

growth and remodeling of cardiac tissue [1]. PLA₂ is another phospholipid involved in angiotensin signaling. The active PLA₂ is responsible for arachidonic acid release from the plasma membrane followed by the generation of eicosanoids [1]. The primary role of eicosanoids is to regulate blood pressure [1].

3.2.2.2. Protein Kinase C (PKC) Activation

The activation of PKC depends on diacylglycerol (DAG) generation, which is produced via angiotensin-induced PLC and PLD phosphorylation [1,130]. PKC is a member of a family of serine/threonine kinase proteins, consisting of eleven distinct isoenzymes [125,130]. This family involves calcium-sensitive cPKCs (α , β_1 , β_2 , γ), novel nPKCs (δ , ϵ , η , θ) and atypical aPKCs (ζ , λ , ι) [130]. Angiotensin stimulation triggers the translocation of cytosolic PKC to membrane structures where they become activated [125]. The activated forms of PKCs interact with membrane-associated anchoring proteins (RACKs), which direct the PKC isoforms to their molecular targets [130]. Many of the known PKC actions are mediated through the phosphorylation of mitogen kinases (e.g., p42/p44 Erk MAPK) and tyrosine kinase cascades [125,131]. The PKCs play a pivotal role in a spectrum of adaptive and maladaptive cardiac responses, including cardiac hypertrophy and structural remodeling [125,130,131]. Different isoforms of PKC family are selectively and differentially modified in the hypertrophic heart, whereby nPKCs are particularly affected in the angiotensin-induced hypertrophy [131]. However, the PKC activity also influences the TGF- β pathway, targeting the phosphorylation of R-Smad 2 and R-Smad 3 [111]. As downstream signaling effectors of TGF- β , R-Smads are known to mediate the increased synthesis of collagen [32,111]. The

phosphorylation of R-Smads by PKC is primarily associated with the activation of PLC- γ [111].

3.2.2.3. Receptor-induced Tyrosine Kinases (RTKs)

Ligand binding to RTKs triggers signaling pathways that affect tissue growth and cell proliferation. These pathways involve mitogen activated protein kinase (MAPK) activation, and tyrosine phosphorylation of intracellular proteins, including PLC- γ and PI3K [1,24,100]. RTKs are also activated by angiotensin via the process of transactivation, where the angiotensin-induced AT₁ receptor stimulation activates distant RTKs [100]. The RTKs involved in angiotensin-induced transactivation signaling include PDGFR (platelet-derived growth factor receptor), EGFR (epidermal growth factor receptor), and IGF1R (insulin-like growth factor receptor), all of which are primarily stimulated by growth factors and cytokines [1]. As the angiotensin-mediated RTK transactivation is also operative in growth-promoting signaling pathways, it suggests that angiotensin has in addition to its pressor properties inflammatory-like characteristics [1,100]. Angiotensin triggers the secondary release of various growth factors, including TGF- β ₁ cytokine that participates in tissue remodeling [4,8,100]. In cardiac fibroblasts and myofibroblasts, angiotensin stimulates TGF- β ₁ mRNA expression, which when released can trigger collagen expression in these cells [4].

3.2.2.4. Nonreceptor-induced Tyrosine Kinases (nonRTKs)

NonRTKs constitute a family of various intracellular tyrosine kinases, involving PLC- γ , Src family kinases, JAK and TYK, FAK, Ca²⁺-dependent tyrosine kinases (e.g., Pyk 2), Crk-associated substrate (p130^{Cas}) and PI3K [125]. Although these kinases are typically

activated by RTKs, they can also be activated by AT₁ [24,100]. The angiotensin-induced phosphorylation of nonRTKs occurs in minutes, which in turn triggers the molecular cascades that affect the activation of PKC and mitogen activated protein kinases (MAPKs) [1,100]. NonRTKs regulate processes such as integrin-mediated cell migration, cytoskeletal rearrangement, proliferation and cell survival [1,125]. Consequently, these kinases play an essential role in cardiac development and remodeling in cardiovascular disease [100].

3.2.2.5. Phosphatidylinositol 3-kinase (PI3K) Activation

PI3K is a nonreceptor-induced tyrosine kinase that is activated by receptor tyrosine kinases (RTKs) and AT₁ [125]. This kinase is a heterodimer composed of 85- and 110-kDa subunits [1]. The regulatory p85 subunit has no intrinsic catalytic activity, and it forms an association complex with the activated receptor to trigger the catalytic activity of p110 [24]. Concomitantly, p110 translocates from the soluble fraction to the vesicular fraction in Golgi membranes, catalyzing the synthesis of 3-phosphorylated phosphoinositides PI(3)P, PI(3,4)P₂ and PI(3,4,5)P₃ [24,100]. These lipids act as key intermediates in receptor-stimulated mitogenesis that determine cell response to a particular stimulating factor [24]. The PI3K activity regulates receptor-induced endocytosis, membrane and intracellular vesicular trafficking, cell survival, metabolism and cytoskeletal reorganization [24,125]. The molecular targets in PI3K signaling involve the activation of various kinases, including atypical PKCs, PLC- γ , mitogen activated protein kinases (MAPKs), and Akt/PKB [125]. However, PI3K also targets TGF- β signaling pathway. This kinase binds the FYVE domain of SARA, which is a membrane-bound protein that forms association complexes with nonphosphorylated R-Smad 2 and R-Smad 3 in the basal state [31]. R-Smads are downstream signaling effectors of TGF-

β , which actions are involved in mediating the increased synthesis of collagen [32,111]. The PI3K pathway in angiotensin signaling is proposed to serve as a balance between mitogenesis and apoptosis, which represents an important process in the regulation of vascular structure in health and disease [125].

3.2.2.6. Small G Protein Activation and Oxygen Radical Generation

In addition to heterotrimeric G proteins that regulate the activity of AT₁, monomeric small (21 kDa) guanine nucleotide-binding proteins (small G proteins) may also participate in the modulation of the AT₁-stimulated signaling pathways [1]. Small G proteins comprise a family of proteins divided into five subfamilies, which include Ras, Rho, ADP ribosylation factors, Rab and Ran [1]. The Rho subfamily of proteins is involved in angiotensin-induced regulation of cellular responses, resulting in cellular growth and Ca²⁺ mobilization [1]. These processes are mediated via mitogen activated protein kinases (i.e. JNK MAPK), PLD activation, and generation of reactive oxygen species [1]. The reactive oxygen species act as intracellular secondary messengers that contribute to the angiotensin-induced long-term signaling events [1].

3.3. Mitogen-activated Protein Kinase (MAPK) Signaling Pathway

MAPKs belong to a superfamily of serine/threonine kinases that regulate intracellular signal transduction pathways involved in cell differentiation, growth, and apoptosis [93]. The activation of MAPK signaling pathways is triggered by growth factors, cytokines and stress via the stimulation of receptor tyrosine kinases (RTKs) and G-protein-coupled receptors [132]. Signaling initiated by the MAPK pathways is sequential, where the phosphorylation of

MAPK kinase kinase (MAPKKK) activates MAPK kinases (MAPKK) that in turn phosphorylate and activate MAPK [1]. Once phosphorylated, MAPKs translocate from the cytosol into the nucleus, where they target other kinases and transcription factors involved in the program of cell proliferation. The molecular targets of MAPKs include the cytosolic 70/90 kDa ribosomal S6 kinase (RSK), EGFR (endothelin growth factor receptor), PLA₂ (phospholipase A₂), and transcription factors such as *c-myc*, *c-jun*, *c-fos* and Elk-1 [132]. Angiotensin differentially activates three MAPKs, including extracellular signal-regulated kinases of 42 kDa and 44 kDa (p42/p44 Erk MAPKs), c-Jun NH₂-terminal kinases of 46 kDa and 55 kDa (JNK MAPKs) and 38 kDa MAPK (p38 MAPK) [93]. TGF-β₁ also signals via MAPKs in addition to the Smad group of proteins. The MAPK pathway activated by TGF-β₁ involves the phosphorylation of TAK 1 (TGF-β₁-activated kinase 1), which belongs to the group of MAPKKK family of kinases [133]. TAK 1 signaling pathway is implicated in the activation of JNK MAPKs and p38 MAPK [133].

3.3.1. Extracellular Signal-regulated Kinases (p42/p44 Erk MAPKs)

The Erk MAPKs are activated in response to growth and differentiation factors, including angiotensin [1]. These kinases play a central role in cell proliferation, differentiation, cytoskeletal reorganization, and synthesis of various cytokines [1,125]. The angiotensin-induced activation of p42/p44 Erk MAPKs is mediated through atypical group of PKCs, and requires dual phosphorylation at threonine and tyrosine residues [1]. Phosphorylated Erk MAPKs translocate into the nucleus where they regulate the activation of various transcription factors, including AP-1 (activator protein 1) [1]. Angiotensin-induced increased activity of AP-1 is linked to TGF-β₁ mRNA expression, collagen type I synthesis,

and development of cardiac hypertrophy [23,93,134,135]. In TGF- β pathway, the Erk cascade interferes with collagen type I synthesis, which is due to binding of Erk MAPKs to specific phosphorylation sites in the linker region of R-Smads [23,30]. This in turn inhibits R-Smad nuclear accumulation and subsequent collagen synthesis in response to TGF- β_1 [30,32].

3.3.2. c-Jun NH₂-terminal Kinases (JNK MAPKs)

The JNK MAPKs are phosphorylated and activated by various environmental stress stimuli, inflammatory cytokines and growth factors [13]. The angiotensin activation of JNKs is mediated via novel PKCs, comprising a signaling pathway that regulates apoptosis and cell growth [1]. Following phosphorylation, the JNKs translocate to the nucleus where they regulate the activation of transcription factors such as c-Jun, ATF-2 and Elk-1 [1]. ATF-2 phosphorylation is also a common nuclear target of Smad and TAK 1 MAPKKK pathways, which are involved in TGF- β signaling [133]. In infarcted heart, the myocardial JNKs are highly activated, which suggests the possible involvement of JNKs in the development of cardiac diseases [88].

3.3.3. p38 MAPK

p38 MAPK is activated by various environmental stress stimuli, inflammatory cytokines and growth factors [13]. This kinase plays an important role in inflammatory responses, apoptosis and inhibition of cell growth [1]. p38 MAPK and Erk MAPKs are major mediators of the angiotensin mitogenic actions [23]. Angiotensin increases the phosphorylation and activation of p38 MAPK, which in turn contributes to the increased collagen type I and collagen type III synthesis [135]. The p38 MAPK signaling pathway is

significantly elevated in various pathological conditions, including cardiac ischemia, MI, tissue remodeling and cardiac hypertrophy [1,88].

4. Synopsis of Literature Review

TGF- β_1 is a potent stimulus of collagen synthesis in cardiac fibroblasts and myofibroblasts [13, 14]. Smad proteins are the major effectors of TGF- β signaling pathway, and are highly expressed in myofibroblasts [6,14,29]. However, these cells also display a crosstalk between Smad and angiotensin signaling pathways [6]. In the present study, we characterized the crosstalk between the activated TGF- β_1 and angiotensin postreceptor mechanisms, influencing the expression of P-Smad 2 in cultured primary cardiac myofibroblasts.

IV. MATERIALS AND METHODS

1. Materials

Culture media (Dulbecco's Modified Eagle Medium, DMEM/F-12, and Minimum Essential Medium, MEM), fetal bovine serum (FBS), and antibiotics (penicillin, streptomycin) were purchased from GIBCO BRL (Grand Island, NY). Culture plates and multiwell culture dishes were obtained from Fisher Scientific (Whitby, CA). Prestained low-molecular-weight marker, anti-mouse HRP and anti-rabbit horseradish peroxidase (HRP) conjugated IgGs were supplied by Bio-Rad (Hercules, CA). Polyvinylidene difluoride (PVDF) blotting membranes were obtained from Roche Diagnostics (Laval, CA). The enhanced chemiluminescence (ECL+ Plus) detection system, anti-mouse IgG Texas Red antibody, Streptavidin fluorescein, and anti-sheep/goat Ig, anti-rabbit Ig, and anti-mouse IgG biotinylated antibodies were from Amersham (Arlington Heights, IL). Human angiotensin II, anti-Flag antibody, and the protein assay kit were purchased from Sigma-Aldrich (Oakville, CA). Anti-human TGF- β_1 neutralizing antibody (AF-101-NA) and anti-human TGF- β RII neutralizing antibody (AF-241-NA) were obtained from R&D (Minneapolis, MN). Primary antibodies against phospho-specific p42/p44 Erk MAPKs, actin, and TGF- β_1 were acquired from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific p38 MAPK primary antibody was purchased from Calbiochem (Cambridge, MA). Primary antibody against phospho-specific R-Smad 2 was obtained from Calbiochem (San Diego, CA) and Upstate Biotechnology (Lake Placid, NY). Total R-Smad 2 primary antibody was obtained from Cell Signaling (Beverly, MA). The anti-type I collagen primary antibody was purchased from Southern Biotechnology Associates (Birmingham, AL). Procollagen (SP1.D8) primary antibody was kindly provided by Dr. H. Furthmayr (New Haven, CT). Rhodamine-phalloidin

was obtained from Cedarlane (Hornby, CA). α -smooth muscle actin primary antibody was purchased from Sigma (St. Louis, MO). LY 294002 and wortmannin (i.e. the inhibitors of phosphoinositide 3-kinase), chelerythrine chloride and bisindolylmaleimide I (i.e. the inhibitors of protein kinase C), SB 203580 (the inhibitor of p38 MAPK), and PD 98059 (the mitogen-induced extracellular kinase 1 inhibitor, and the inhibitor of p42/p44 Erk MAPKs) were all purchased from Calbiochem. Losartan (i.e. the angiotensin II type I receptor blocker) was obtained from MERCK (Rahway, NJ). Procollagen type I carboxy-terminal propeptide (PICP) ^{125}I radioimmunoassay (RIA) kit was purchased from DiaSorin (Stillwater, MN). The Effectene kit was obtained from Qiagen (Mississauga, CA). All other materials used were commercially available.

2. Preparation and Culture of Adult Rat Cardiac Myofibroblasts

Adult cardiac myofibroblasts cultures were prepared from male 200-250 g Sprague-Dawley rats according to the methods of Brilla et al. with minor modifications [36,111,136]. Rat hearts were subjected to Langendorff perfusion at a flow of 5 ml/min at 37°C with recirculatory Joklik's medium containing 0.1% collagenase and 2% bovine serum albumin (BSA) for 25-35 min. Liberated cells were collected by centrifugation at 2,000 rpm for 10 min, and resuspended in DMEM/F-12. Cells were seeded on a 100-mm noncoated culture flasks at 37°C with 5% CO₂, and grown in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 1 $\mu\text{l}/\text{ml}$ ascorbate. The cardiac fibroblasts attached to the bottom of the culture flasks during the two-hour primary incubation period, and were further maintained in the enriched DMEM/F-12. Nonadherent myocytes were removed by changing the culture medium. In this study we used myofibroblast cells from the first passage (P1) or second passage (P2). Based on preliminary studies (Figure 15),

the growth of subconfluent cells (70-90%) was arrested for 48 h by incubating the cells in DMEM/F-12 in the absence of serum.

