The Status of ERK1/2 in Volume Overload-Induced Cardiac Hypertrophy and Heart Failure

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Presented to the
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For the Degree of
Master of Science

By

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree of

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Dedicated to:

My parents Xiangli and Zhaolan

My husband Jiasheng

My Daughter Ruisi

My sisters Jinling and Xiaodong

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ABBREVIATION

AP-1: the activated protein 1

ANF: atrial natriuretic factor

Ang II: angiotensin II

ACE: angiotensin converting enzyme

ACEI: angiotensin converting enzyme inhibitor

ASP: arterial systolic pressure

AT_1: angiotensin II type 1 receptor

AT_2: angiotensin II type 2 receptor

AVE: aortocaval shunt with enalapril treatment

AVEL: aortocaval shunt with both enalapril and losartan treatment

AVL: aortocaval shunt with losartan treatment

CHF: congestive heart failure

DAG: diacylglycerol

DD: double distilled water

+d*P*/d*t*: rate of pressure development

-d*P*/d*t*: rate of pressure decay

EGF: epidermal growth factor

ERK: extracellular signal-regulated protein kinase

ET-1: endothelin-1

HR: heart rate

$InsP_3$: inositol 1, 4, 5 trisphosphate

JNK: c-Jun N-terminal protein kinase

Liver wet/dry wt ratio: Liver wet/dry weight ratio

Liver wet wt: liver wet weight

Lung wet/dey wt ratio: lung wet/dry weight ratio

Lung wet wt: lung wet weight ratio

LV: left ventricle

LV wt: left ventricle weight

LVH: left ventricular hypertrophy

LVEDP: left ventricular end diastolic pressure

LVSP: left ventricular systolic pressure

MAPK: mitogen activated protein kinase

MAPAPK2: MAP kinase-activated protein kinase 2

MEK: extracellular signal-regulated protein kinase kinase

MI: myocardial infarction

MKK: MAP kinase kinase

MKKK: MAP kinase kinase kinase

MLC-2: myosin light chain-2

NE: norepinepherine

PDGF: platelet-derived growth factor

PI3K: PI3 kinase

PKA: protein kinase A

PKC: protein kinase C

PMA: phorbol myristate acetate

RAS: renin-angiotensin system

RV: right ventricle

RV wt: right ventricle weight

SAPK: stress-activated protein kinase

SH: sham

SW: septal wall

TF: transcription factor

TGF-β: transforming growth factor β

ABSTRACT

Although volume overload induced by aortocaval shunt in rats has been shown to result in cardiac hypertrophy in 4 weeks and heart failure in 16 weeks, mechanisms involved in these conditions are not known. Since mitogen-activated protein kinase (MAPK) pathway is an important signaling transduction mechanism that participates in cell growth, differentiation, and proliferation by transducing extracellular stimuli into intracellular response, it is likely that MAPK is altered in cardiac hypertrophy and heart failure. Although changes in MAPK in end stage heart failure human as well as experimental models for the low cardiac output heart failure have been reported, very little information is available regarding the status of MAPK signal transduction in high-output model of heart failure. Therefore, the objective of this study was to examine the status of MAPK in volume overload-induced cardiac hypertrophy and heart failure. For this purpose, we induced volume overload in rats by creating aortocaval (AV) shunt for 4 and 16 weeks and the status of both ERK1 and ERK2, two widely studied MAPK in relation to cardiac growth, was examined. Although the renin-angiotensin system (RAS) is activated in cardiac hypertrophy and heart failure, no information regarding the relationship between RAS and MAPK in high-output cardiac hypertrophy and heart failure is available in the literature. Therefore, an interaction between RAS and ERK1/2 was studied by examining the effects of treatment with an angiotensin converting enzyme inhibitor (ACEI), enalapril, and/or an angiotensin type 1 receptor (AT_1) antagonist, losartan, in AV shunt model.

Our results showed that at 4 and 16 weeks, heart weight and heart to body weight ratio increased in the AV shunt animals as compared to the sham (SH) controls indicating cardiac hypertrophy and heart failure. Circulation congestion as reflected by increased lung and liver wet weight and incresed liver wet to dry weight ratio indicating heart failure was observed to occur at 16 weeks. Left ventricular end diastolic pressure (LVEDP) was decreased in both cardiac hypertrophy and failure whereas both $+dP/dt$ and the $-dP/dt$ were not changed at 4 weeks but

were depressed at 16 weeks. All these changes were partially prevented by treatment with enalapril and/or losartan. Cellular protein content of the phosphorylated ERK1/2 was increased without any changes in the total ERK1/2 content in the 4 weeks hypertrophied hearts. On the other hand, protein contents of both total and phosphorylated forms of ERK1/2 were markedly increased at 16 weeks. The ERK1/2 activities, estimated by measuring changes in phospho-Elk1, were markedly increased at both 4 and 16 weeks. These changes in MAP kinase activities and protein content in both 4 and 16 weeks volume overloaded animals were attenuated by treatment with enalapril and losartan; combination of enalapril and losartan did not show any additional beneficial effect. These results suggest that activation of ERK1 and ERK2 may contribute to the development of cardiac hypertrophy and heart failure due to AV shunt-induced volume overload. The blockade of RAS may partially prevent the development of cardiac hypertrophy and heart failure through ERK1 and ERK2 signaling transduction mechanisms.

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LITERATURE REVIEW

1. Introduction

Cardiac hypertrophy and congestive heart failure (CHF) are very common clinical conditions, and CHF is well known as the final outcome of different types of heart diseases. During cardiac hypertrophy in response to various growth stimuli including classical growth hormones, various neuroendocrine factors [endothelin-1 (ET-1), angiotensin II (Ang II), phenylephrine, etc] and the increased mechanical load, cardiomyocytes of adult myocardium increase their cellular mass. This involves alterations in both cell structure and protein expression that are mediated by changes at the transcriptional and translational levels (1). In contrast to immature cardiomyocytes, the adult cardiomyocytes only undergo hypertrophic growth but do not proliferate. Studies with failing hearts from human and animal models have identified many signaling pathways which play crucrial roles in CHF. However, mechanism(s) that participate in the progress of cardiac hypertrophy and heart failure is still far from clear. Therefore, studies explorating the functional link between the activation of mediators, their interacellular response(s) and clinical consequence may be of great importance.

The renin-angiotensin system (RAS) is one of the important pathways that regulate the cardiac function. It is believed that the increased activity of RAS for a prolonged period of time constitutes to the pathogenesis of cardiac hypertrophy and CHF. Ang II is the most important component of RAS.

Mitogen-activated protein kinase (MAPK) is also involved in cardiac hypertrophy and heart failure. It is activated in response to a wide variety of extracellular stimuli and induce changes in the critical intracellular processes promoting cell growth, differentiation, proliferation, apoptosis and transformation (2-4). These extracellular stimulis include cell deformation, secretion of adhesion molecules, and some neurohomones [AngII, ET-1 and

norepinepherine (NE)] that bind to heptahelical G protein-coupled receptors (5). The available evidence indicates that Ang II generated either from the circulation or by the paracrine and autocrine secretion has the regulatory effect on MAPK activity in the heart. Treatment of patients with CHF or animals in different experimental models with Ang II blockers, including different types of angiotensin-converting enzyme inhibitors (ACEI) or Ang II type I receptor (AT1) antagonists exerts beneficial effects in terms of preventing the clinical signs, improving heart function, and reducing mortality.

2. Cardiac hypertrophy and heart failure

Cardiac hypertrophy is a complex compensatory mechanism of the heart to adapt to excessive workload (pressure overload/volume overload), heart dysfunction and/or genetic mutation. In response to various extracellular stimuli such as the mechanical stress and stress-induced release of growth-promoting factors such as Ang II, ET-1 and transforming growth factor- (TGF-β), myocardium adapts to the increased workload through hypertrophy of individual terminally differentiated myocytes. Many signal transduction pathways that link extracellular hypertrophic stimuli to the nuclear transcription factors participate in this process.