The purity of fibroblasts was found to be $\geq 95\%$ using routine phenotyping methods as previously described [136,137]. Briefly, endothelial cells were labeled with the use of a monoclonal antibody against factor VIII, and we found that less than $\sim 1\%$ of cultured cells stained positively for this protein. Less than 1% of cells were positive for desmin, a protein specific for smooth muscle cells (SMCs), and more than 95% of cells stained positively for procollagen type I, which is a major protein product in fibroblast cells. However, based on results in Figure 19, passaged fibroblasts (P1 and P2 cells) that are, in particular, initially plated at low relative density, express markers for myofibroblast phenotype (i.e., α -smooth muscle actin, α -SMA) as previously described by Masur, et al. [138], and stained positively for procollagen type I, which is a major protein product in myofibroblast cells. It is emphasized that these results indicate that fibroblasts phenotypically modulate to myofibroblasts in culture when plated out at initial low density.

3. Protein Extraction and Assay

Inhibition or stimulation of cells was terminated by rinsing them twice with ice-cold phosphate buffered saline (PBS) prior the addition of 120 μ l RIPA lysing buffer (pH= 7.6), containing 150 mM NaCl, 1.0% nonidet P-40 (NP-40), 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4 , and 1 mM EGTA), and protease inhibitors (4 μ M leupeptin, 1 μ M pepstatin A, and 0.3 μ M aprotinin). Collected samples were allowed to lyse in RIPA buffer for 1h on ice, and were subsequently sonicated for 5 sec. Insoluble pellet (membrane fraction) was removed by

centrifugation for 15 min at 14,000 rpm at 4°C. Supernatant was collected, and stored at -20°C. The total protein concentration of all samples was measured using the bicinchoninic acid (BCA) method as previously described [139].

4. Western Blot Analysis of Target Proteins

Aliquots from cell lysates were mixed with Laemmli loading buffer (final concentration; 125 mM Tris-HCl (pH=6.8), 5 % glycerol, 2.5 % SDS, 5 % 2-mercaptoethanol, and 0.125 % bromophenol blue), and boiled for 5 min. Equal amounts of protein samples (15 µg) were resolved by 10 % SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with the help of prestained low-molecular-weight marker (10 µl). Separated proteins were electrophoretically transferred onto a 0.45 µM polyvinylidene difluoride (PVDF) membranes. PVDFs were blocked overnight at 4°C in Tris-buffered saline with 0.2 % Tween 20 (TBS-T) containing 5 % skim milk, and probed with primary antibodies for 1h at room temperature. Primary antibodies were diluted 1:250 in 0.2 % TBS-T with 5% skim milk, and included the antibodies against phosphorylated R-Smad 2, total R-Smad 2, phosphorylated p42/p44 Erk MAPKs, phosphorylated p38 MAPK, and actin. The incubation period of secondary antibodies was 1 h at room temperature with the dilution 1:10 000 in 0.2 % TBS-T containing 1% skim milk. Secondary antibodies included horseradish peroxidase (HRP)-labeled anti-mouse IgG for detection of total R-Smad 2, phosphorylated p42/p44 Erk MAPKs and actin, and anti-rabbit IgG for detection of phosphorylated R-Smad 2 and phosphorylated p38 MAPK. Protein bands on Western blots were visualized by ECL+ Plus according to the manufacturer's instructions, and developed on film. Even protein loading was confirmed by immunoblotting analysis against actin, by incubating PVDF membranes into Ponceau S solution (0.1 % Ponceau S in 5 % acetic acid), or by Coomassie blue staining.

5. Immunofluorescence Assay

Adult primary cardiac myofibroblasts were plated on coverslips, and allowed to grow for 24 h in DMEM/F-12 containing 10 % FBS until ~60% confluent. Cell growth was arrested by the addition of serum-free media for 48 h. Immunofluorescent staining was performed as previously described [6]. This technique was applied to detect R-Smad 2 nuclear translocation in cells being exposed to (1) TGF- β_1 stimulation; (2) angiotensin stimulation; and (3) pretreatment with chelerythrine or LY 294002 inhibitors followed by TGF- β_1 or angiotensin stimulation. Immunostaining method was also used to detect TGF- β_1 release in cells treated with angiotensin. Briefly, cells were washed twice with cold PBS, fixed in 4 % paraformaldehyde, rendered permeable with 0.1 % Triton X-100 in PBS, and incubated with total R-Smad 2 or TGF- β_1 primary antibodies over night at 4°C. After being washed with cold PBS, cells were incubated with the biotinylated anti-mouse IgG secondary antibody (for R-Smad 2 detection) or anti-rabbit IgG secondary antibody (for TGF- β_1 detection) followed by the incubation with FITC-labeled streptavidin tertiary antibody. The incubation period of the secondary and tertiary antibodies was 90 min. All antibodies were diluted at 1:20 in PBS containing 1 % bovine serum albumin (BSA). After being washed with cold PBS, cells were immersed in the nuclear dye Hoescht No. 33342 (10 $\mu\text{g}/\text{ml}$) for visualization of cellular nuclei, and subjected to additional wash with PBS. The slides were examined under a microscope equipped with epifluorescence optics, and photographed on Provia Fujichrome 400 color film or with a digital camera.

6. Transient Transfection of Cardiac Myofibroblasts, and Immunofluorescence Double Staining

Primary cardiac myofibroblasts were plated on coverslips, and allowed to grow in DMEM/F-12 containing 10 % FBS until ~50 % confluent. Cells were transiently transfected with a Flag epitope-tagged R-Smad 2 expression vector by using the Effectene kit. Twenty-four hours after the transfection, the immunofluorescence analysis was performed. Briefly, cells were washed twice with cold PBS, fixed in 4 % paraformaldehyde, rendered permeable with 0.1 % Triton X-100 in PBS, and double stained with anti-Flag antibody (1:1000 with 1% BSA in PBS) and anti-collagen type I antibody (1:100 with 1% BSA in PBS) over night at 4°C. After being incubated with the primary antibodies, cells were subjected to wash with cold PBS. The anti-Flag antibody was detected with anti-mouse Ig Texas Red (1:20 with 1% BSA in PBS for 90 min), whereas the anti-collagen type I antibody was detected with the anti-goat IgG biotinylated secondary antibody (1:20 with 1% BSA in PBS for 90 min) followed by the incubation with FITC-labeled streptavidin tertiary antibody (1:20 with 1% BSA in PBS for 90 min). After being washed with cold PBS, cells were immersed in the nuclear dye Hoescht No. 33342 (10 µg/ml) for visualization of cellular nuclei, and subjected to additional wash with PBS. The slides were examined under a microscope equipped with epifluorescence optics, and photographed on Provia Fujichrome 400 color film or with a digital camera.

7. Quantitative Determination of Procollagen Type I Carboxy-terminal Propeptide (PICP)

Adult cardiac myofibroblasts were grown in multiwell culture dishes in DMEM/F-12 with the presence of 10% FBS, and allowed to grow until ~90 % confluent. The cells were

then starved for 24 h in DMEM/F-12 with 0.5 % FBS, and stimulated with angiotensin (1 μ M) for 24 h. PICP 125 I RIA was performed according to the manufacturer's instructions. Beckman coulter gamma counter was used to detect the bound tracers from culture samples.

8. Cell Number Counting

The cell number counting was performed to normalize the PICP experiment, and thereby we used the same cell sample cultures as in the PICP experiment. Briefly, myofibroblasts were washed from the 10% serum media with PBS solution, removed from the bottom of the multiwell culture dishes by the addition of trypsin, and transferred into PBS. The final volume of all the samples was the same. The cell counting was carried out by using a Coulter counter.

9. Statistical Analysis

All values are expressed as means \pm SE. One-way analysis of variance (ANOVA) followed by the Student –Newman-Keuls method was used for comparing the differences among multiple groups (SigmaStat). Significant differences among groups were defined by a probability ≤ 0.01 or ≤ 0.05 .

V. RESULTS

1. Phosphorylated R-Smad 2 (P-Smad 2) Increases in TGF- β_1 - and Angiotensin-Stimulated Primary Cardiac Myofibroblasts

As TGF- β_1 is known to exert its effects through the R-Smad 2, we first examined the kinetics of the TGF- β_1 -induced P-Smad 2 expression. The experiments were performed within a 60 minute time-frame to observe the onset of immediate signaling events that could be involved in R-Smad 2 phosphorylation. We stimulated cells with TGF- β_1 (10 ng/ml) for 5, 15, 30, and 60 minutes, and measured P-Smad 2 by Western blot analysis (Figure 1A). TGF- β_1 -induced P-Smad 2 was noticed as early as at 5 minutes, and continuously increased at every subsequent time point relative to a control (Figure 1B). Concomitantly, we investigated the kinetics of the angiotensin-induced P-Smad 2 by stimulating the cells with angiotensin (1 μ M) at the same time points as indicated above (Figure 2A). In contrast to TGF- β_1 , angiotensin-induced P-Smad was phasic in a time-dependent manner (Figure 2B). Amounts of P-Smad 2 rapidly increased at 5 minutes, decreased by 15 minutes, increased once more at 30 minutes, and decreased again by 60 minutes. The pattern depicted showed a high degree of reproducibility. These results indicated that both TGF- β_1 - and angiotensin-treated primary cardiac myofibroblasts induced P-Smad 2, where the phosphorylation kinetics profile was unique to each of the signaling pathways at least within the first 60 min of stimulation.

2. Phosphorylated MAP Kinases are Expressed in TGF- β_1 - and Angiotensin-treated Primary Cardiac Myofibroblasts

To determine whether TGF- β_1 or angiotensin could stimulate the phosphorylation of MAP kinases in addition to R-Smad 2, we used immunoblotting to probe for phosphorylated p42/p44 Erk MAPKs (P-p42 Erk MAPK and P-p44 Erk MAPK) and phosphorylated p38 MAPK (P-p38 MAPK) at the indicated times (Figures 1A and 2A). In TGF- β_1 -treated cells, amounts of P-p42 Erk MAPK and P-p44 Erk MAPK increased in parallel with P-Smad 2 expression, whereas the amounts of P-p38 MAPK decreased, were minimal at 30 minutes, and returned to basal levels at 60 minutes (Figure 1B). In angiotensin-treated cells, amounts of P-p42 Erk MAPK and P-p44 Erk MAPKs were maximal at 15 minutes, and gradually decreased at every subsequent time point (Figure 2C). Amounts of P-p38 MAPK decreased, were minimal at 30 minutes, and returned to nearly basal levels at 60 minutes (Figure 2B). Thus, the phosphorylation of MAPKs occurred in both TGF- β_1 - and angiotensin-treated cells; however, the phosphorylation profiles of these signaling pathways were unique within the each type of stimulation.

3. Angiotensin-induced P-Smad 2 is Mediated via AT₁ Receptor in Primary Cardiac Myofibroblasts

Angiotensin increased P-Smad 2 accumulation. To test whether the angiotensin-induced P-Smad 2 was mediated via AT₁ or AT₂ receptors, we pretreated quiescent cells with losartan (10 μ M) or PD 123319 (10 μ M) for 1 h, respectively. Following inhibition, cells were stimulated with angiotensin (1 μ M) for 30 minutes. We observed the accumulation of P-Smad 2 by Western blotting (Figure 3A and 3B). Losartan treatment significantly attenuated

the angiotensin-induced P-Smad 2 increase relative to angiotensin (Figure 3C). In contrast to losartan, PD 123319 inhibitor did not decrease P-Smad 2 relative to angiotensin control values (Figure 3C). Thus, the increases in P-Smad 2 in the angiotensin-treated cardiac myofibroblasts are mediated via AT₁ receptors.

4. Angiotensin-induced P-Smad 2 is Independent of TGF- β ₁ Signaling in Cultured Cardiac Myofibroblasts

The phosphorylation of R-Smad 2 was induced in both TGF- β ₁- and angiotensin-treated cells. However, as angiotensin is known to trigger the synthesis of TGF- β ₁, then these observations could suggest that TGF- β ₁ is responsible for the angiotensin-mediated increase of P-Smad 2. To investigate this possibility, we used anti-TGF- β ₁ neutralizing antibody (TGF- β ₁ NA) to neutralize the biological activity of TGF- β ₁ in cultured cells. Following the pretreatment with TGF- β ₁ NA (1.5 ng/ml) for 1h, cells were stimulated with TGF- β ₁ (10 ng/ml) or angiotensin (1 μ M) for 30 minutes. P-Smad 2 was immunoblotted at indicated time points (Figure 4A and 4B). As shown in Figure 4C, TGF- β ₁ NA significantly reduced the P-Smad 2 accumulation in TGF- β ₁-treated cells. However, TGF- β ₁ NA did not affect angiotensin-induced P-Smad 2, which was still significantly increased relative to both non-treated cells and cells treated with TGF- β ₁ NA only (Figure 4C). Based on these observations, the angiotensin-induced P-Smad 2 appeared to be independent of TGF- β ₁-induced signaling pathway. To confirm this, we examined whether anti-TGF- β RII neutralizing antibody (T β R_{II} NA) affected P-Smad 2 expression in angiotensin-stimulated cell as T β R_{II} NA has the ability to neutralize the cell surface TGF- β RII mediated bioactivity. Therefore, we pretreated

cultured cells with T β RII NA for 1h at various concentrations followed by the stimulation with angiotensin (1 μ M) for 30 minutes. Regardless of dose, T β RII NA could not attenuate the angiotensin-induced P-Smad 2 expression as demonstrated by Western blotting (Figure 5). In addition, we also tested the release of TGF- β ₁ from cardiac myofibroblasts after stimulation with angiotensin (1 μ M) for 30 min. To immunostain the cells we used TGF- β ₁ primary antibody (Santa Cruz, CA), which detected granules of stored TGF- β ₁ in the cytosol of cells in both non-stimulated and stimulated cells. Based on these data, TGF- β ₁ release was not induced in angiotensin-treated cardiac myofibroblasts at least within the first 30 min of stimulation (Figure 20).

5. Effects of PD 98059 and SB 203580 MAPK Inhibitors on P-Smad 2 Expression in TGF- β ₁- and Angiotensin-stimulated Cardiac Myofibroblasts

The increases in P-Smad 2, and changes in p38 MAPK and p42/p44 Erk MAPKs phosphorylation in cardiac myofibroblasts were induced by TGF- β ₁ and angiotensin. These observations suggested that the induction of P-Smad 2 might be mediated via MAPK pathway. To test this possibility, we pretreated cells with PD 98059 (10 μ M) or SB 203580 (50 μ M) for 1h, and stimulated them with either TGF- β ₁ (10 ng/ml) or angiotensin (1 μ M) for 30 minutes. P-Smad 2 accumulation was measured by Western blot analysis (Figures 6A and 6B, and 7A and 7B). As demonstrated, PD 98059 inhibitor had no significant effect on the R-Smad 2 phosphorylation in either TGF- β ₁- or angiotensin-treated cardiac myofibroblasts at investigated time points (Figure 6C). In contrast to PD 98059, SB 203580 pretreatment decreased the angiotensin-induced P-Smad 2 at 30 minutes, but had no effect on the P-Smad 2 accumulation in TGF- β ₁-stimulated cells (Figure 7C). Thus, p38 MAPK might be involved in

modulating the phosphorylation level of R-Smad 2 in angiotensin-stimulated cardiac myofibroblasts. Stock solutions for inhibitors were suspended following the manufacturer's guidelines, and were mixed with DMSO. Therefore, we also tested the effect of DMSO on P-Smad 2 accumulation- no effect was observed as indicated by the Western blot analysis (Figure 16).

6. Effects of Chelerythrine Chloride and LY 294002 Inhibitors on P-Smad 2 Expression in TGF- β_1 - and Angiotensin- stimulated Primary Cardiac Myofibroblasts

As recent studies showed that phosphorylation of R-Smad 2 was dependent on PKC or PI3K activation [31,111], we also wished to examine how these kinases affected P-Smad 2 formation in TGF- β_1 - or angiotensin-treated cardiac myofibroblasts. To this end, we stimulated cells that had been pretreated with chelerythrine (1 μ M) or LY 294002 (10 μ M) for 1 h with TGF- β_1 (10 ng/ml) or angiotensin (1 μ M) for 30 minutes. P- Smad 2 expression was measured by Western blot analysis (Figures 8A and 8B, and 9A and 9B). As demonstrated, neither chelerythrine nor LY 294002 had any effect on R-Smad 2 phosphorylation in TGF- β_1 -treated cells at investigated time points (Figures 8C and 9C). However, in the angiotensin-treated cells the same inhibitors significantly decreased P-Smad 2 relative to control values (Figures 8C and 9C). The effects of chelerythrine and LY 294002 on P-Smad 2 in the angiotensin-stimulated cells were confirmed with bisindolylmaleimide I (i.e., PKC inhibitor) and wortmannin (i.e., PI3K inhibitor), respectively (Figures 17 and 18). These results demonstrated that PI3K and PKC might be possible mediators in modulating R-Smad 2 phosphorylation in the angiotensin, but not in TGF- β_1 signaling pathway. Stock solutions for the inhibitors were prepared following the manufacturer's guidelines, were mixed with

DMSO, and tested as in section 5. No effect of DMSO on P-Smad 2 expression was observed (Figure 16).

7. Effects of Chelerythrine chloride and LY 294002 on P-Smad 2 Nuclear Translocation in TGF- β_1 -and Angiotensin-stimulated Primary Cardiac Myofibroblasts

In these set of experiments, we sought to further investigate the effects of chelerythrine and LY 294002, and examine their roles on the nuclear translocation of P-Smad 2 in TGF- β_1 -or angiotensin-treated cells. The P-Smad 2 nuclear translocation was detected by immunocytochemical staining. For the staining we used the total R-Smad 2 primary antibody that recognized both phosphorylated and non-phosphorylated state of R-Smad 2. In the basal state (i.e. control group), anti-R-Smad 2 immunostaining was distributed to the cytoplasm proximal to nuclei (Figures 10A and 10B). In cells stimulated with TGF- β_1 (10 ng/ml) or angiotensin (1 μ M) anti-R-Smad 2 immunostaining was detected in the nuclei, suggesting that P-Smad 2 accumulated in the nuclei. Increased P-Smad 2 nuclear accumulation was noted at 15 and 30 min after TGF- β_1 treatment (Figure 10A), and at 5 and 30 minutes after angiotensin treatment (Figure 10B). These observations indicated that both TGF- β_1 and angiotensin could drive the nuclear translocation of P-Smad 2 in a time dependent fashion. However, in cells pretreated with chelerythrine (1 μ M) or LY 294002 (10 μ M) for 1 h, and thereafter stimulated with TGF- β_1 or angiotensin for 30 minutes, anti-R-Smad 2 staining remained in the extracellular space, suggesting that the P-Smad 2 nuclear translocation was attenuated in both pretreated groups (Figure 11). LY 294002 appeared to attenuate this process more potently compared to chelerythrine.

8. Collagen Type I Concentration Increases in Angiotensin-treated Cardiac

Myofibroblasts

To verify that angiotensin induces collagen type I synthesis in primary cardiac myofibroblasts, we stimulated cells with angiotensin (1 μ M) for 24 h, and performed the PICP experiment. As shown in Figure 12, angiotensin induced a 2.4-fold increase of collagen type I expression in primary cardiac myofibroblasts relative to control ($P^* < 0.01$).

9. Overexpression of R-Smad 2 in Cardiac Myofibroblasts Increases Collagen Type I

Expression

Angiotensin increased both phosphorylation of R-Smad 2 and collagen type I synthesis. These observations suggested that P-Smad 2 could exert a direct influence on collagen I expression in cultured cardiac myofibroblasts. To begin addressing a cause and effect relationship between P-Smad 2 and collagen synthesis, we transiently transfected nonquiescent cells with Flag epitope-labeled R-Smad 2. The transfection efficiency was estimated at 10%, which was obtained by calculating the positively stained cells after probing with anti-Flag antibody. The ectopic Flag-R-Smad 2 was localized mainly in the cytoplasm as detected by immunofluorescence analysis (Figure 13). The relative expression of collagen type I in the R-Smad 2-transfected cells was also detected by immunofluorescence analysis (Figure 13). The R-Smad 2-transfected cells showed increased expression of collagen type I relative to neighboring nontransfected cells. This result was consistent between transfected cells, indicating that R-Smad 2 overexpression was associated with the increased level of collagen type I protein synthesis in primary cardiac myofibroblasts.

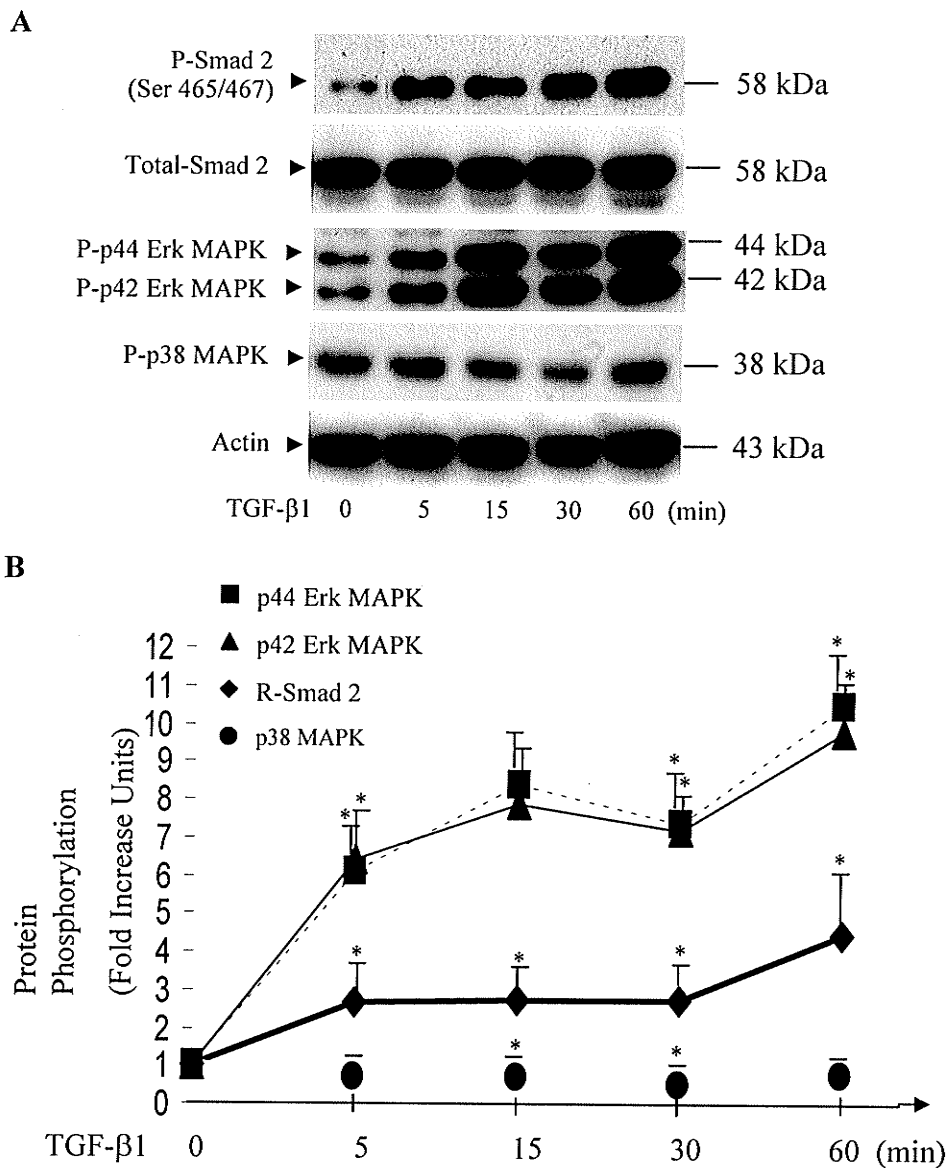


Figure 1. Panel A: Representative Western blot analysis of phosphorylated R-Smad 2 (P-Smad 2) and phosphorylated cytoplasmic kinases in cultured primary cardiac myofibroblasts treated with TGF- β 1 (10 ng/ml) at indicated times; 0% serum starvation for 48 h; total cell lysates. Western blots show P-Smad 2 (~58 kDa) protein, total Smad 2 (~58 kDa) expression, phosphorylated p42 Erk MAPK or P-p42 Erk MAPK (~42 kDa), phosphorylated p44 Erk MAPK or P-p44 Erk MAPK (~44 kDa), and phosphorylated p38 MAPK or P-p38 MAPK (~38 kDa). Western blots of actin (~43 kDa) indicate relatively even protein loading among the lanes. **Panel B:** Graphical representation of time-dependent changes in phosphorylation profiles of signaling cascades shown in the panel A; $P \leq 0.05$ vs. control; data was quantified by densitometric scanning, and expressed as mean \pm SEM ($n \geq 3$).

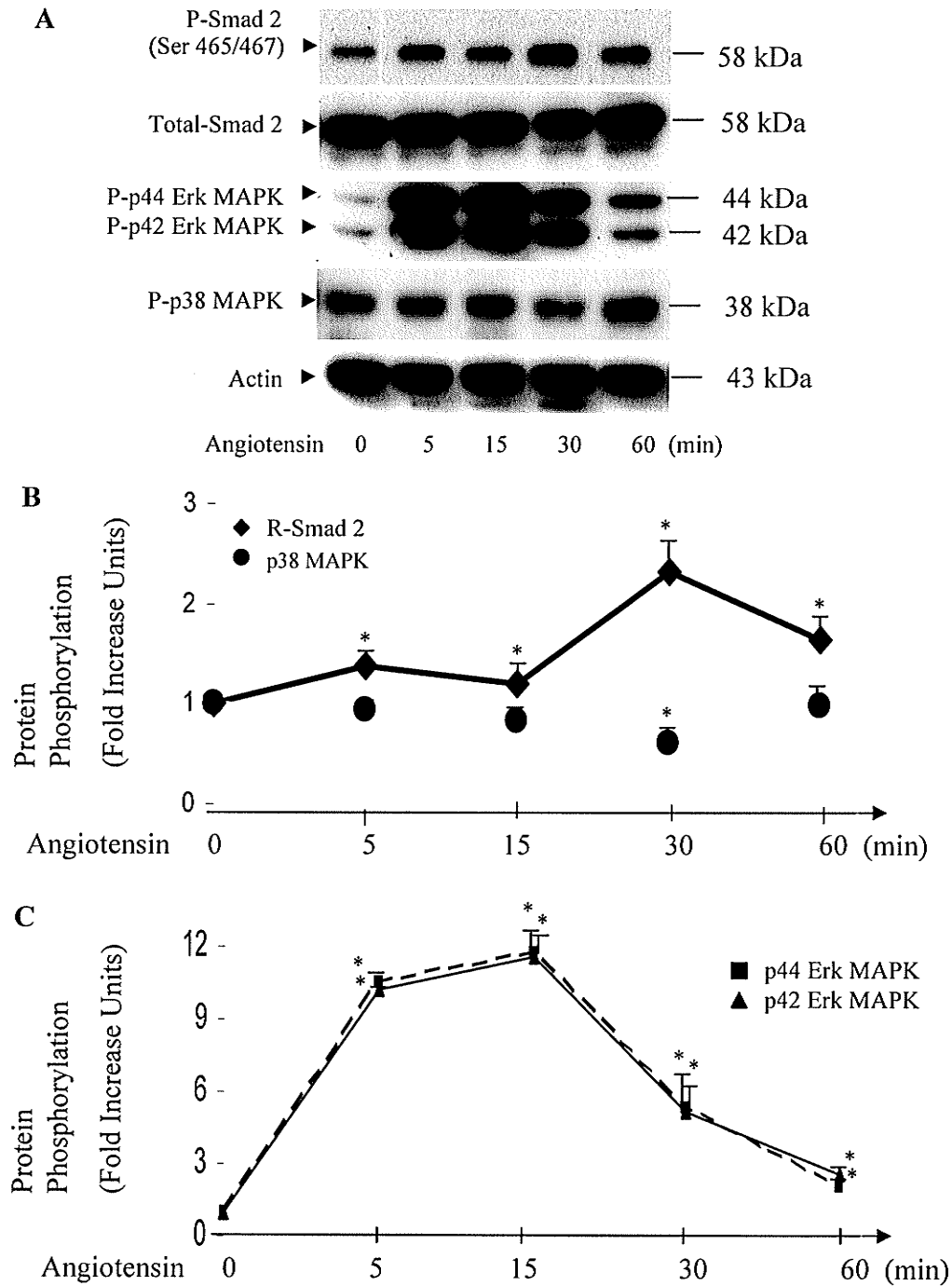


Figure 2. Panel A: Representative Western blot analysis of phosphorylated R-Smad 2 (P-Smad 2) and phosphorylated cytoplasmic kinases in cultured primary cardiac myofibroblasts treated with angiotensin (1 μ M) at indicated times; 0% serum starvation for 48 h; total cell lysates. Western blots show P-Smad 2 (~58 kDa) protein, total Smad 2 (~58 kDa) expression, phosphorylated p42 Erk MAPK or P-p42 Erk MAPK (~42 kDa), phosphorylated p44 Erk MAPK or P-p44 Erk MAPK (~44 kDa), and phosphorylated p38 MAPK or P-p38 MAPK (~38 kDa). Western blots of actin (~43 kDa) indicate relatively even protein loading among the lanes. **Panels B and C:** Graphical representation of time-dependent changes in phosphorylation profiles of R-Smad 2 (B), p38 MAPK (B), p44 Erk MAPK (C), and p42 Erk MAPK (C) signaling cascades shown in the panel A; $P^* \leq 0.05$ vs. control; data was quantified by densitometric scanning, and expressed as mean \pm SEM ($n \geq 3$).