It is generally accepted that cardiac hypertrophy is one of the most critical risk factors of heart diseases. The hypertrophic response is characterized by an increase in the myofibril/sarcomeres, and myosin light chain-2 (MLC-2v), and an increase in the content of contractile proteins so as to induce an enlargement of volume of individual cells without cell division. It is also characterized by induction of the embryonic genes such as the cardiac specific atrial natriuretic factor (ANF) (6). Both pressure- and volume-overload can induce a characteristic change in the morphology of the myocytes and different patterns of myocytes enlargement due partially to reorganization of the molecular composition of individual cardiac myocytes. This results in concentric and eccentric hypertrophy, leading to progressive dilatation of the failing heart (7, 8). It has been demonstrated that myocytes diameter is markedly increased

in compensated concentric hypertrophy (9, 10), which had been found in early or mild pressure overload (commonly seen in aortic stenosis and hypertension). Because of the efforts made by the ventricular wall to normalize wall stress, the cardiac myocytes lay down sarcomeres predominantly in parallel to the increase in myocytes cross-sectional area which in turn leads to a relative thickening of the wall of ventricule (11). On the other hand, eccentric hypertrophy had been found in early or mild volume overload such as aortic and mitral insufficiency, diffuse myocardial damage (in viral or toxic myocarditis), and localized myocardial damage (such as myocardial infarction). The available evidence show that the relative lengthening and thickening of cardiac myocytes go proportionately by sarcomere accumulation at both series and parallel in end-stage ischemic cardiomyopathy and decompensated dilated cardiomyopathy (12, 13).

Many studies have been carried out in different models examining the hemodynamic and biochemical changes in either pressure overload or volume overload. Using rats with heart failure after myocardial infarction, Raya and colleagues reported similar hemodynamic changes in this rat model treated with either Ang II blocker DuP 753 or with ACEI captopril (14). Schunkert et al indicated that both ACE activity and the expression of ACE mRNA were increased in the pressure overload-induced hypertrophied left ventricule in rats with the chronic aortic stenosis (15). Qing and Gaicia have reported that both ACEI (captopril) and AT1 antagonist (losartan) could partially reverse cardiac hypertrophy induced by the aortocaval shunt in rats and thus, improve the hemodynamic performance of the heart. It could also restore the ANF in both plasma and tissue to level close to normal (16). A recent study has shown that AT1 receptor blocker YM358 and ACEI enalapril produce similar effects on the hemodynamic performance in AV shunt rat model (17). Using this high-output heart failure model, Gealekman et al stated that the expression and activity of endothelial NO synthase (eNOS) were unchanged. However, both the inducible NO synthase (iNOS) expression and activity were approximately doubled as compared to the control group, suggesting that the depression of myocardial

contractility and beta-adrenergic hyporesponsiveness may be due to an increment in iNOS activity (18). Liu and Ma reported disorder of microtubular structure in volume overload-induced cardiac hypertrophy, which may govern the hypertrophic response of cardiacmyocytes (19). Petretta and colleagues reported that after treatment of the cardiac autonomic adaptation of idiopathic dilated cardiomyopathy (DCM) patients with losartan, their condition in response to saline load induced volume overload was improved and the urine output increased (20). Studies from our laboratory also indicate that during the heart failure stage induced by volume overload in rats, myocardial contractility both *in vivo* and *in vitro* was decrease, whereas, the contractile response to β-adrenergic stimulation was enhanced (21). It was also reported that treatment with imidapril or losartan might improve hemodynamic function at least partially by reversing the depressed bioactivity of Gsα during heart failure stage in volume overload (21).

The whole process of hypertrophy can be divided into three phases which include developing hypertrophy phase, the compensatory hypertrophy phase and the decompensatory phase, which is also referred to heart failure. Although patients with advanced heart failure can move back and forth from the clinical heart failure depending on their diets and appropriate therapy, the whole process during these three stages can be viewed as a progression (22).

Heart failure represents an enormous clinical problem and is a symptomatic syndrome in which cardiac output is inadequate to meet the metabolic needs of the human body. Because of its high incidence, the prevalence and the mortality of congestive heart failure (CHF) have increased dramatically during the last few decades. As a result, it has become the major social and economic burden. Therefore, extensive efforts are made to develop promising therapeutic procedures/protocols to alleviate some of these clinical heart problems.

3. Role of the renin-angiotensin system in cardiac hypertrophy and heart failure

a. The renin-angiotensin system

The renin-angiotensin system is unique in that it does not only regulates blood pressure, but has direct effects on the heart, kidneys and blood vessels (23, 24). It is critically involved in the regulation of the cardiovascular function. The RAS includes different components that participate in the regulatory processes.

One of the components of RAS system involves renin which is an enzyme liberated from the juxtaglomerular cells of the afferent arterioles in kidneys in response to a reduced renal perfusion, low blood volume, low blood pressure or left ventricular failure. In the liver, renin catalyzes the conversion of angiotensinogen to angiotensin I (Ang I). Ang I is relatively inactive and converted to angiotensin II by the activated angiotensin-converting enzyme (ACE). ACE is a member of the family of zinc metallopeptidases, which is mainly derived from the capillary bed of the lung and elsewhere in the body. Ang II is a powerful vasoconstrictor (25), and exerts a great adaptive response in restoring fluid volume after hemorrhage. However, it shows maladaptive effect on its long-term activation during cardiac hypertrophy and heart failure (26). Alternatively, Ang II can also be released from Ang I via chymase, a chymotrypsin-like proteinase, without affected by ACE inhibition (27).

b. The angiotensin II receptors

Angiotensin II mediates its downstream second messenger-pathways through binding to its two different subtypes of receptor, angiotensin II type 1 (AT_1) and type 2 (AT_2) receptor, which link to guanine nucleotide-binding protein (G protein) (28). Both of them belong to the seven transmembrane domain receptor family, share only 34% homology of protein sequence and have distinct functional and pharmacological properties (29). AT_1 receptors predominate in

the adult heart, mediate the vasoconstrictor actions of Ang II and thus are viewed as activator/regulatory receptors (30). Activation of AT_1 recepors also accounts for the increasing contractility of myocardium (31, 32), the proliferation of fibrous tissue and blood tissue, and the hypertrophic response of cardiac myocytes (8), aldosterone secretion and catecholarmine release (33). AT_1 receptors are blocked by its specific antagonist, losartan (34). The downstream targets of AT_1 receptor include Gq/Gi coupled receptors, phospholipase C, PKC, Ca^{2+}, receptor tyrosine kinases, Src family, tyrosine kinases, JAK2 and MAP kinases. AT_2 receptors exert different effects compared to AT_1 receptors. Their downstream targets include Gi coupled receptors, kinin/NO/cGMP, protein phosphatase, MAPK and ceramide. AT_2 receptors are relatively highly expressed in the fetal hearts and are critical for the embroynic heart development and therefore they are viewed as fetal phenotype (35). Activation of AT_2 receptors generally exert inhibitory/counter regulatory effects which includes vasodilatation, apoptosis, negative chronotropy, natriuresis and cell growth inhibition (36-38).

c. Role of angiotensin II in cardiac hypertrophy and heart failure

There is compelling scientific evidence that RAS is critically involved in the regulation of cardiovascular function because its (RAS) activation results in activation of hemodynamic defence reaction, impaired endothelial cell function, stimulation of growth and protooncogenes, apoptosis, oxidative stress, and an enhancement of the paracrine and autocrine vascular production of many factors that induce remodeling and restructuring (24, 39, 40). Moreover, Ang II is the most important component of RAS, which either directly or indirectly involved in regulating the above mentioned cardiovascular functions (41). It has been demonstrated that Ang II along with other extracellular mediators (vasopressin, endothelin and norepinephrine) stimulate sodium and water retention (24), vasoconstriction, and cardiac inotropic action (42) in hemodynamic defence reaction, and further modify gene expression and promote growth of cardiomyocytes. Many investigators have addressed to changes in gene expression and protein

component of RAS in cardiac volume overload model (43-46). Ang II is one of those changes and thus plays a major role in the development of volume overload-induced cardiac hypertrophy (44, 47). Mechanical stretch has been demonstrated to be the activation factor for the release of Ang II, and thus acts as an initial mediator of the hypertrophic response in cardiac myocytes (48). In addition, experimental studies have further illustrated that Ang II generated either from the circulation or by the paracrine and autocrine secretion, all have the effects of regulating MAPKs activity in the heart (49, 50), yet angiotensin II generated in the circulation and tissue systems appear to serve different functions: circulating angiotensin II is probably most significant in regulating the vasomotor tone, whereas angiotensin II produced in the tissues participates in signaling pathway that regulates gene expression resulting in the proliferation (42). Many short-term adaptive response happened during a brief circulation challenge all will turned into maladaptiven response when sustained for a long periods as in patients with heart failure. Extensive studies have provided increasing evidence that blockade of angiotensin II formation also inhibits maladaptive growth (51, 52). Patient with severe heart failure are treated with increasing dose of diuretics, which activates the renin-angiotensin system, resulting in the elevation of Ang II in both circulation and tissue levels, and thus stimulating the release of aldosterone. Aldosterone not only promotes retention of sodium and water, but also has growth-promoting effects, and may be involved in the maladaptive hypertrophy and heart failure (42). Therefore, Ang II is also involved in the maladaptive hypertrophy and heart failure. In cultured adult cardiac fibroblasts, Ang II has been found to induce collagen synthesis, and to inhibit the collagenase activity, and therefore may be involved in the pathologic myocardial fibrosis (28, 53). In addition, Ang II has also been implicated in apoptosis during the onset of diabetes, and in response to mechanical deformation (54).

4. Mitogen-activated protein kinases

a. General information and function of MAP Kinases

Mitogen-activated protein kinases (MAPK) are an essential part of the signal transduction pathway and play a critical role in cell growth, differentiation and transformation (55). MAPKs are encoded by a multigene family and are activated in response to a wide variety of extracellular stimuli and induce changes in cell growth, proliferation and apoptosis (2-4, 38). MAPK was initially identified as a protein serine/threonine kinase which can regulate cell growth and proliferation because of its capacity to phosphorylate microtubule-associated protein when activated by binding of peptide growth factors to their tyrosine kinase receptors (56). More recently, the MAPK signaling pathway has been reported to be activated by cell deformation, which may induce secretion of adhesion molecules or by release of some neurohomones which activated heptahelical G protein-coupled receptors (5).

Several parallel kinases pathways lead to the activation of different family members of mammalian MAP kinases. In general, growth factors and stress can activate the MAP kinase pathways, whereas osmosensing may also activate MAP kinase.

There are three MAPKs, which have been identified, namely the extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal protein kinase [JNK also known as stress-activated protein kinase (SAPK)] and p38 (also known as Mkp2/CBSP) (57). Activation of these three classes of MAP kinases is characterized by particular stimuli. For instance, while growth factors and phorbol myristate acetate (PMA) strongly activate ERK1/2. Their effect on JNK and p38 kinases is weak (58).

There is increasing evidence showing that MAPKs are critically implicated in the regulation of signaling pathways that ultimately lead to cardiac hypertrophy. The involvement of ERK in cardiac hypertrophy has been investigated using different models, including cultured

cardiac myocytes (59), human failing heart (60), hypertensive mice (61) and the neonatal rat cardiomyocytes (62 ,63). The involvement of other members of the MAPK family such as p38 and JNK in cardiac hypertrophy has also been reported (62, 64-66). In addition, the involvement of other members of the MAPK family such as p38 and JNK in cardiac hypertrophy has also been reported. Several reports suggest that MAPKs are critically involved in the regulation of signaling pathways that ultimately lead to cardiac hypertrophy. Therefore, MAPKs play important an role in the ventricular remodeling in different cardiovascular diseases by regulating the transactivating activity of a variety of transcription factors, and thereby, control the rate and the specificity of the expression of gene transcription factors that are considered integral part in the hypertrophic response.

Mitogen-activated protein kinase (MAPK) cascade consists of a three-tiered module, which are MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK) and MAP kinase (MAPK). MKKK activated the downstream extracellular signal-regulated protein kinase (MAPK/ERK) kinase (MEK), which in turn, activates the MAP kinase by phosphorylation on threonine and tyrosine residues within the catalytic domain (67, 68). After activation, the MAP kinase moves through large pores on the nuclear membrane and translocates into the nucleus, where the transcription factors are located (69). These transcription factors regulate the induction of sets of genes, which largely determine the ultimate biological response of the cell, including the hypertrophic response of cardiac myocytes (63).

b. Extracellular Signal-Regulated Protein Kinase

The first subgroup of MAP kinase, which includes extracellular signal-regulated protein kinases (ERK1 and ERK 2) was identified in cell extracts by Sturgill and Wu in 1991 (70). The ERK pathways are coupled by a GTP-binding protein which is composed of G_{α} and $G_{\beta\gamma}$ subunits. The most important isoform of G_{α} in the heart which activates ERKs is $G_{\alpha q}$ (71, 72). Once

stimulated by Ang II, ET-1 or α-adrenergic agonists, $G_{\alpha q}$ will activate phospholipase C to hydrolyze phosphoinositides in the cytoplasmic membrane to produce diacylglycerol (DAG) and inositol-1, 4, 5 triphosphate ($InsP_3$). DAG further stimulates the lipid-dependent serine/threonine kinases PKC, along with $G_{\beta\gamma}$ to activate ERKs signaling cascades (73, 74). $InsP_3$, on the other hand, causes release of calcium that activates ERK pathways *via* both Ras dependent and independent mechanisms (75, 76). Stimulation of β-adrenergic agonists results in the ativation of adenylate cyclase which catalyzes the hydrolysis of ATP to cyclic AMP. Cyclic AMP then activtates PKA which stimulates the transcriptional factors that will go through MAP kinase pathways and further promote protein synthesis and cell growth (77, 78). By using three-dimensional digital imaging microscopy, Gonzalez et al found majority of the subcellular ERKs in quiescent cells surrounding the nucleus, but some present within the nucleus lumen. On activation ERKs are translocated into the nucleus, which supports the view that MAP kinase carries a signal from the cell cytoplasm into nucleus (79). Following translocation into nucleus, phosphorylated ERKs catalyzes the phosphorylation of Elk1 which may play an important role in regulating atrial natriuretic factor (ANF) expression (80, 81).

Studies of others have revealed that ERK is related to a 42kDa protein that becomes transiently phosphorylated at tyrosine residues following stimulation of fibroblasts by a variety of mitogens, including EGF, PDGF, phorbol ester and insulin-like growth factor II. These findings suggest that MAP kinase plays an important role in the signaling pathway that is responsible for the G0-G1 transition in the cell cycle (82). The ERK cascade is composed of Ha-Ras, Raf-1, MEK and ERK and Rsk, spans from the plasma membrane to the nucleus and transduces mitogenic signals downstream from membrane receptor tyrosine kinases (82). ERK is stimulated by multiple extracellular stimuli and oncogenes. The signaling transduction cascade is triggered by the binding of a ligand to its receptor, resulting in the activation of Ras which plays the central role in the activation of ERK1/2. Activation of the mitogen-activated protein kinase

(MAPK) results in the activation of several cellular protein kinases such as the ribosomal S6 kinase, MAP kinase-activated protein kinase 2 (MAPAPK2), all of which catalyze the phosphorylation of some nuclear transcription factors (TF), such as Elk/p62^{TCF}, ATF-2, c-Myc and C/EBPβ (81-85). MAPK can also promote the transcription of genes required for the growth response through activation of another Ser/Thr kinase, Rsk (86). Taken together, these results further demonstrate the importance of MAP kinase in the modulation of cellular signal transduction which leads to cardiac hypertrophy.