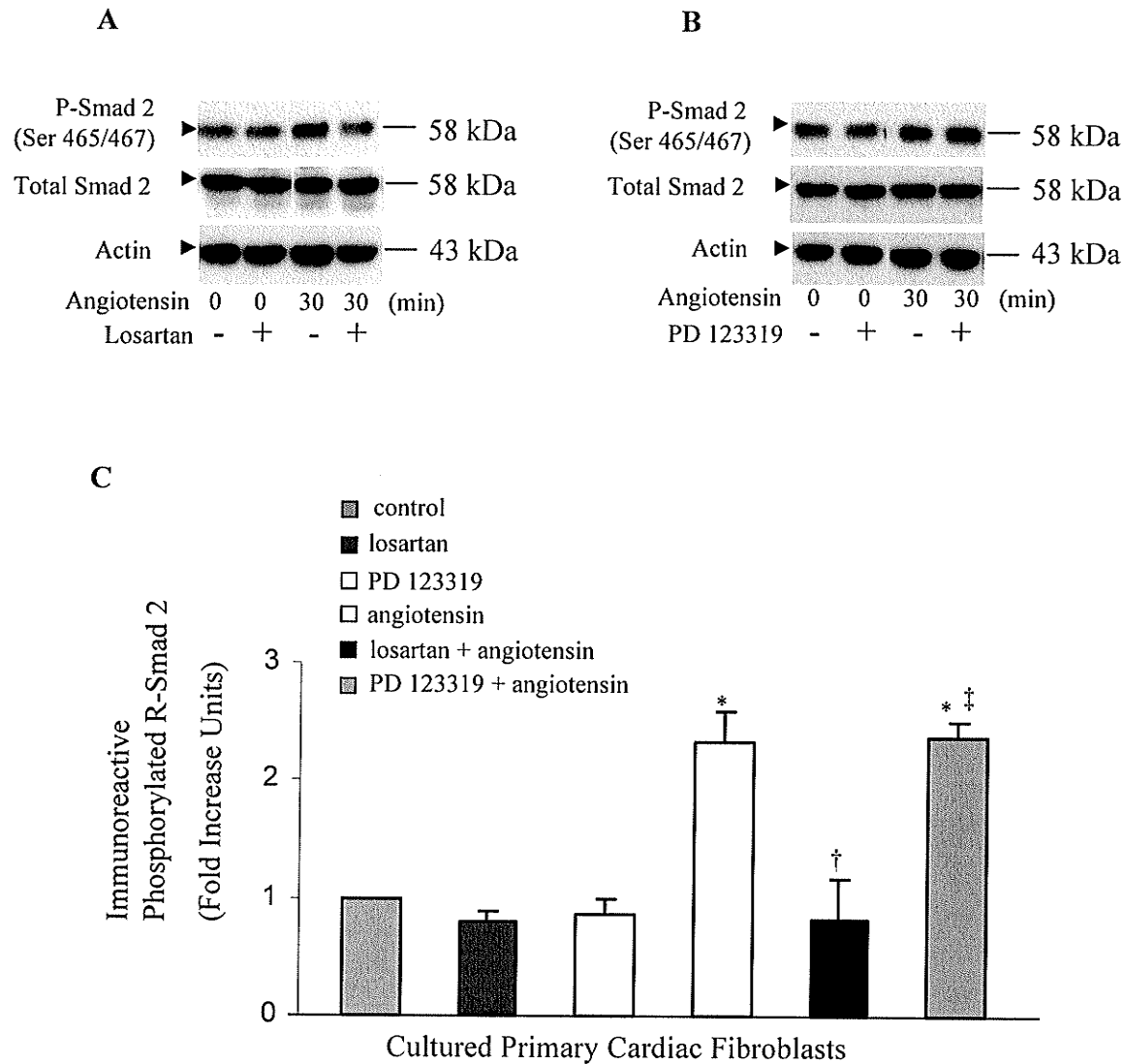


Figure 3. Panels A and B: Western blot analysis of phosphorylated R-Smad 2 (P-Smad 2) in cultured primary cardiac myofibroblasts pretreated with losartan (antagonist of angiotensin type I receptor) or PD 123319 (inhibitor of angiotensin type II receptor); 0% serum starvation for 48 h; total cell lysates. Cells were pretreated with losartan (10 μ M) or PD 123319 (10 μ M) for 1h prior the stimulation with angiotensin (1 μ M) for 30 min. Representative Western blots show P-Smad 2 (~58 kDa) protein, total Smad 2 (~58 kDa), and actin (~43 kDa). Actin is an indicator of relatively even protein loading among the lanes. **Panel C:** Histogrammic representation of quantified data of immunoreactive P-Smad 2 from the groups of samples shown in the panels A and B (quantified by densitometric scanning); $P^* \leq 0.05$ vs. control; $P^\dagger \leq 0.05$ vs. angiotensin; $P^* \dagger \leq 0.05$ vs. PD 123319; data are expressed as mean \pm SEM ($n \geq 3$).

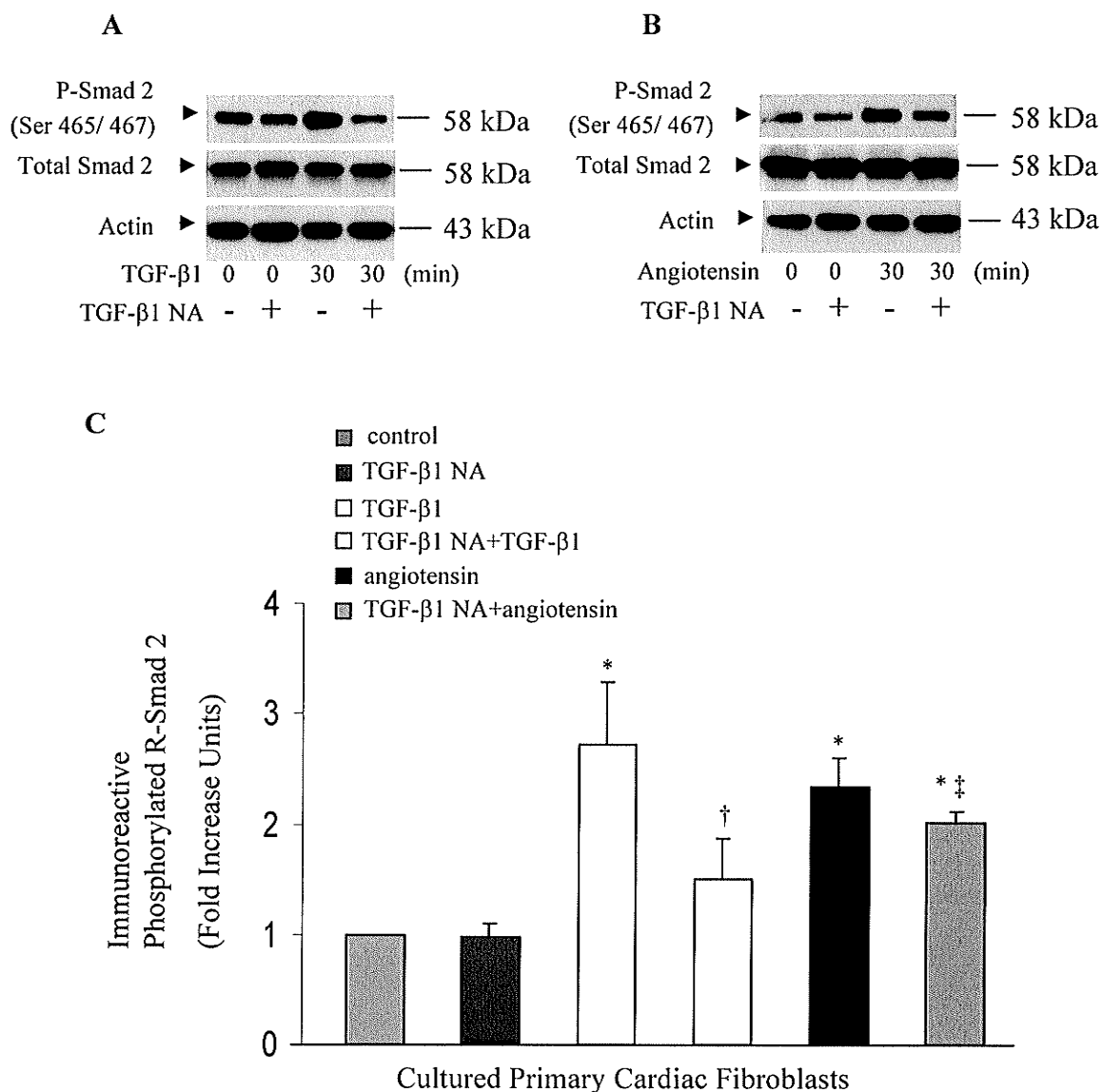


Figure 4. Panels A and B: The effects of TGF- β 1 neutralizing antibody (TGF- β 1 NA) on TGF- β 1- and angiotensin-mediated R-Smad 2 phosphorylation (P-Smad 2) in cultured primary cardiac myofibroblasts; 0% starvation for 48 h; total cell lysates. Cells were pretreated with TGF- β 1 NA (1.5 ng/ml) for 1 h followed by stimulation with TGF- β 1 (10 ng/ml) or angiotensin (1 μ M) for 30 min. Representative Western blots show P-Smad 2 (~58 kDa) and total Smad 2 (~58 kDa) expression. Western blots of actin (~43 kDa) in panels A and B indicate relatively even protein loading among the lanes. **Panel C:** Histogrammic representation of quantified data of immunoreactive P-Smad 2 from the groups of samples shown in the panels A and B (quantified by densitometric scanning); P* \leq 0.05 vs. control; P† \leq 0.05 vs. TGF- β 1; P‡ \leq 0.01 vs. TGF- β 1 NA ; data are expressed as mean \pm SEM (n \geq 3).

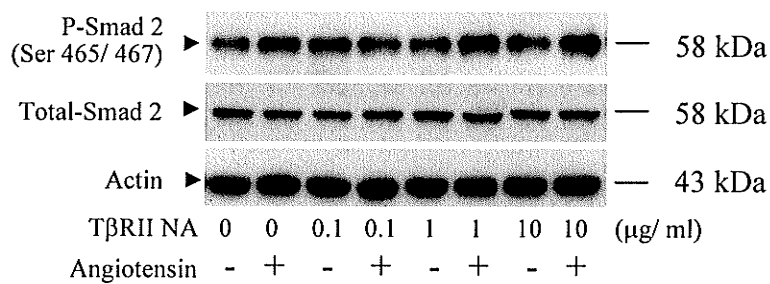


Figure 5. Dose-dependent effect of TGF- β RII neutralizing antibody (TBR II NA) on R-Smad 2 phosphorylation (P-Smad 2) in cultured primary cardiac myofibroblasts stimulated with angiotensin; 0% starvation for 48 h; total cell lysates. Cells were pretreated with TBR II NA with indicated doses for 1 prior to stimulation with angiotensin (1 μ M) for 30 min. Representative Western blots show P-Smad 2 (~58 kDa) and total Smad 2 (~58 kDa). Western blot of actin (~43 kDa) indicates relatively even protein loading among the lanes.

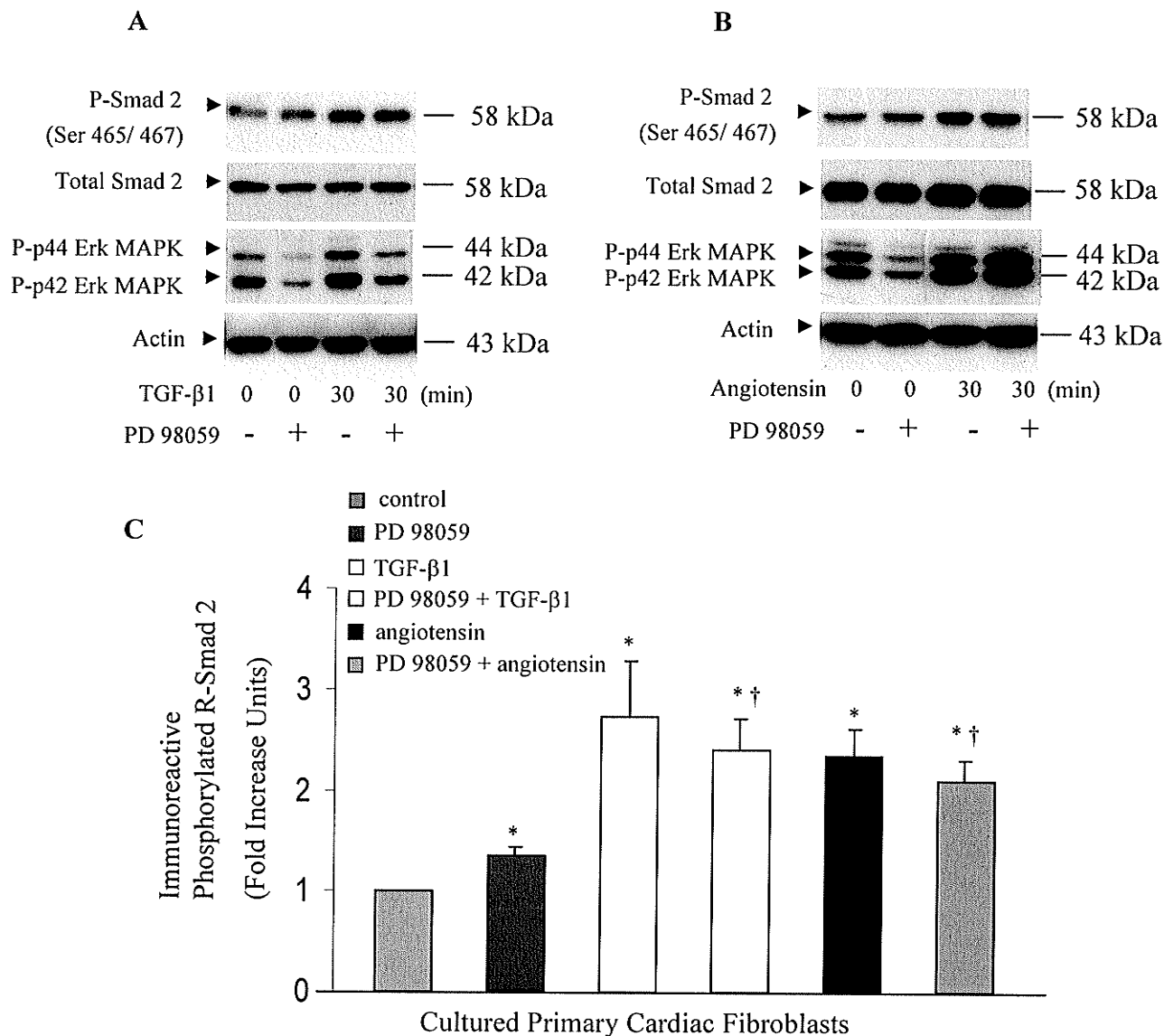


Figure 6. Panels A and B: Western blot analysis of phosphorylated R-Smad 2 (P-Smad 2) in cultured primary cardiac myofibroblasts pretreated with PD 98059 (the upstream inhibitor of p42/p44 Erk MAPKs, i.e. MEK-1); 0% serum starvation for 48 h; total cell lysates. Cells were pretreated with PD 98059 (10 μ M) for 1h prior the stimulation with TGF- β 1 (10 ng/ml) or angiotensin (1 μ M) for 30 min. Representative Western blots show P-Smad 2 (~58 kDa) protein, total Smad 2 (~58 kDa), phosphorylated p42 Erk MAPK or P-p42 Erk MAPK (~42 kDa), and phosphorylated p44 Erk MAPK or P-p44 Erk MAPK (~44 kDa). Western blots of actin (~43 kDa) indicate relatively even protein loading among the lanes. **Panel C:** Histogrammic representation of quantified data of immunoreactive P-Smad 2 from the groups of samples shown in the panels A and B (quantified by densitometric scanning); $P^* \leq 0.05$ vs. control; $P^\dagger \leq 0.05$ vs. PD 98059; data are expressed as mean \pm SEM ($n \geq 3$).