Ras plays a critical role in the MAP kinase pathway. A signaling transduction cascade triggered by the binding of a ligand to its receptor activates Ras, followed by the activation of MAPK. MAPK in turn catalyzes the phosphorylation of transcription factors (TF) either directly or through activation of another Ser/Thr kinase, Rsk, thus promoteing the transcription of genes required for tissue growth (86). Activation of Ras initiates the phosphorylation cascade, involving Raf-1 (MKKK) to MEK (MAP kinase kinase), and finally activate the MAP kinase family (66). Therefore, activation of the Raf-mitogen-activated/ERK kinase (MEK)-ERK protein kinase appears to be an important cascade in cardiac hypertrophy.

As a third part of Ras-Raf-MEK-ERK cascade, activation of MEK-1 will subsequently activate the downstream target, ERK1/2 which will catalyzes the phosphorylation and activation of transcription factors such as c-myc, c-jun, ATF-2 and Elk1 that form some of the final targets of the cascade. The Rek (p90^{s6k}) contributes to the phosphorylation of ribosomal protein S6 which can also be activated by ERKs and resulting in the phosphorylation of ribosomal protein S6 and some other transcription factors, and is therefore involved in the induction of cardiac hypertrophy (87). ERKs may also phosphorylate upstream components in the signaling cascade that lead to their activation, thus, demonstrating a feedback regulation (88).

There is some controversy regarding the activation of ERKs in the ischemia-induced cardiac dysfunction. Bogoyevitch et al reported that ERKs activation was not observed either

following ischemia or ischemia/reperfusion. It would appear that activation of ERKs is probably coupled preferentially to the activation of Gq protein receptors and receptor tyrosine protein kinase, but not to stress receptors stimulated JNK/SAPK and p38 (89, 90). Marie and colleagues demonstrated that although ERK1 and ERK2 were not activated by the global ischemia, they stimulated a p38 activator, the MAPK-activated protein kinase-2 (MAPKAPK2). These authors also investigated the role of stress-activated c-Jun N-terminal kinase (JNK/SAPKs) and indicated that although JNK was not activated by ischemia alone, it was activated by the reperfusion following ischemia. However, Yoshida et al investigated changes in MAP kinases activities in an acute myocardial infarction in rats in either ischemic or non-ischemic myocardiam. Results of their study indicated that p44, JNKs and p38MAPK were all significantly activated although some variations were observed in the activation patterns of each group of MAPK in different regions, including the ischemic myocardium, non-ischemic septal wall (SW), and the right ventricular wall (RV). Their echocardiographic results suggest that MAPKs activation might be partially induced by the acceleration of workload and/or stretch (91). These data suggest that activation of multiple parallel MAPK signaling transduction cascades may be involved in response to cellular stresses in hearts (92). Therefore, MAP kinase is critically involved in cellular events regulating of cardiac hypertrophy.

c. c-Jun N-terminal kinase (JNK) /Stress-Activated Protein Kinase(SAPK)

The c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) was identified on the basis of its capacity to bind to the c-Jun transactivation domain or its ability to phosphorylate MAP2 (82). JNK/SAPK was first identified as a 54-kDa protein Ser/Thr kinase activated in necrotic livers of cycloheximide-treated rats (93) and recognized as a cellular stress activated protein kinase. JNKs are represented by more than 10 different isozymes encoded by three genes (JNK1-3). The c-Jun N-terminal kinase (JNK) 1 and 2 are two distant relatives of

MAP kinase. They are JNK-1 (46 kDa) and JNK-2 (55 kDa) and they all bind to the transactivation domains of c-Jun, and phosphorylate the transactivation domains of c-Jun, which is a component of the activator protein 1 (AP-1) transcription factor (68, 94), and Elk1(83). Transcription factor ATF2 has been identified as another target for JNK protein kinase. The phorphorylation of ATF2 by JNK can further increase transcriptional activity and gene expression (84, 85, 95). However, unlike ERKs, growth factors, phorbol esters or activated Ras only weakly activate JNK/SAPK, whereas JNK/SAPK is strongly activated by inflammatory cytokines and cellular stresses such as the viral infection, toxin, TNF, IL-1, UV radiation, heat or osmotic shock or at low concentration of protein synthesis inhibitors (67, 68, 96-98).

As for MAP kinases, activation of the JNKs requires phosphorylation of both threonine and tyrosine in subdomain VIII of the catalytic domain which are different from that in ERK1 and ERK 2. Since UV irradiation, hyperosmolarity and inflammatory cytokines such as tumor necrosis factor (TNF) stimulate JNK activity, it is proposed that the JNK pathway is invoked by the stress-induced cellular responses.

JNK pathway consists of Ha-Ras, Rac/Cdc42Hs, MEKK-1, MKK4/7 or JNK kinase (JNKK) and JNK. Rac is a monomeric G protein, which is related to Ras, and links the stress-activated receptor to downstream phosphorylations. Analogous to ERKs, once phosphorylated, JNK will cross into the nucleus and activates several transcription factors, including c-Jun, ATF2 and Sap-1 (82, 86, 99, 100). c-Jun, the earliest known target of the JNK pathway is a part of the AP-1 transcription factor that regulates the genes involved in cell proliferation. However, the biological function of JNK remains obscure. Some evidence show that the mitogenic signals such as the growth factors and phorbol esters stimulate the ERK pathway, but not the JNK pathway. JNKs regulate a partially overlapping set of transcription factors compared with those governed by ERK1 and ERK2. JNKs are unable to phosphorylate and activate p90RSK, the

target of ERKs. However, activity of JNKs in phosphorylating and activating the N-terminal region of the c-jun family members is higher than ERKs (94).

In non-myocytic cells, it is frequently found that SAPKs are associated with apoptosis (101, 102). Therefore, SAPKs is more likely to be involved in decompensated hypertrophy. Andreka, Zang et al reported that transient activation of JNK in cardiac myocytes serves both to delay and attenuate the total extent of apoptosis (103). SAPK has also been shown to produce either pro-apoptotic or anti-apoptotic effects in many cell types and activation of JNK/SAPK in response to mechanical stress, cytokines and oxidative stress, showed correlations with apoptosis of cardiac myocytes (104, 105). As reported lately, the JNK pathway which is regulated by MKKK1 is critical for the protection against oxidative stress-induced apoptosis in cardiac hypertrophy (106). Therefore, the pro-apoptotic and anti-apoptotic effects of JAN/SAPK may be related to the downstream effectors of these kinases. However, the direct cause and effect relationship between JNK activation and apoptosis remain unclear.

d. p38 MAP kinase

p38 is a relatively new member of the MAP kinase family. It was cloned in 1994 by Han et al and Rouse et al independently (107, 108). The p38 enzyme was identified for being required for restoring the osmotic gradient across plasma membrane. It is a mammalian homologue of the yeast osmosensing protein kinase, HOG-1. Similar to JNK, p38 can be activated by UV light, and regulated by the osmotic stress. It can also be activated by the proinflammatory cytokines such as IL-1 and tumor necrosis factor (TNF). However, growth factors such as phorbol ester and EGF can only cause modest changes in p38 MAP kinase activity (94).

p38 has a dual phosphorylation site motif which is distinct from those of ERK and JNKs. The activation loop containing these phosphorylation sites in the kinase structure is shorter by six amino acids in p38 than in ERKs and JNKs, suggesting that phosphorylation of p38 may occur

through mechanisms distinct from those that control the phosphorylation of ERKs and JNKs (82).

The signaling pathway that leads to p38 activation is not completely understood. The MAP kinase kinases (MKKs) for p38 are JNKK1 (Sek1), MKK3, MKK4 and MKK6. MKK3 is specific for p38 while Sek1 can phosphorylate both p38 and JNKs. However, co-transfection assays *in vivo* showed that Sek1 can activate JNK but not p38 (58). Although the biological function of p38 remains to be determined, the p38 pathway is indeed involved in the regulation of inflammatory cytokine biosynthesis. Therefore, activation of p38 is likely leads to the activation of the JNK pathway. The autocrine loop may represent a common way of crosstalk between different MAP kinase pathways in mammalian cells. Like JNK, p38 kinase also phosphorylates transcription factors (e.g. ATF2, CHOP and MEF2C) and thus, increasing their trans-phosphorylation activities (92).

Pro-apoptotic or anti-apoptotic actions have been described for p38 MAPK. The pro-apoptotic action of p38 was found on treatment of the hypoxic myocytes with selective p38 and JNK2 inhibitors, and following reduction in apoptosis (100, 109). It has also been reported that the oxidative stress-induced apoptosis of myocytes was partially due to the activation of p38 (110, 111). However, the anti-apoptotic action of p38 has also been reported in cardiomyocytes (112, 113).

Although many studies have focused on the roles of MAP kinase pathways in the development of the hypertrophic response, but the underlying mechamisms still remains to be defined. Results of some studies have shown that proto-oncogene Ha-Ras, the Gαq-containing heterotrimeric G protein, and the interleukin-6 (IL-6) receptor gp130, are important mediators for the hypertrophic response *in vitro* and *in vivo*, and may do so *via* activation of their downstream signaling pathways such as MAP kinase cascade. Endothelin-1, phenylephrine and PMA are powerful hypertrophic agonists in cultured cardiac myocytes. Related studies have shown that

activation of the ERK signal transduction cascade by either of these might mediate the hypertrophic response. JNK and p38 have also been suggested to affect cardiac hypertrophy by inhibiting the cell proliferation and promoting of apoptosis. The involvement of different MAP kinase signaling cascades probably provides both the flexibility and integration of gene expression that governs normal tissue growth. However in patients with cardiac hypertrophy and heart failure, they probably contribute to the maladaptive growth response that is critical in cardiomyopathy of overload (114).

5. Interaction between MAPK and RAS

Due to high prevalence and mortality, congestive heart failure (CHF) has become the major social and economic burden. Therefore, the development of potential drug therapy and improved management of heart failure patients has become a very demanding task. It has been demonstrated that the neuroendocrine stimuli for cardiac function arise from the sympathetic nervous system and the renin-angiotensin system (RAS). These stimuli induce cardiac dilatation, myocytes dysfunction and remodeling. Innovative drug therapy or procedures modulating the signal transduction systems that generate abnormal cardiac phenotypes, may therefore make it possible to prevent the transition from adaptive to maladaptive hypertrophy. Although the cellular mechanism(s) are still poorly understood, findings from experimental studies and clinical trials in heart failure patients indicate that the neuroendocrine blockers (e.g.angiotensin-converting enzyme inhibitors, angiotensin II type1 receptor blockers and β-adrenergic blockers) have been successfully used in patients with heart failure. These drugs at least partially, reverse the cardiac remodeling, and improve myocytes function (115).

ACEIs were initially identified in the snake venom. Considerable evidence has demonstrated that this group of drugs provide superior capabilities as compared to other drugs in the control of blood pressure, reduction of proteinuria, and in slowing the progression of renal disease. These drugs were introduced for the treatment of heart failure because of their

vasodialatory, antihypetrensive and antiproteinuric effects, and their demonstrated ability to delay progression of renal disease in conjunction with their ability to reduce systemic blood pressure. Their ability to prolong survival is very unique among the vasodilator drugs. Although mechanism(s) by which these drugs improve cardiac function and clinical outcome remains poorly defined, it is clear that their beneficial clinical effects depend on their ability to reduce the plasma level of angiotensin II and to increase the plasma bradykinin levels and thereby to inhibits maladaptive growth (116). Earlier studies suggested that as a result of their ability to inhibit kininase II or ACE, ACEIs reduced plasma Ang II levels and increased plasma bradykinin levels. However, there is a continning debate about the ability of ACE inhibitors to produce long-term suppression of Ang II levels. There is growing evidence that alternative pathways of conversion of Ang I to Ang II, including a chymase pathway, may restore the circulating or tissue levels of Ang II and bradykinin, despite ACE inhibition. Indeed, clinical studies have releavled that the circulating level of Ang II tends to return toward the pre-treatment levels during long-term ACEI therapy (117). In addition, data from some experimental models of ventricular dysfunction suggest that at least some of the beneficial effects of ACE inhibitor are mediated by kinin stimulation rather than by Ang II suppression. Results of acute studies in humans have demonstrated that a bradykinin receptor antagonist inhibited the blood pressure-lowering effects of ACEI (118). Experimental studies in the rat model of myocardial infarction suggest that long-term treatment with ACEIs decreases the abnormal accumulation of myocardial collagen, the fibrosis which increases the myocardial stiffness and hasten the remodeling process (28). The data from other clinical studies suggest that ACEIs prolong survival in patients with severe heart failure by inhibiting Ang II-induced apoptosis (119, 120). Furthermore, ACEI treatment can attenuate myosin heavy chain content and the isoform shift in heart failure. The preventative effect on remodeling of the sarcoplasmic reticulum membrane and

sarcolemmal membrane after myocardial infarction has also been demonstrated in rat experimental model (121).

It is well established now that treatment with ACEIs improves prognosis of patient with heart failure (28, 87, 122, 123), in that their survival rates are much improved (124). The reduced hospitalization rates for the heart failure patients in several subgroups, including age, sex, and presumed etiology, support the view that ACEIs slow down the progression of the overload-induced cardiomyopathy and delay the development of more severe heart failure (125-127). The clinical trials of treatment with enalapril showed consistenly the reduction in morbidity and mortality in patient with heart failure (128). These studies also demonstrated that the long-term treatment of patients with ACEI favourably altered the left ventricular loading conditions and repressed the progressive remodeling of ventricle, and thus prolonging the survival period (28, 128-131). In the rat cardiac infarction model, although the infarct size was not reduced, the treatment with ACEI did reduce the left ventricular fill pressure and distension, improved the ventricular performance, and repressed the dilatation of the left ventricle. Results of the experimental study in our laboratory have also demonstrated the beneficial effects of ACEIs on the rat heart failure model, which were associated with the improved myofibrillar ATPase activities as well as prevented changes in the protein contents and gene expression of myosin heavy chain isozyme (132). More studies are warranted to clearly define the mode and site of ACEIs action in the hearts of patients with heart failure.

In myocardial infarction (MI), the activated renin-angiotensin system is the hallmark of congestive heart failure (CHF) (133) and it plays an important role in cardiac remodeling and/or the prognosis following MI (134). Blocking the production of Ang II inhibits maladaptive growth (51, 135, 136). These cellular responses mediated by heptahelical receptors that are coupled to $G_{\alpha q}$ and stimulate JAK/STAT-controlled MAP kinase pathways. This pathway is also activated by the enzyme-linked cytokine receptors (49, 137). Activation of these mitogenic

signaling pathways almost all exert their worsening effects on the failing heart (138). A significant reduction in the cardiac hypertrophy has been demonstrated in ACEI treated groups in different pressure overload rat aortic models, such as the aortic stenosis, aorta banding and the constriction of abdominal aorta. Treatment with ACEIs prolonged the survival rate (52, 139-142). Moreover, in volume-overload induced heart failure, ACEI therapy significantly reduced the LVEDP, suggesting that RAS may exert some effects on volume-overload induced cardiac hypertrophy and failure (143-146). The available ACEIs include captopril, benazepril, enalapril, fosinopril, lisinoprol, quinaprol, ramipril, perindopril erbumine and trandolapril.