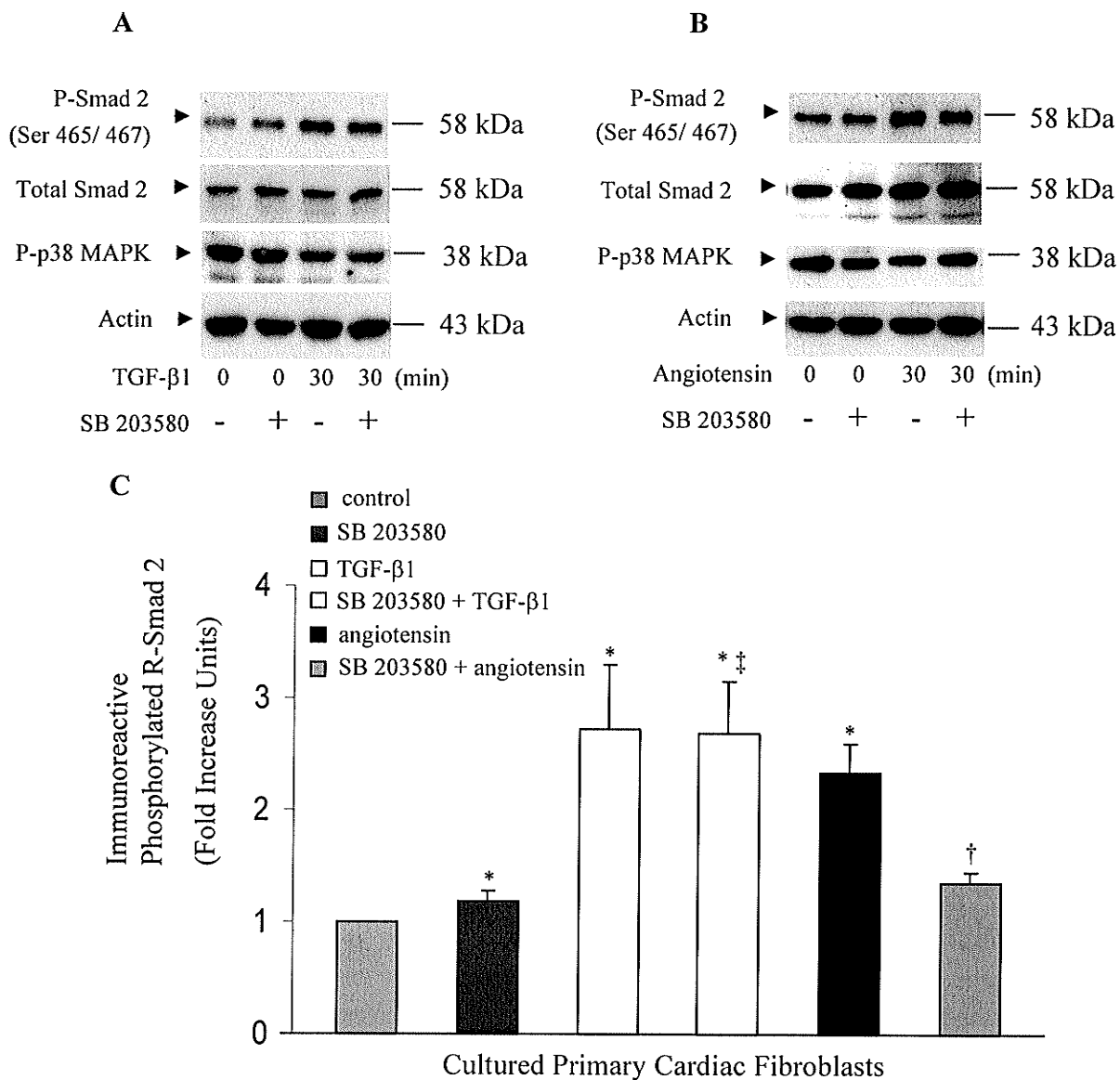


Figure 7. Panels A and B: Western blot analysis of phosphorylated R-Smad 2 (P-Smad 2) in cultured primary cardiac myofibroblasts pretreated with SB 203580 (p38 MAPK inhibitor); 0% serum starvation for 48 h; total cell lysates. Cells were pretreated with SB (50 μ M) for 1h prior the stimulation with TGF- β 1 (10 ng/ml) or angiotensin (1 μ M) for 30 min. Representative Western blots show P-Smad 2 (~58 kDa) protein, total Smad 2 (~58 kDa), and phosphorylated p38 MAPK or P-p38 MAPK (~38 kDa). Western blots of actin (~43 kDa) indicate relatively even protein loading among the lanes. **Panel C:** Histogrammic representation of quantified data of immunoreactive P-Smad 2 from the groups of samples shown in the panels A and B (quantified by densitometric scanning); $P^* \leq 0.05$ vs. control; $P_{\dagger} \leq 0.05$ vs. SB 203580; $P^{\ddagger} \leq 0.05$ vs. angiotensin; data are expressed as mean \pm SEM ($n \geq 3$).

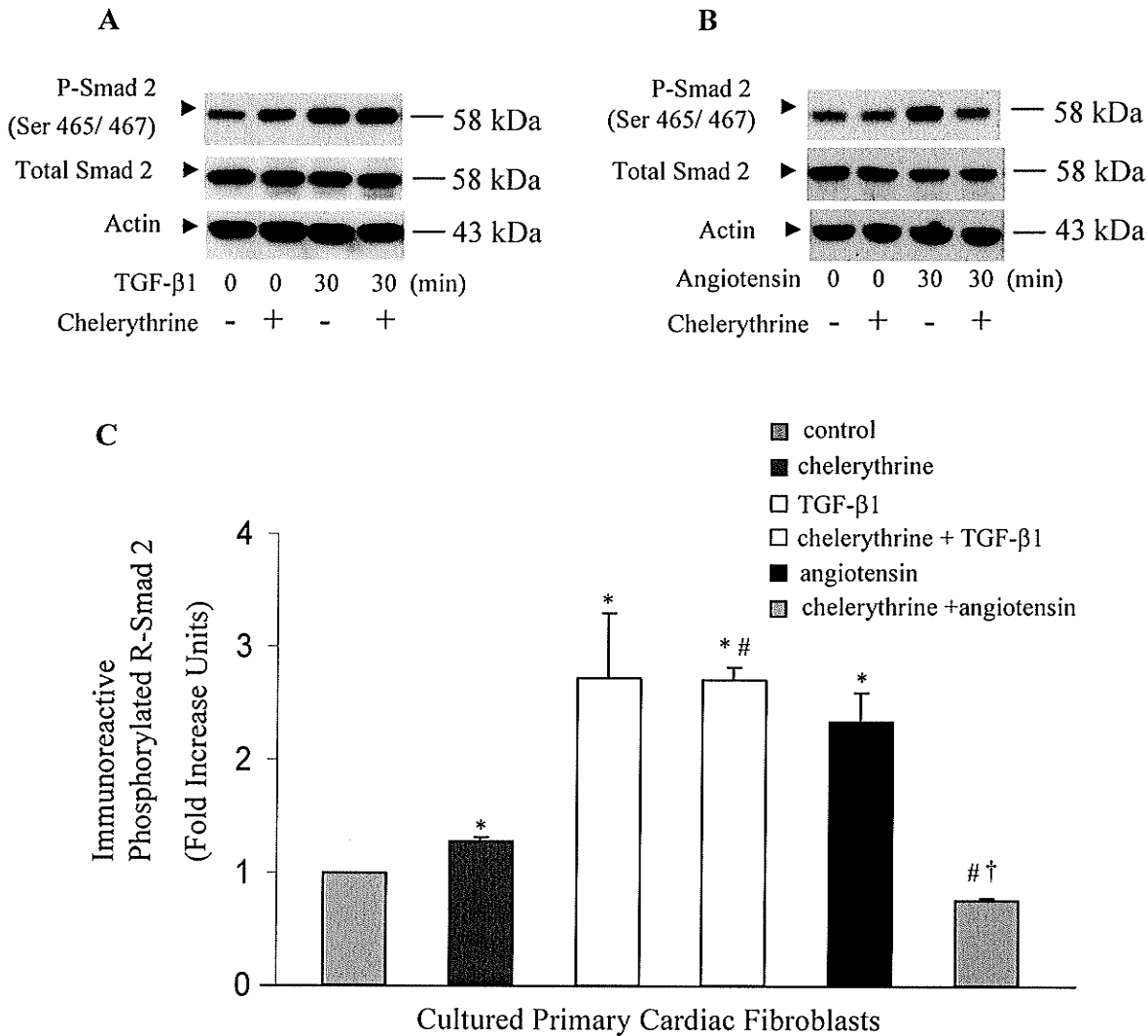


Figure 8. Panels A and B: Western blot analysis of phosphorylated R-Smad 2 (P-Smad 2) in cultured primary cardiac myofibroblasts pretreated with chelerythrine chloride (PKC inhibitor); 0% serum starvation for 48 h; total cell lysates. Cells were pretreated with chelerythrine (1 μ M) for 1h prior the stimulation with TGF- β 1 (10 ng/ml) or angiotensin (1 μ M) for 30 min. Representative Western blots show P-Smad 2 (~58 kDa) protein and total Smad 2 (~58 kDa). Western blots of actin (~43 kDa) indicate relatively even protein loading among the lanes. **Panel C:** Histogrammic representation of quantified data of immunoreactive P-Smad 2 from the groups of samples shown in the panels A and B (quantified by densitometric scanning); $P^* \leq 0.05$ vs. control; $P^\# \leq 0.05$ vs. chelerythrine; $P^\dagger \leq 0.05$ vs. angiotensin; data are expressed as mean \pm SEM (n=3).

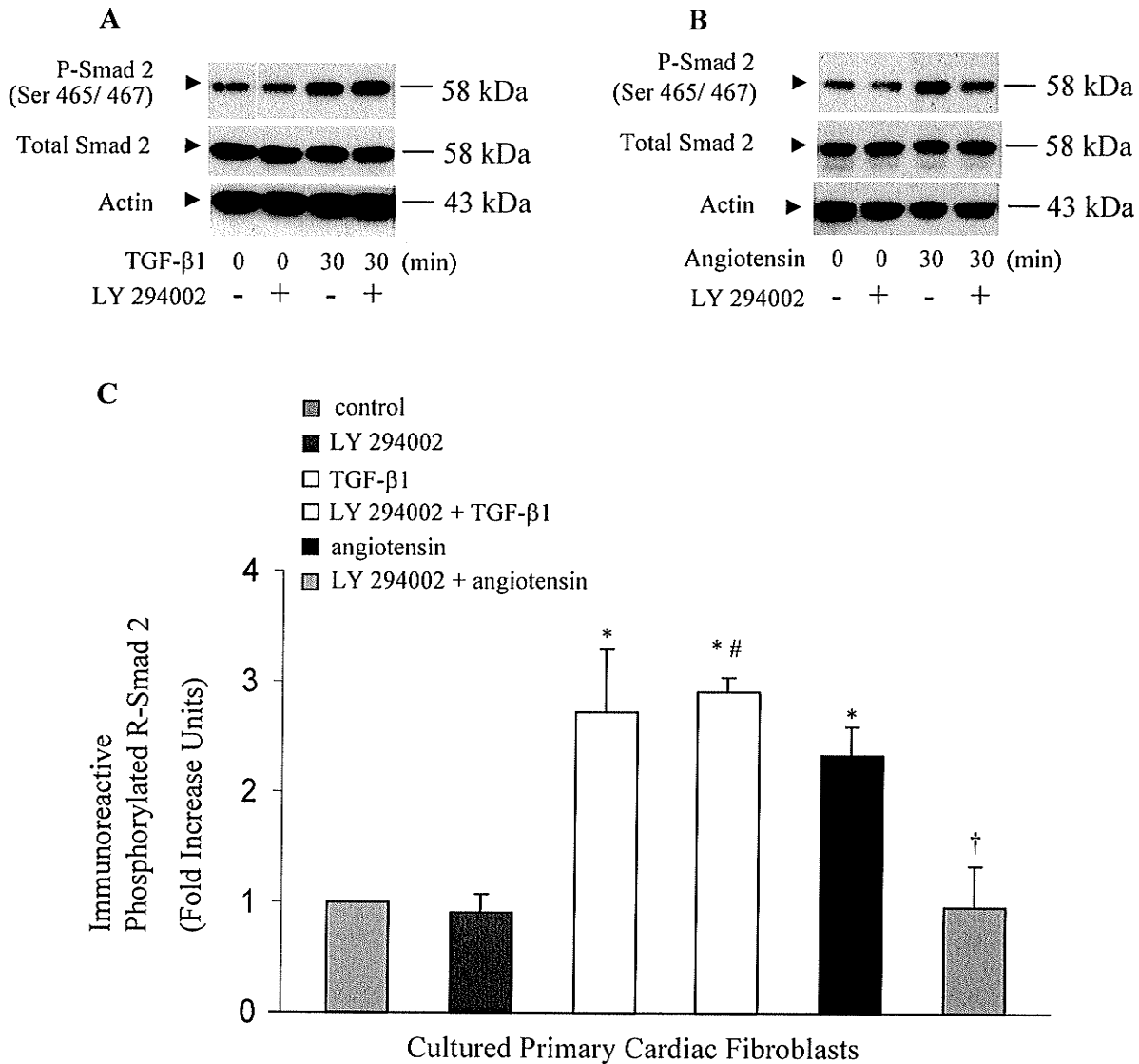


Figure 9. Panels A and B: Western blot analysis of phosphorylated R-Smad 2 (P-Smad 2) in cultured primary cardiac myofibroblasts pretreated with LY 294002 (PI3K inhibitor); 0% serum starvation for 48 h; total cell lysates. Cells were pretreated with LY 294002 (10 μ M) for 1h prior the stimulation with TGF- β 1 (10 ng/ml) or angiotensin (1 μ M) for 30 min. Representative Western blots show P-Smad 2 (~58 kDa) protein and total Smad 2 (~58 kDa). Western blots of actin (~43 kDa) indicate relatively even protein loading among the lanes. **Panel C:** Histogrammic representation of quantified data of immunoreactive P-Smad 2 from the groups of samples shown in the panels A and B (quantified by densitometric scanning); $P^* \leq 0.05$ vs. control; $P\# \leq 0.05$ vs. LY 294002; $P^\dagger \leq 0.05$ vs. angiotensin; data are expressed as mean \pm SEM ($n \geq 3$).

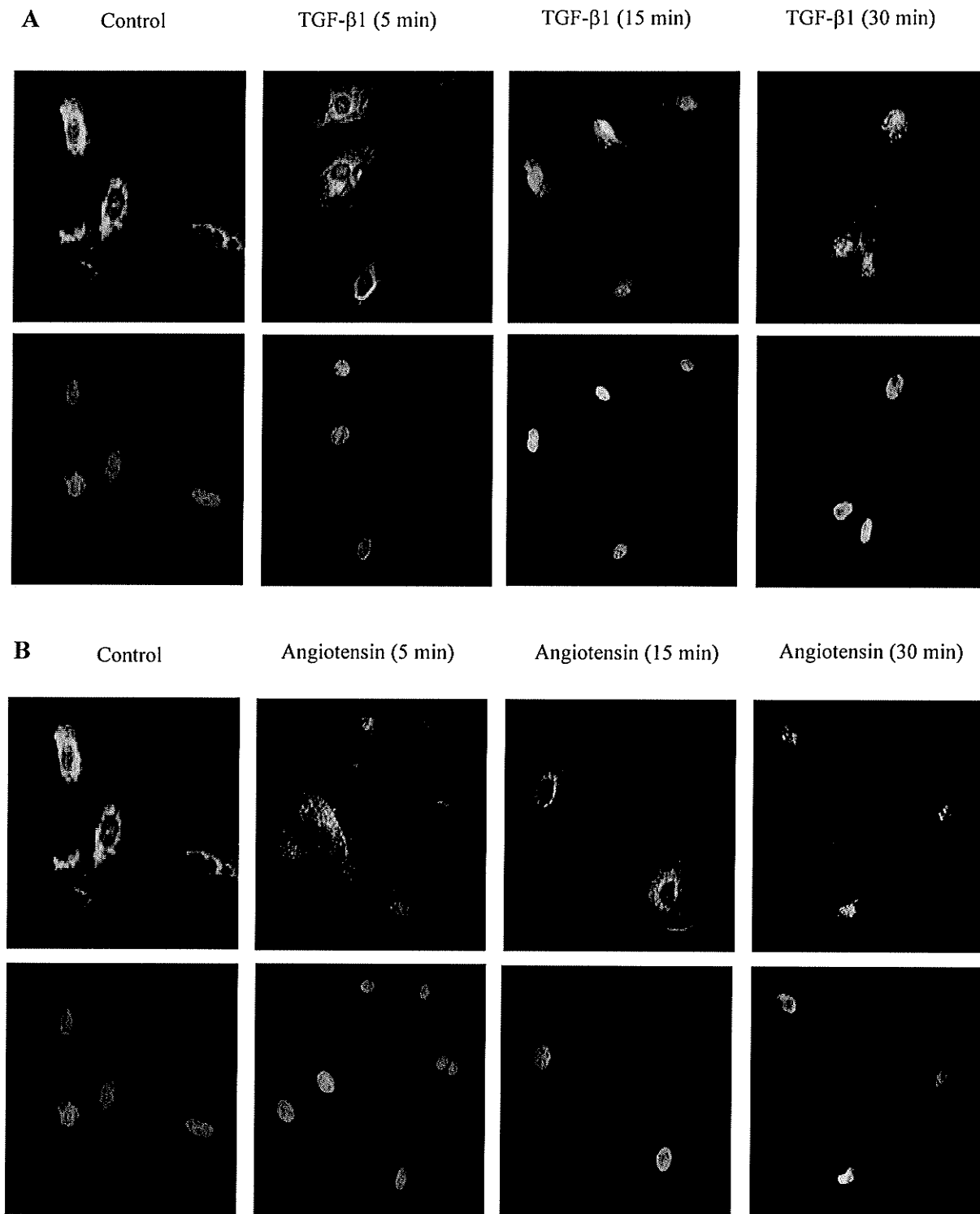


Figure 10. R-Smad 2 localization in cultured primary cardiac myofibroblasts treated with TGF- β 1 or angiotensin. Panel A: Immunofluorescent staining of total R-Smad 2 (nonphosphorylated and phosphorylated R-Smad 2) in cells treated with TGF- β 1 (10 ng/ml) for 30 min. **Panel B:** Immunofluorescent staining of total R-Smad 2 in cells treated with angiotensin (1 μ M) for 30 min. Nuclei of identical fields in (A) and (B) were detected with Hoechst 33342 staining. Original magnification, \times 400.

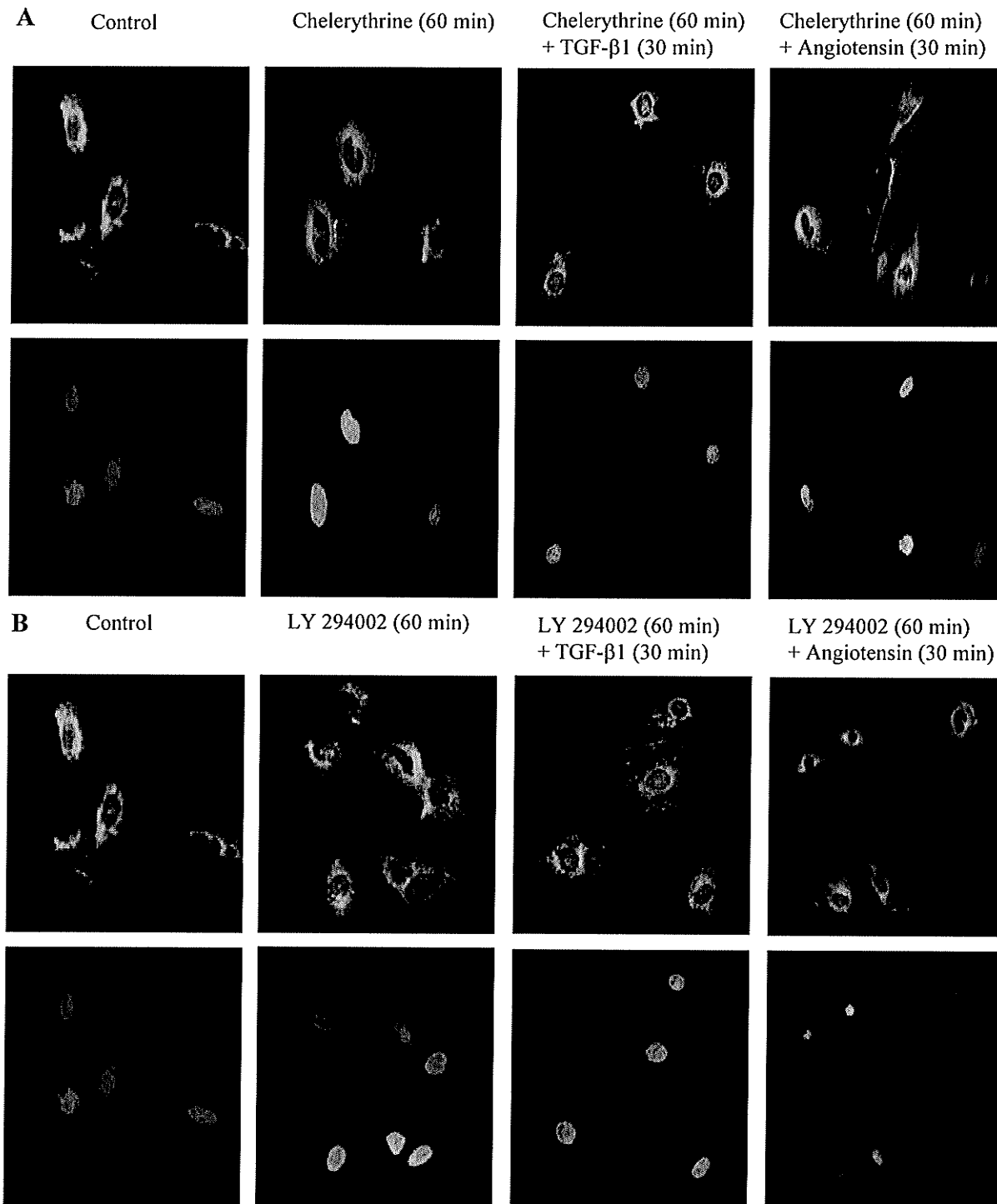


Figure 11. R-Smad 2 localization in cultured primary cardiac myofibroblasts pretreated with chelerythrine (PKC inhibitor) or LY 294002 (PI3K inhibitor), and stimulated with TGF- β 1 or angiotensin. Panel A: Immunofluorescent staining of total R-Smad 2 (nonphosphorylated and phosphorylated R-Smad 2) in cells treated with chelerythrine inhibitor (1 μ M) for 1h, and then stimulated with TGF- β 1 or angiotensin for 30 min. **Panel B:** Immunofluorescent staining of total R-Smad 2 in cells treated with LY 294002 (10 μ M) for 1h, and then stimulated with TGF- β 1 or angiotensin (1 μ M) for 30 min. Nuclei of identical fields in (A) and (B) were detected with Hoechst 33342 staining. Original magnification, \times 400.

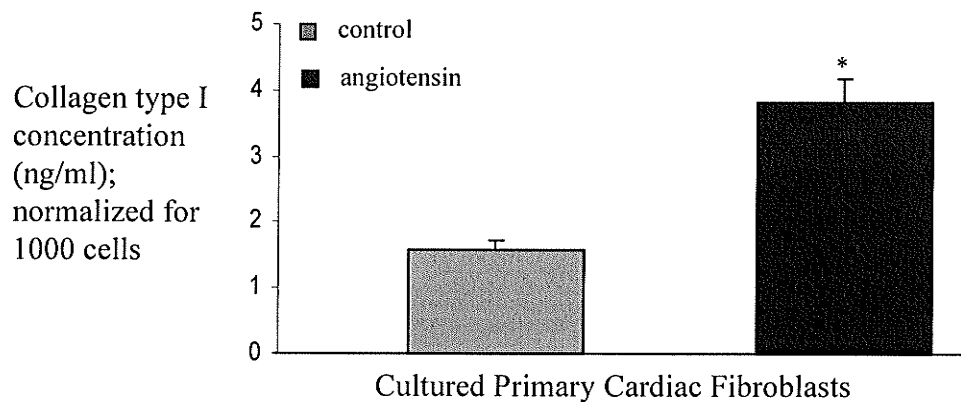


Figure 12. PICP assay. The effect of angiotensin on collagen type I concentration in cultured primary cardiac myofibroblasts; 0.5% serum starvation for 24 h; stimulation with [angiotensin]= 1 μ M for 24 h; $P^* < 0.01$; data is expressed as mean \pm SEM (n=6).

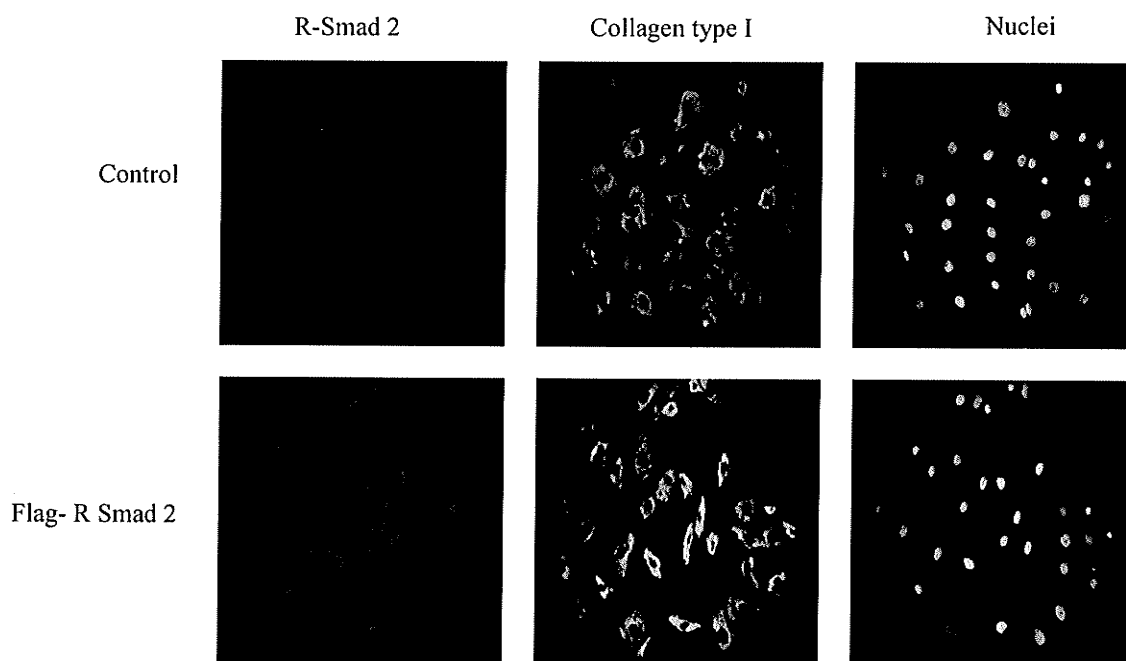


Figure 13. R-Smad 2 overexpression and collagen type I expression in cardiac myofibroblasts. Representative cultured primary cardiac myofibroblasts stained for immunoreactive exogenous (transfected) Flag-R Smad 2 protein, and identical fields stained for collagen type I and for cellular nuclei (Hoechst 33342 staining). Original magnification, $\times 400$.

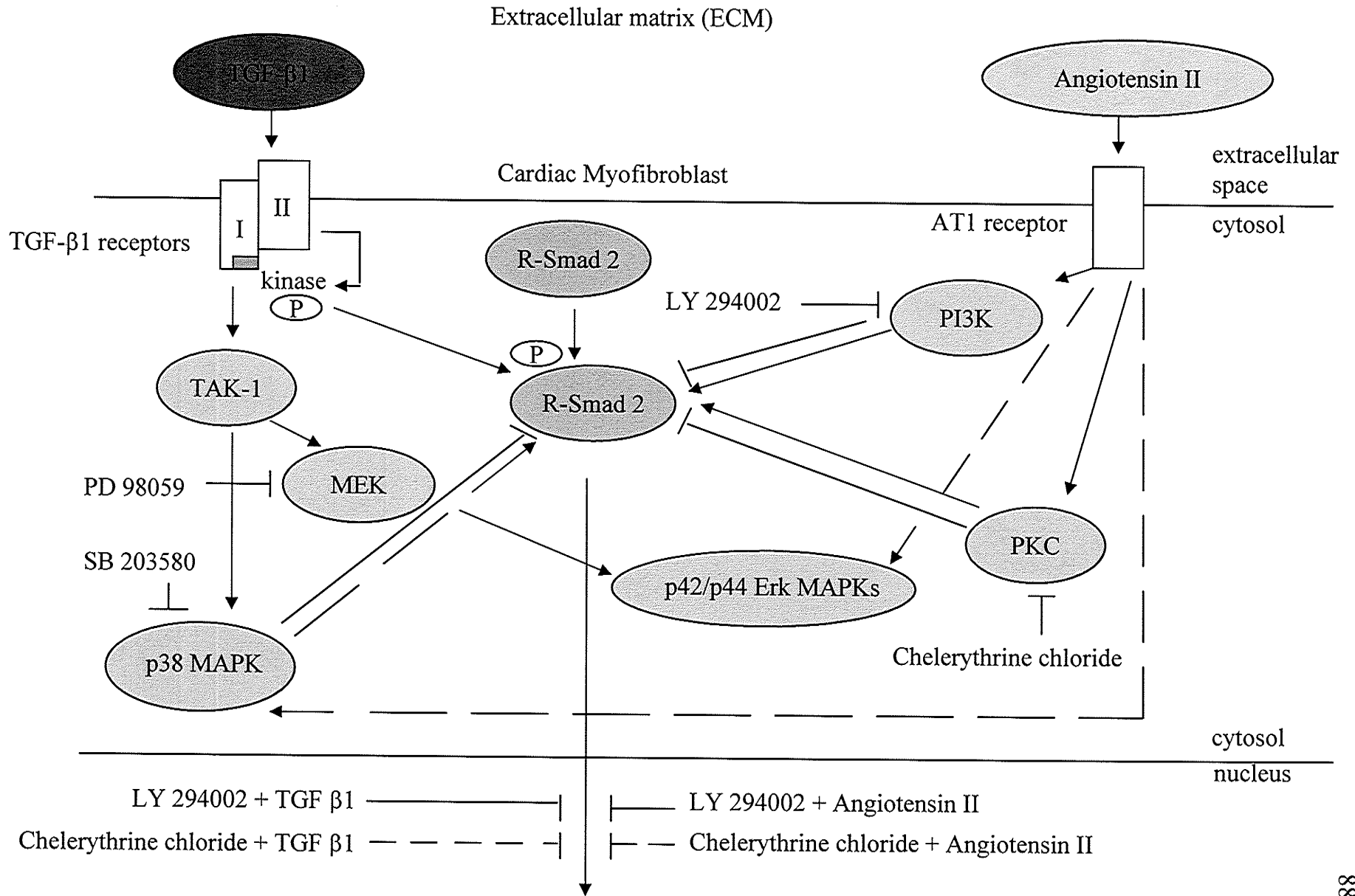


Figure 14. Graphical representation of TGF-β1- and angiotensin-induced kinase signaling cascades involved in the process of phosphorylation of R-Smad 2 at Ser 465/467 in cardiac myofibroblasts.