The mechanism of Angiotensin II receptor subtype 1 (AT_1) blockers is different from the ACEIs. They are more specific blockers for the RAS and have no effect on any of the other known enzymatic systems that result in the formation of Ang II. These blockers primarily bind to AT_1 receptors and block the binding of angiotensin II to its high affinity type 1 receptors. AT_1 receptor blockers inhibit vasoconstriction, and the hormone-stimulating and growth-promoting effects of Ang II. Their antihypertensive and hemodynamic effects appear to be similar to those of the ACEIs. Daily oral intake of losartan, the specific AT_1 receptor blocker, has shown its efficiency and safety in the treatment of hypertension (147). Candesartan, another blocker of AT_1 receptor, which binds to receptors tightly, produce long-lasting effects which cannot be overcome by even high concentration of AngII. It provided the control of blood pressure for 24 hours without negative cardiovascular effects of Ang II (148-150). There is increasing scientific evidence that when AT_2 receptors are not blocked, the serum level of Ang II was elevated by AT_1 receptor blocker, indicating the counter regulatory effects of AT_2 receptors. The experimental studies have also revealed that the stimulation of AT_2 receptor result in the activation of bradykinin-nitric oxide-cyclic GMP vasodilator cascade in addition to directly activating kininogenase, and further production of bradykinin (151, 152). Mediators of antiproliferative actions of angiotensin II blockade have also been reported (153). Multiple lines

of evidence suggest that Ang II antagonist had beneficial effect on the heart failure (151, 152, 154). It has also been reported that losartan favourably increases the coronary flow in experimental dogs (155). Furthermore, because losartan is devoid of the side effects, including cough, angioedema, renal dysfunction and first-dose hypotension evoked by ACEIs (121, 156, 157) it is considered to be an alternative therapeutic method for patients who cannot tolerate the side effects of ACEI. The available AT_1 receptor blockers include losartan, valsartan, irbesartan, candesartan cilexetil, telmisartan and eprosartan.

Because of the dissimilar mechanisms of action of ACEIs and AT_1 blockers, their effects on blood pressure, reduction in proteinuria and attenuation of glomerulosclerosis and tubulointerstitial fibrosis have been studied in different experimental models. Moreover, both drugs inhibit apoptosis and limit the production of many soluble mediators of fibrosis, including TGF-β, fibronectin, laminin, endothelin and type 4 collagen (158-161). Schieffer and colleagues reported an equal effect of ACEIs and AT_1 receptor blocker on reducing cardiac hypertrophy, restoring minimal coronary vascular resistance in postinfarction reactive hypertrophy and attenuating the noninfarcted left ventricular myocardial interstitial fibrosis. Their study also suggested that ACEIs and AT_1 receptor blockade probably have similer effect on inhibiting the generation of angiotensin II and in preventing ventricular remodeling in postmyocardial infarction (162). Similar effect of losartan and enalapril on improving exercise tolerance in patients with heart failure has been addressed by demonstrating the reduction of myosin heavy chain isozyme 2a and 2b and a shift to myosin heavy chain isozyme 1, which is the slower and more fatigue-resistant isoform (163). However, Linz and Scholkens reported that in pressure-overload rat model of aortic banding, losartan was less effective in preventing cardiac hypertrophy as compared with ACE inhibition (164). Ruzicka and Leenen suggested that in a volume-overload rat model, AT_1 receptor blockade was more efficient than ACEI in blunting the hypertrophic response (47). Theoretically, different mechanisms of these two classes of drugs

suggest that combination therapy of these blockers might be an ideal approach to completely blocking the renin-angiotensin system. Results of clinical and experimental studies support the assumption of obtaining an additive effect of the combination therapy with the two classes of drugs in kidney protection (165). In pig heart failure model, the reduced densities of L-type Ca^{2+}-channels and diminished activities of SR Ca^{2+}-pump were recovered more by the combination therapy with ACE inhibitor benazepril and losartan than either of the drug administered independently (166). In patients with congestive heart failure, the studies using combination therapy are in progress. The available data indicate that the combined use of ACEI and AT_1 blockers is capable of further reducing end-diastolic volume in the left ventricle and improving exercise tolerance as compared with the administration of each drug individually (167, 168). Hamroff and colleagues reported that the combined AT_1 receptor blockade by losartan at maximal recommended dose and tolerated dose of ACEI enhanced peak exercise capacity and alleviated symptoms in the congestive heart failure patients (169). In a random double-blind multicentre trial, the RESOLVE Pilot Study (Randomized Evaluation of Strategies for Left Ventricular Dysfunction), left ventricular dimensions were measured after the combinated treatment with candestartan cilexetil and enalapril for 43 weeks. Results show that attenuation of the ventricular remodeling was greater than after treatment with the individual drug (170). However, different combinations of drugs produced different results. The combination therapy of these two classes of drugs in experimental models did not produce additive beneficial effect as compared with the usage of each drug individually in terms of delaying progression of renal disease in short period of treatment (171, 172). In view of these conflicting data, it would be unwise to advocate the combined treatment of patients with blockers of AT_1 receptors and ACEI. Apparently, further experimental and clinical studies are needed to resolve this medical issue.

It has been well recognized that activation of RAS is one of the important upstream signaling that is critically involved in the regulation of MAP kinase signal transduction

pathways. Result of a previous study indicated that after infusion of angiotensin II, activity of the cardiac p44-ERK (ERK1) was greater than that of p42-ERK (ERK2). Further study has revealed that the infusion of high-dose of Ang II induced differential ERKs activation between the left and right ventricles. The authors proposed that ERK1 and ERK2 may play different role in cardiac diseases through RAS cascade (55). The relationship between Ang II and MAP kinases was investigated in hypertrophied neonatal rat cardiac myocytes by Aoki and colleagues (62). They found that Ang II activated ERKs, while PD98059, a specific inhibitor of MAPK/ERK kinase (MEK), inhibited Ang II-induced expression of atrial natriuretic factor (ANF) at both the mRNA and polypeptide levels. Dominant-negative Ras inhibited both ERK activation and ANF up-regulation by Ang II, whereas constitutively active forms of Ras and MEK were sufficient to activate the ANF promoter. These results suggest that Ang II regulates ANF expression through ERK pathways. The ERK pathway mediates an agonist-specific and phenotype-specific response in cardiac hypertrophy (62). In an experimental study where the neonatal rat cardiomyocytes were stretched, Yamazaki reported that CV-11974, the AT_1 receptor antagonist, completely blocked the activation of MAP kinases, by the stretch-conditioned medium. These results demonstrate that Ang II, secreted from the stretched cardiomyocytes, was involved in activation of Raf-1-MKK-MAPK signaling pathways (173). The mechanical stress, which initiates secretion of Ang II, has been suggested to be the possible activator of MAP kinase (174-176). In another study using the neonatal rat cardiac myocytes, Zou reported that PKC and Raf-1, but not tyrosine kinases or Ras, are critical for Ang II-stimulated ERKs activities (66). Takemoto et al also reported activation of myocardial MAP kinases in rat heart *in vivo* after applying either Ang II or α-adrenergic or β-adrenergic agonists (177).

The data from the experimental and clinical studies demonstrate an important relationship between MAP kinases and the rennin-angiotensin system in cardiac hypertrophy and heart failure. Therefore, ACEIs and AT_1 antagonists may exert their beneficial effects on heart

functions by inhibiting the activation of MAP kinases signaling pathways. Although there are many reports showing the relationships between RAS and MAP kinase, it should be noted that most of these results are based on different cultured cell types *in vitro*, or cardiac tissue samples which includes blood vessels, muscle, connective tissue and nerves. Although cardiac myocytes constitute 76% volume of the myocardial tissue, some reservation should be exercised in the interpretation of results in terms of heart functin *in vivo*. Furthermore, a considerable portion of the myocardium has undergone significant changes by the fibroblasts, inflammatory cells, and the deposition of collagen, which occurred in almost all cardiomyopathies and the experimental models of heart failure. Studies with the isolated neonatal cardiac myocytes may not be suitable for formulating treatment of heart disease in adults, since the heart undergoes significant biochemical and functional changes during development (178). Thus, caution must be exercised in the interpretation of data obtained from different clinincal and experimental studies using the diverse population of patients and tissue preparations. Further studies investigating the relationship between MAP kinases and RAS in isoloated adult cardiac myocytes and experimental models of heart failure will provide useful information concerning the heart disease and future development of therapeutic strategies.

STATEMENTS OF THE PROBLEM AND HYPOTHESES TO BE TESTED

Cardiac hypertrophy is a complex compensatory response of the heart to increased pressure overload or volume overload. Although it is initially beneficial for the generation of more contractile force, excessive and/or prolonged hypertrophic stimulation leads to the dysfunction of cardiomyocytessubsequent to heart failure. The work from several laboratories has indicated that the development of cardiac hypertrophy involves many signaling transduction pathways linking the extracellular hypertrophic stimuli to nuclear transcription factors, eventually leading to myocytes hypertrophy. However, the cellular events leading to pathophysiological alterations in the heart tissue are not well understood. As MAPK is an essential part of the cell signal transduction pathway that play an important role in cell growth of the heart tissue, the involvement of extracellular signal-regulated kinase (ERK) in cardiac hypertrophy and low-output heart failure has been investigated using different experimental models. However, very little information regarding changes in ERK during the high-output heart failure is available in the literature. This study was therefore undertaken to examine changes in the MAP kinase pathway in a high-output heart failure induced by AV shunt in rat. In view of the fact that RAS is important for the development of cardiac hypertrophy and heart failure, we have examined the effects of an ACE inhibitor, enalapril, and an AT_1 receptor antagonist, losartan, treatments on the AV shunt induced changes in MAP kinase activity and protein contents. Combination of both enalapril and losartan was also used to eatablish if the effect of their combination therapy are additive. It was anticipated that this investigation would provide comprehensive information regarding the pathogenesis of cardiac hypertrophy and heart failure induced by volume overload and the data would be useful for evaluating the therapeutic potential

of combination treatment with an ACE inhibitor and AT_1 receptor antagonist in congestive heart failure.

MATERIALS AND METHODS

1. Experimental model

Male Sprague-Dawley rats weighing from 250 g to 300 g were used in this study. Animals were provided with tap water and rat chow *ad libitum*. They were kept in animals quarters with a 6:00 am to 6:00 pm light/dark cycle at controlled temprature. Experiments were performed according to the *Guidenlines to the Care and Use of Experimental Animals* issued by the *Canadian Council on Animal Care*. The aortocaval shunt was produced following the protocol generated by Garcia and Diebold (179). Briefly, animals were anaesthetized with the isofluorane, the ventral abdominal laparotomy was performed. The intestine was positioned laterally and it was kept moistened using normal saline. Using blunt dissection, the aorta and vena cava between the renal arteries and iliac bifurcation were exposed. Vessels proximal and distal to the intended puncture site were temporarily ligated. In an effort to creat the shunt, an 18-gauge needle was inserted into the exposed abdominal aorta and advanced through the medial wall into the vena cave. The puncture site was then sealed with a drop of inocyanate (Krazy glue) following the withdrawal of the needle. A successful shunt was confirmed by the pulsatile flow of oxygenated blood into vena cava from the abdominal aorta. Absorbable suture and autoclips were utilized to properly close the abdominal musculature and the skin incisions. Age-matched animals were subjected to the same surgical procedures without creating the shunt sham operated group.

2. Experimental design

In this study, three series of experiments were carried out. The first series was designed to characterize the general parameters and hemodynamic changes for development of cardiac hypertrophy at 4 weeks and heart failure at 16 weeks in aortocaval shunted rats treated with or

without enalapril and/or losartan. In both 4 and 16 weeks groups, animals were randomly separated into five groups: sham–operated (SH), aortocaval shunt (AV), AV + enalapril (AVE) , AV + losartan (AVL), and AV + enalapril + losartan (AVEL). Enalapril (10mg/kg/day) and losartan (20mg/kg/day) were dissolved in tap water and administered orally by a gastric tube starting at 3 days after surgery. The SH and AV groups were provided tap water *ad libitum*. The second series of experiments was devoted to determine alterations in the ERK1 and ERK2 in different groups to find out the interaction between MAPK signaling pathway and the renin-angiotensin system. Changes of both non-phosphorylated and phosphorylated ERK1 and ERK2 protein content were examined in order to define their role in altering the cardiac responses to enalpril and/or losartan treatment. The third series of experiments was carried out to further define the interaction of MAPK signaling pathway with renin-angiotensin system during development of both cardiac hypertrophy and heart failure. For this purpose, we chose phosphorylated-Elk1, which is the downstream substrate of ERK1/2 and also is the transcription factor activated by ERK1/2 that further regulate downstream gene expression related to protein synthesis and cardiomyocytes proliferation, to serve as the marker of alterations of the activities of ERK1/2.

3. General characteristics

Rats were weighed and sacrificed for measuring general parameters. After removal of the connective tissue and atria, total heart, LV and RV weights were measured, then LV and RV was put into liquid nitrogen and stored at -70°C. In order to assess the existence of circulatory congestion, the wet/dry weight ratio of the lung and liver were also calculated. In each case lungs and liver were removed and freed from the connective tissue and weighed, chopped into smaller pieces, then placed into oven (65°C) until a constant weight was obtained. This usually need more than 24 hours.

4. *In vivo* hemodynamic assessment

After animals were anethetized with ketamine (90 mg/kg) and xylazine (9mg/kg), an ultraminiature catheter (Millar Instruments, Texas) connected to a pressure transducer was inserted into the right carotid artery and advanced into the LV. The left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), heart rate and rate of pressure development (+d*P*/d*t*) and pressure decay (-d*P*/d*t*) were recorded with Biopac data Acquisition system (Biopac System Inc., Goleta, CA). The catheter was subsequently withdrawn to the aorta and the arterial systolic pressure (ASP) and arterial diastolic pressure (ADP) were measured.

5. Preparations of tissue extract for ERK determination

The preparation of tissue extract for ERK1/2 was carried out by the method described in a commercial kit purchased from NewEngland BioLab, Cell Signaling Technology Commpany. Briefly, measure 100mg left ventricular tissue, which was frozen in liquid nitrogen and stored at –70°C, powdered in liquid nitrogen, then suspended in 750μl cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%Triton-X100, 2.5 mM sodium pyrophosphate, 1 mM β -Glycerolphosphate, 1mM Na_3VO_4, 2μM Leupeptin, freshly added 1mM PMSF before use, homogenized twice with politron at 8,000 RPM for 15 second, this step is for isolating total proteins from rat hearts. The solution was placed into a 1.5 ml Eppendorf tube, sonicated four times at 40 amplitude for 5 seconds. The homogenated was centrifuged at 15,000 x g (Eppendorf centrifuge 5415C) for 10 minutes at 4°C. The supernatant was carefully removed, aliquoted into small volume and stored at -70°C for future use.

6. Protein concentration determination

Protein concentration of tissue extract was estimated in triplicates by the microassay procedure of Bradford. Tissue samples were diluted appropriately, made to a final volume of 800

µl, and treated indentical to standars. A standard curve was obtained by taking differents samples of BSA solution containing 2.5, 5, 7.5, 10 µg/ml in total volume of 800 µl. The colour reagent (200 µl) was added to each tube and the mixture incubated for 15 minutes at 22°C. The colour intensity was measured in a spectrophotometer (SPECTRAmax® PLUS^{384}, Molecular Devices) at 595nm.

7. Immunoprecipitation

The immunoprecipitation for the estimation of phospho-Elk1 was followed as described in the kit bought from the NewEngland BioLab. To the tissue extract containing approximately 200µg total protein, 15µl of immobilized Phospho-p44/42 MAP Kinase (Thr202/Tyr204) Monoclonal Antibody was added. The samples were incubated at 4°C with gentle rocking for 4 hours to overnight. The immunoprecipitated sample was centrifuged at 10000 x g (Eppendorf centrifuge 5415C) for 30 seconds at 4 °C. The pellet was washed twice with 500 µl of 1X cell lysis buffer. The pellet was then washed twice with 500 µl of 1X kinase buffer [25mM Tris (pH 7.5), 5mM β-Glycerolphosphate, 2mM DTT, 0.1mM Na_3VO_4, 10 mM $MgCl_2$]. The mixture was centrifuged at 10000 x g (Eppendorf centrifuge 5415C) for 30 seconds at 4°C.