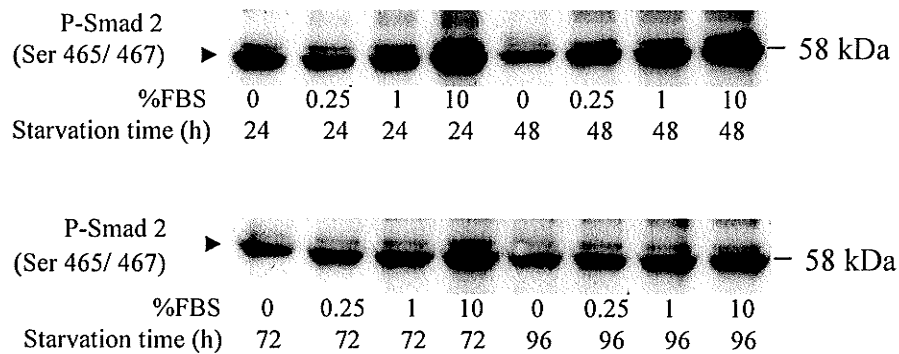


Figure 15. Representative Western blot analysis showing P-Smad 2 (~58 kDa) protein expression from nuclear lysates; cultured primary cardiac myofibroblasts were grown for 24h, 48h, 72h, or 96 h in DMEM/F-12 culture media, containing various concentration of fetal bovine serum (FBS). Based on these results the growth of subconfluent cells (70-90%) was arrested after 48h of incubation in DMEM/F-12 with 0% of serum.

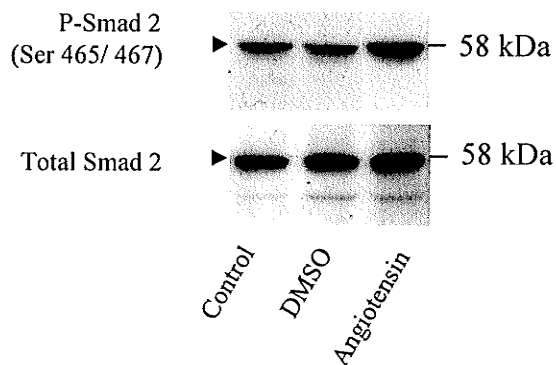


Figure 16. Representative Western blot analysis showing P-Smad 2 (~58 kDa) and total Smad 2 (~58 kDa) expression in cultured primary cardiac myofibroblasts treated with dimethylsulfoxide (DMSO) solvent for 1h; 0% starvation for 48h; total cell lysates; [DMSO]=1 μ l/ml for 60 min; [angiotensin]=1 μ M for 30 min.

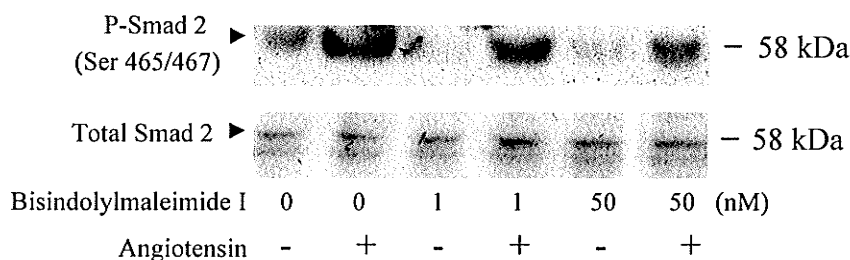


Figure 17. Western blot analysis showing phosphorylated P-Smad 2 (~58 kDa) and total Smad 2 (~58 kDa) expression in cultured primary cardiac myofibroblasts pretreated with bisindolylmaleimide I (PKC inhibitor); 0% serum starvation for 48 h; total cell lysates. Cells were pretreated with bisindolylmaleimide I at indicated doses for 15 min prior the stimulation with angiotensin (1 μ M) for 30 min. Based on these results bisindolylmaleimide I decreased angiotensin-induced P-Smad 2 by 1.7 fold at a dose of 1 nM, and 3 fold at a dose of 50 nM as quantified by densitometry scanning. Chelerythrine chloride is another inhibitor of PKC, which also produces a decrease in P-Smad 2 in angiotensin-stimulated cells (Figure 8).

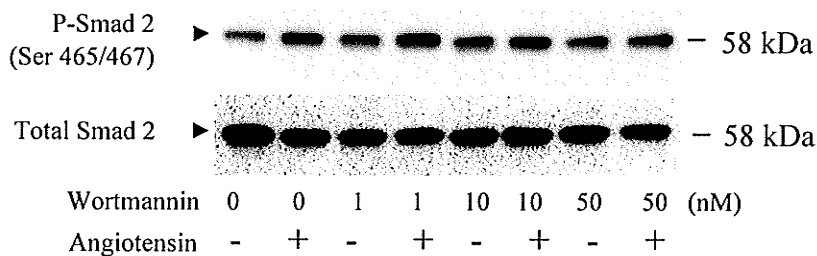


Figure 18. Western blot analysis showing phosphorylated P-Smad 2 (~58 kDa) and total Smad 2 (~58 kDa) expression in cultured primary cardiac myofibroblasts pretreated with wortmannin (PI3K inhibitor); 0% serum starvation for 48 h; total cell lysates. Cells were pretreated with wortmannin at indicated doses for 15 min prior the stimulation with angiotensin (1 μ M) for 30 min. Based on these results wortmannin at a dose of 50 nM decreased angiotensin-induced P-Smad 2 by one fold as quantified by densitometry scanning. LY 294002 is another inhibitor of PI3K, which also produces a decrease in P-Smad 2 in angiotensin-stimulated cells (Figure 9).

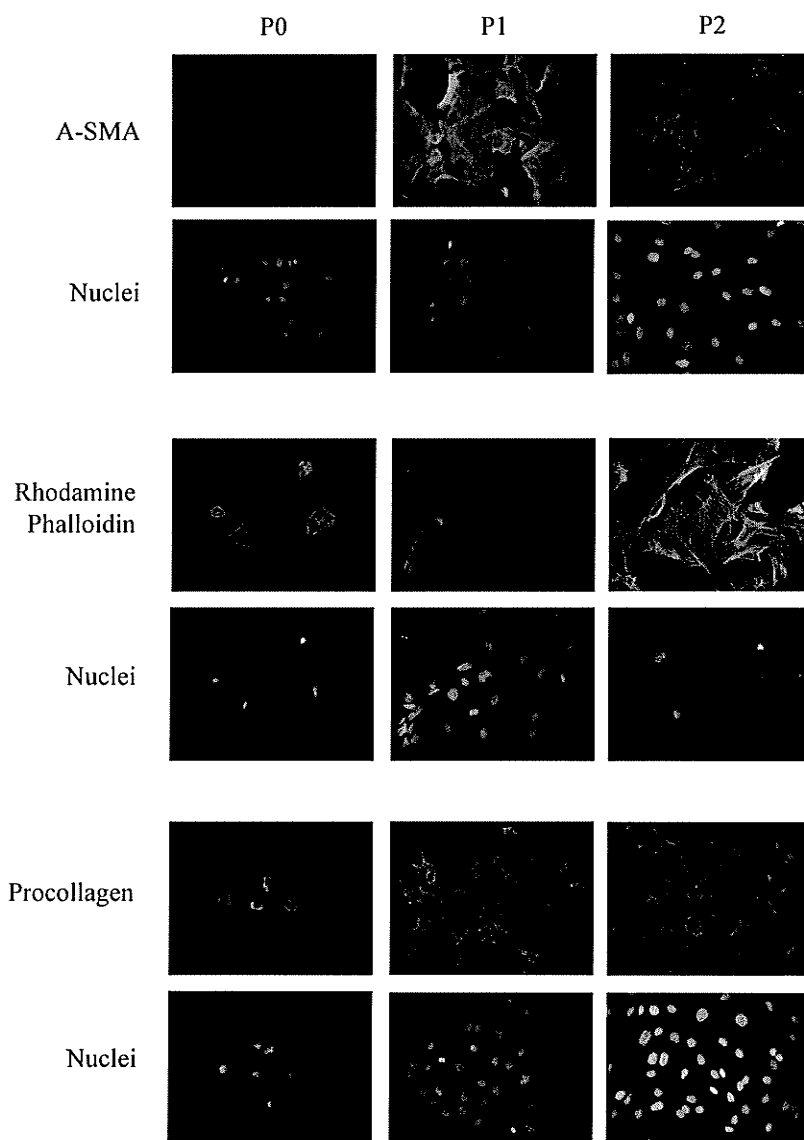


Figure 19. Fibroblasts phenotypically modulate to myofibroblasts in culture. Primary adult cardiac fibroblasts at Passages 0, 1 and 2 (P0, P1 and P2). 3 days following isolation (P0) or passage (P1, P2), cells were stained for immunoreactive α -smooth muscle actin (α -SMA, a myofibroblast marker), rhodamine phalloidin (F-actin stain) and immunoreactive procollagen (SP1.D8). Cells were stained for nuclei (Hoescht 33342) and the identical corresponding fields are shown. The phenotype shift occurs rapidly; both P1 and P2 express markers of myofibroblast phenotype in > 95% of cells observed. Original magnification x400.

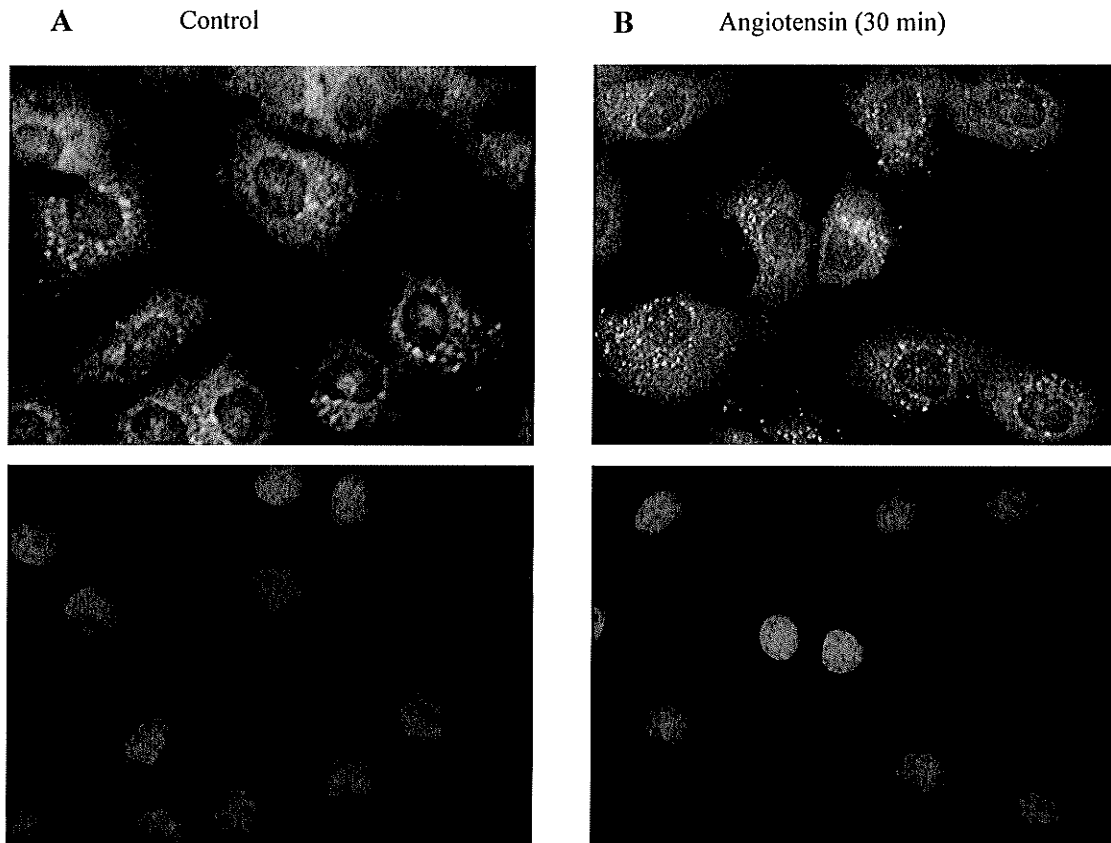


Figure 20. TGF- β 1 localization in cultured primary cardiac myofibroblasts. Panel A: Immunocytochemical localization of TGF- β 1 in quiescent non-stimulated cells (control group). **Panel B:** Immunocytochemical localization of TGF- β 1 in cells stimulated with angiotensin (1 μ M) for 30 min. Nuclei of identical fields in (A) and (B) were detected with Hoechst 33342 staining. Original magnification, \times 400.

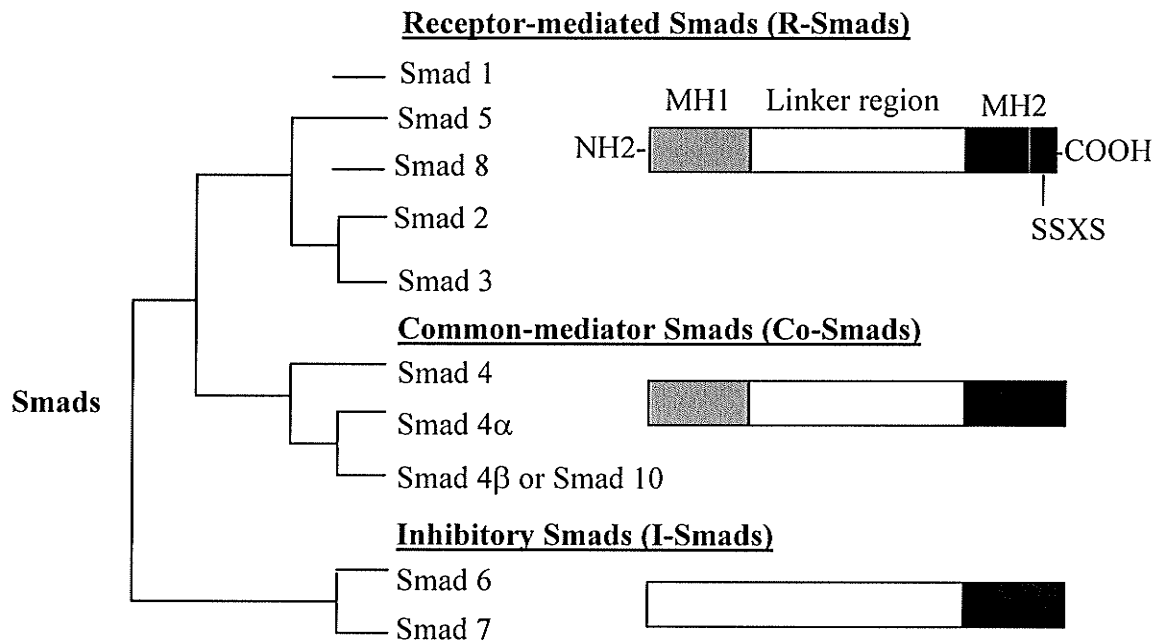


Figure 21. The Smad Family of Proteins. Phylogenetic tree of Smad effectors with their schematic structures ([31]- modified).

VI. DISCUSSION

Although first described as being primarily a vasoconstrictor hormone, angiotensin is now considered to play a role as a cytokine-like peptide [1]. Angiotensin is produced either systemically via the classical renin-angiotensin system (RAS) or locally via the tissue RAS [124]. Activation of the circulating RAS with sustained elevations in plasma angiotensin induces vascular remodeling of small arteries and arterioles [140]. Locally produced angiotensin from these vascular sites is involved in development of perivascular fibrosis of coronary vasculature of non-infarcted myocardium [140]. Development of fibrous tissue remote to infarct comprises a major aetiological factor involved in the appearance of chronic cardiac failure [140]. The increased activation of intracardiac RAS plays an important role in the remodeling of the extracellular matrix [7,141]. Evidence that angiotensin contributes to the development of cardiac fibrosis is obtained from reports that it increases collagen synthesis in cultured cardiac fibroblasts *in vitro*, and stimulates mRNA collagen type I expression in hearts *in vivo* [7,19,36]. Significant elevation of cardiac collagen observed in post MI-hearts is accompanied by the presence of myofibroblasts [6,7,9]. Myofibroblasts are the phenotypic cell variants derived from quiescent fibroblasts [10,22], representing the major cells involved in the synthesis and deposition of collagen proteins [8,13,15]. The phenotypic modulation of fibroblasts to myofibroblasts is known to be affected by TGF- β_1 [13,142]. Decorin is a natural inhibitor of TGF- β_1 , and thereby may function as a suppressor of TGF- β_1 -mediated tissue fibrosis by binding this cytokine in the extracellular matrix [143,144]. In experimental heart failure in rats, decorin mRNA is significantly increased in border and infarct scar [19]. Recent evidence would indicate that angiotensin is also involved in the phenotypic modulation of fibroblasts to myofibroblasts [129]. While angiotensin-mediated

modulation of TGF- β_1 ligand expression occurs both *in vitro* and *in vivo* [4,6,17,145], crosstalk between angiotensin and TGF- β_1 signal cascades in the development of cardiac fibrosis and heart failure remains unclear.

In the present study, we characterized the crosstalk between the activated TGF- β_1 and angiotensin postreceptor mechanisms, influencing the accumulation of P-Smad 2 in cultured primary cardiac myofibroblasts. We demonstrated that, in these cells, angiotensin stimulation is associated with (1) R-Smad 2 phosphorylation via AT₁ activation; (2) R-Smad 2 phosphorylation independent of TGF- β_1 signaling; and (3) R-Smad 2 phosphorylation possibly occurring via the integrated crosstalk between the PI3K pathway, the PKC pathway, and the activation of p38 MAPK. Furthermore, these events are associated with (1) nuclear translocation of R-Smad 2 that appears to be independent process from phosphorylation of R-Smad 2, and (2) fibrillar collagen type I synthesis.

The phosphorylation of R-Smad 2 by traditional TGF- β mediated mechanisms occurs on the Ser-Ser-X-Ser (SSXS) motif that is found at the MH2 region near the C-terminus [29]. Using Western blot analysis we showed that both TGF- β_1 (10 ng/ml) and angiotensin (1 μ M) significantly increased the phosphorylation of R-Smad 2 at Ser 465/467, which are key residues in the SSXS motif. In cardiac fibroblasts and myofibroblasts, angiotensin mediates intracellular signaling pathways via angiotensin type I (AT₁) and AT₂ receptors [6,126]. In an effort to characterize the angiotensin-induced P-Smad 2 expression in myofibroblasts, we pretreated cultured cells with losartan to induce the AT₁ blockade, or PD 123319 to inhibit AT₂ activation. In contrast to PD 123319, losartan administration was associated with decreased immunoreactive P-Smad 2 in cardiac myofibroblasts, indicating that AT₁ activation plays a significant role in the process of phosphorylation of this protein. This result also

supports our previous study, which shows that losartan inhibits P-Smad 2 nuclear translocation in isolated cardiac myofibroblasts [6]. As evidenced by immunofluorescent staining, nuclear translocation of P-Smad 2 is driven by both TGF- β_1 and angiotensin although the kinetics of the P-Smad 2 nuclear translocation may be different between the two modes of signaling. These differences may arise from the extent of R-Smad 2 phosphorylation that varied between TGF- β_1 and angiotensin treatments as indicated by Western blot analysis. In addition, a recent study of Smad coactivator binding using co-immunoprecipitation methods (unpublished data from our lab) indicates that angiotensin stimulation caused formation of the complex between R-Smad 2 and FOXH1, a Smad transcriptional coactivator. This result supports the hypothesis that angiotensin receptor activation precedes and may induce P-Smad 2 nuclear translocation and binding to a tertiary transcription factor. Together, these data indicate a linkage between angiotensin receptor activation, and subsequent R-Smad 2 phosphorylation as well as nuclear translocation of P-Smad 2.

Angiotensin is known to stimulate TGF- β_1 ligand expression [4], and is also involved in conversion of latent TGF- β_1 ligand to an active form in cardiac myofibroblasts [6]. Thus, it may be argued that angiotensin acts on fibroblasts indirectly via secondary TGF- β_1 release. To address and clarify this issue, we used inhibitors of TGF- β signaling including i/ anti-TGF- β_1 neutralizing antibody (TGF- β_1 NA) that specifically neutralizes the biological activity of TGF- β_1 , and ii/ anti-TGF- β type II receptor (R-II) neutralizing antibody (T β R-II NA), which abrogates T β R-II function. As the administration of TGF- β_1 NA (1.5 ng/ml) or T β R-II NA (1 μ g/ml) did not alter angiotensin-mediated increase of cellular P-Smad 2, we suggest that R-Smad 2 phosphorylation by angiotensin is specific and independent of TGF- β_1 signaling pathway in cardiac myofibroblasts. In addition, our study of TGF- β_1 release using

immunocytochemical method detects granules of stored TGF- β_1 in the cytosol of non-stimulated cells (i.e., control group) and after stimulation with angiotensin, suggesting that angiotensin could not induce a TGF- β_1 release within the first 30 min of stimulation. This result supports the hypothesis that angiotensin receptor activation may phosphorylate R-Smad 2 independently of TGF- β_1 in cardiac myofibroblasts.

Our investigation of signaling crosstalk between the activated postreceptor mechanisms in TGF- β_1 - and angiotensin-treated myofibroblasts revealed that the phosphorylation of R-Smad 2 expression was associated with apparent differences in the phosphorylation of p42/p44 Erk MAPKs and p38 MAPK. As we sought to characterize the signaling events associated with the modulation of P-Smad 2, we used pharmacological inhibitors to elucidate the biological functions of different kinases on the phosphorylation of this protein in TGF- β_1 - or angiotensin-stimulated cardiac myofibroblasts. We hypothesized that the R-Smad 2 phosphorylation occurred via a specific kinase mediator, involving extracellular signal regulated kinases p42/p44 Erk MAPKs, p38 MAPK, protein kinase C (PKC), and/or phosphoinositide 3-kinase (PI3K). MAPKs regulate a variety of cellular functions; however, their roles in the development of cardiac fibrosis are unknown. Further, TGF- β_1 is known to influence MAPK phosphorylation and activation. The biochemical link between TGF- β receptors and MAPKs might occur via TGF- β -activated kinase 1 (TAK-1), which is a MAPK kinase kinase member (MAPKKK) [133]. Thus, the activated MAPKs may converge on R-Smads, modulating their phosphorylation and biological function [31]. p38 MAPK and p42/p44 Erk MAPKs are also characterized as major mediators of the mitogenic action in the angiotensin signaling pathways [1,23]. The angiotensin-induced activation of Erk MAPKs is linked to TGF- β_1 mRNA expression, collagen type I synthesis, and

development of cardiac hypertrophy [23,93,134,135], whereas phosphorylated p38 MAPK contributes to increased collagen type I and collagen type III synthesis [135]. By analyzing the role of the MAPK superfamily in the TGF- β_1 - or angiotensin-induced P-Smad 2 in primary cardiac myofibroblasts, we confirmed the involvement of phosphorylated forms of p42/p44 Erk MAPKs and p38 MAPK in both signaling systems. We demonstrated that the phosphorylation of these MAPKs varied extensively in kinetics and magnitude between the two modes. To characterize the role of p42/p44 Erk MAPKs and p38MAPK on the phosphorylation of R-Smad 2, we used pharmacological inhibitors PD 98059 and SB 203580, respectively. We noted that a 10 μ M concentration of PD 98059 was associated with nearly complete attenuation of the phosphorylation of Erk MAPKs in TGF- β_1 -treated cells *vs* control values, but it did not significantly alter R-Smad 2 phosphorylation. In angiotensin-stimulated cells, the same inhibitor neither changed the phosphorylation of Erk MAPKs nor the phosphorylation of P-Smads. In contrast to PD 98059, the administration of 50 μ M SB 203580 prior TGF- β_1 treatment did not significantly change the phosphorylation of p38 MAPK *vs* controls, and it did not change the phosphorylation of R-Smad 2. However, in angiotensin-stimulated cells, SB 203580 treatment was associated with decreased accumulation of phosphorylated R-Smad 2 despite the lack of effect on p38 MAPK phosphorylation. Based on these results, we suggest that in primary cardiac myofibroblasts (1) the Erk signaling cascade has no effect on either TGF- β_1 - or angiotensin-induced P-Smad 2 expression at Ser 465/467; and (2) the p38 MAPK cascade participates in regulating angiotensin- (but not TGF- β_1 -) induced phosphorylation of Smad 2 at Ser 465/467 either directly, or indirectly via unknown mediator. This investigation of a putative mediator was not pursued further in the current study.

In addition to MAPK cascade, AT₁ stimulation also activates protein kinase C (PKC) [125], and phosphatidylinositol 3-kinase (PI3K) [1,24]. PKC is a major component in tyrosine kinase signaling pathways that is known to modulate the R-Smad phosphorylation in TGF- β ₁ signaling pathway in fibroblast-like kidney cells [111]. PI3K signaling involves activation of various kinases, including atypical PKCs, PLC- γ , mitogen activated protein kinases (MAPKs), and Akt/PKB [125]. The possibility that PI3K may also indirectly modulate R-Smad 2 phosphorylation via PI(3)P, which is a product of PI3K [31], is a significant issue. Although it still remains unclear how PI(3)P affects the phosphorylation of R-Smad 2, it is known that it binds SARA (Smad anchor for receptor activation) protein, and tethers it to the endosomal membranes [31]. SARA is a membrane-bound protein that forms association complexes with nonphosphorylated R-Smads in the basal state, and presents R-Smads to the activated type I receptor [146]. In our attempts to investigate the functional roles of PKC and PI3K on P-Smad 2 at Ser 465/467 in TGF- β ₁- or angiotensin-treated cardiac myofibroblasts, we examined the effects of chelerythrine chloride (PKC inhibitor) and LY 294002 (PI3K inhibitor) on the accumulation of this protein. The administration of chelerythrine (1 μ M) prior to TGF- β ₁ treatment did not affect P-Smad 2, whereas in angiotensin-treated cells it decreased P-Smad 2 compared to control values. Similarly, LY 294002 (10 μ M) attenuated P-Smad 2 increase in angiotensin-stimulated cells, but it did not decrease TGF- β ₁-induced P-Smad 2. Together, these data may suggest that the angiotensin-induced P-Smad 2 could be mediated via PKC and PI3K. Whether PKC could mediate this effect (1) alone or (2) in a PI3K-dependent manner or (3) in a PKC-isoform specific manner is unknown and remains to be determined. Whether PKC and PI3K directly affect the phosphorylation of Ser 465/467 (MH2 domain), or indirectly via mediators is also unknown at present. Results from current

immunofluorescence studies using myofibroblasts pretreated with chelerythrine or LY 294002 indicate that these inhibitors may abrogate nuclear translocation of P-Smad 2 in both TGF- β ₁- or angiotensin-treated cells. As observed, LY 294002 inhibits this process more effectively than does chelerythrine, where some nuclear translocation of P-Smad 2 was still present. The latter is in agreement with the data obtained from Yakymovych, et al., showing that R-Smad proteins with mutated PKC phosphorylation sites at MH1 domain were translocated to the nucleus upon treatment with TGF- β ₁ [111]. Our data support the suggestion that R-Smad 2 nuclear import is regulated at specific sites on the MH2 region [122], and that phosphorylation of residues at this site is affected by both chelerythrine and LY 294002. Together, results obtained from Western blot analysis of P-Smad 2 accumulation and immunofluorescence studies of nuclear translocation of P-Smad 2 after TGF- β ₁ or angiotensin stimulation may suggest that the processes of phosphorylation and nuclear translocation of P-Smad 2 could be independent of each other.

Increased angiotensin activation is known to alter the balance in the equilibrium between the synthesis and degradation of major fibrillar collagens in the extracellular matrix (ECM). The turnover of matrix in diseased hearts is linked to increased type I and III collagen mRNA expression and increased rates of collagen synthesis at the remnant myocardium and scar by myofibroblasts [7,8,18,19]. Our current work provides results of a collagen secretion study wherein *in vitro* procollagen type I carboxy-terminal propeptide (PICP) was used as a marker; we confirmed that angiotensin stimulation of myofibroblasts is associated with an elevation of collagen type I secretion in the culture media of treated cells. Furthermore, we also observed that R-Smad 2 overexpression in primary cardiac myofibroblasts is associated with increased expression of collagen type I compared with non-transfected cells. These

results confirm linkage between angiotensin receptor activation, R-Smad 2 phosphorylation and collagen type I expression in cardiac myofibroblasts.

VII. CONCLUSIONS

In conclusion, we demonstrated that the angiotensin-induced R-Smad 2 phosphorylation in primary cardiac myofibroblasts is associated with AT₁ activation, which process occurs independently of TGF- β ₁. The accumulation of P-Smad 2 may occur via the integrated crosstalk between the PI3K pathway, the PKC pathway, and the activation of p38 MAPK. This integration may then set the magnitude of R-Smad 2 phosphorylation, nuclear translocation of this protein, and fibrillar collagen type I synthesis in primary cardiac myofibroblasts. Finally, our results provide evidence that phosphorylation of R-Smad 2 is regulated in a distinct manner by both angiotensin and TGF- β ₁ pathways i.e., whereby R-Smad 2 phosphorylation, transactivation by kinases, and nuclear translocation appear to be independent of each other and unique to each pathway.

In order to extend the current data set, a series of future experiments could be carried out to address i/ the investigation of the role of specific PKC isoform involvement in angiotensin-induced R-Smad 2 phosphorylation of Ser 465/467 (MH2 domain); ii/ the effects of PD 98059 and SB 203580 MAPK inhibitors on P-Smad 2 nuclear accumulation after TGF- β ₁ or angiotensin treatment; iii/ putative mediators that might be involved in regulating the effects of p38 MAPK, PKC and PI3K in angiotensin-induced R-Smad 2 phosphorylation of Ser 465/467; and iv/ anti-P-Smad 2 and anti-Flag-R-Smad 2 double immunostaining of primary cardiac myofibroblasts overexpressed with R-Smad 2 (i.e., Flag-R-Smad 2) to illustrate the relationship between the phosphorylated (active) form of R-Smad 2 and collagen type I expression.

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