8. Determination of ERK activity

The ERK activity was determined according to the procedure described in the p44/42 MAP Kinase Assay Kit provided by the New England BioLab. The pellet containing the immunoprecipitate as described earlier was suspended in 50 µl 1X kinase buffer supplemented with 200 µM ATP and 2 µg Elk-1 fusion protein. The mixture was incubated for 30 minutes at 30 °C and reaction was terminated by adding 25µl 3X SDS sample buffer containing 187.5mM Tris-HCl (pH 6.8 at 25°C), 6% SDS (w/v), 30% Glycerol, 150mM DTT, 0.3% Bromphenol Blue (w/v). The sample was boiled for 5 minutes, vortexed, and centrifuged at 10000 x g (Eppendorf

centrifuge 5415C) for 2 minutes. The sample (30 µl) was loaded on 12%SDS-PAGE gel and analyze by western blot (see western immunoblotting protocol as described below in analysis of ERK1, ERK2 and Elk1 protein content). Probe with Phospho-Elk-1 antibody (1:1000 dilution).

9. Analysis of ERK1, ERK2 and Elk1 protein content

The relative protein content of ERK1, ERK2 and Elk1 was obtained by running 12% sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) gels followed by immunostaining by Western blot assay. The concentration of protein in these samples was adjusted to 2mg/ml with the cell lysis buffer and 4X SDS-PAGE loading buffer (1part) was added into the 1X cell lysis buffer (3 parts). The SDS-PAGE loading buffer contained 250mM Tris-HCl (pH 6.8@25°C), 8%w/v SDS, 40% Glycerol, 200mM DTT and 0.4%w/v Bromphenol Blue. The protein loads for ERK1 and ERK2 were the same (15 µl in each well) while for phospho-Elk1 was 30 µl in each well followed by the protocol provided by the commercial kit. The electrophoresis was carried out first at 100 volts for 10 min to separate the proteins in samples followed by 200 volts for 40-45 min. The proteins in either experiment separated by SDS-PAGE were electroblotted at 4°C onto nitrocellulose transfer membrane, 0.22µm. by employing a transfer buffer containing 25mM Tris-HCL, 192 mM glycine and 20% methanol (v/v) for the determination of relative protein content with immunoblotting analysis. After the transfer, the membrane was washed with 25 ml TBS for 5 minutes at room temperature. The membrane was then incubated in 10mM Tris-HCL (pH7.4) containing 150 mM NaCl solution, 5% nonfat milk powder and 0.1% Tween-20 for 1 hour at room temperature on a shaker. Incubate membrane and primary antibody (1:1000) in 10 ml first antibody dilution buffer (for ERK1/2 is 5% fat-free milk TBST, for Elk1 is 5% BSA TBST) with gentle agitation overnight at 4 °C. The membrane was placed at room temperature for 30 min before washing 3 times for 10 minutes each with 15 ml of TBST. Then incubated the membrane with 1:3000 Goat Anti-Rabbit

Gig (H+L) (Human IgG Adsorbed) Horseradish Peroxidase Conjugate, from BIO-RAD diluted in 1% fat-free milk TBST at room temperature for 1 hour. The membrane was washed with TBST again. According to the manufacturer instructions, protein bands were made visible by the ECL system (Amersham-Pharmacia Biotech). Primary antibodies all come with the commercial kit purchased for New England BioLab.

10. Statistical analysis

All data are expressed as mean ± SEM. Protein content was expressed as percentage of SH control group. Difference between two groups was analyzed by using unpaired student *t*-test. Multigroup comparisons were performed using one-way analysis of variance (AVOVA). Statistically significant differences were considered at a level of $P<0.05$.

RESULTS

1. Characterization of the experimental model

a. General characteristics of the rat model

Out of 90 rats that underwent AV shunt surgery, 8 died within 24 to 72 hours after the operation. Autopsy examination demonstrated neither bleeding from the puncture site nor occlusion of either aorta or vena cava in these animals. The heart was dramatically dilated and stopped in the diastolic phase, suggesting that these animals died from acute heart failure, but not due to the surgical procedure itself. There was no mortality between 1 and 8 weeks post-operation, however, 5 rats died between 10 and 16 weeks after the AV shunt operation. Autopsy examination revealed hypertrophied and dilated heart, congested liver and lungs, edema of limb and face, pleural effusion and presence of ascites, indicating severe stage of congestive heart failure as the cause of death. The rats who survived 16 weeks of the AV shunt surgery were relatively slow in physical movements and their fur around the face as well as neck area as stained with blood that most likely due to sputum originating from the congested lungs. About 30 percent of the operated rats that were sacrificed at 16 weeks after AV shunt operation shows ascites. There had no mortality in the sham operated control group during 16 weeks after the surgery.

The general characteristics of AV shunt rats with or without treatment with enalapril, losartan or enalapril plus losartan for 4 weeks are shown in Table 1. There was no significant difference in body weight (BW) after the operation as compared to sham operated control group among all groups. However, the heart weight (HW) increased. The HW to BW ratio increased in the untreated AV shunt groups as compared to the

Table 1: General characteristics and hemodynamic changes in rats following the aortocaval shunt and treatment with or without enalapril, losartan or enalapril plus losartan treatments for 4 weeks

	SH	AV	AVE	AVL	AVEL
BW (g)	436±16	433±17	440±15	444±17	421±19
HW (mg)	1071±34	1766±76*	1493±80#	1233±132#	1245±90#‡
LVW (mg)	805±22	1282±40*	1099±33#	942±85#	931±62#‡
RVW (mg)	262±10	486±23*	400±19#	366±33#	352±22#‡
HW/BW (mg/g)	2.46±0.06	4.12±0.18*	3.37±0.17#	2.78±0.29#	2.71±0.15#‡
HR (beats/min)	341±20	339±15	343±22	338±18	340±14
ASP (mmHg)	116±2	113±2	111±3	109±3	110±3
ADP (mmHg)	83±4	68±4*	59±4	65±5	66±6
LVSP (mmHg)	113±2	115±2	110±1	112±2	110±2
LVEDP (mmHg)	4.8±1.0	13.3±0.8*	10.4±0.8#	10.9±1.6#	10.9±0.9#

Data are expressed as mean ±SEM from 8 rats in each group. BW: body weight; HW: heart weight; LVW: left ventricular weight; RVW: right ventricular weight; HW/BW ratio: heart weight to body weight ratio (mg/g); HR: heart rate; ASP: arterial systolic pressure; ADP: arterial diastolic pressure; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure; SH: sham control; AV: aortocaval shunt; AVE: aortocaval shunt with enalapril (10 mg/kg/day) treatment; AVL: aortocaval shunt with losartan (20 mg/kg/day) treatment; AVEL: aortocaval shunt with enalapril (10 mg/kg/day) and losartan (20 mg/kg/day) treatment. Enalapril and Lostartan were given daily by gastric gavage. *$P<0.05$ vs SH group; #$P<0.05$ vs AV group; ‡$P<0.05$ vs AVE group.

sham control group. The increased HW, HW/BW ratio, LV wt and RV wt were attenuated by treatment with enalapril, losartan or their combination for a period of 4 weeks. The general characterises of AV shunt rats with or without enalapril, losartan or enalapril plus losartan treatments for 16 weeks are shown in Table 2. The increased HW, HW/BW ratio, LV wt and RVwt in the AV shunt group were partially prevented by these drug treatments. These results show that the heart is hypertrophied at 4 and 16 weeks of AV shunt and that this cardiac hypertrophy is attenuated by the blockade of RAS. The attenuation effect of combination therapy on cardiac hypertrophy was not greater than that observed for enalapril but not for losartan.

b. Circulatory congestion

The lung and liver wet wt, and their wet to dry wt ratios were measured at 4 and 16 weeks after surgery and the results are given in Table 3. No significant change in liver wet wt and liver wet to dry wt ratio among different groups as compared to sham-operated control group was observed at 4 weeks; however, the liver wet wt was significantly increased in AV shunt group without 16 weeks after the operation. These changes in liver wt were partially reversed by the treatment with either, enalapril and/or losartan. At 16 weeks of AV shunt, liver wet to dry wt ratio incressed significantly as compared to the sham group and this was partially reversed by treatment with enalapril and/or losartan. Although the reversal effects of these drugs were evident, treatment with enalapril plus losartan did not produce additive effects. At 4 weeks, lung wet wt was increased in AV shunt group in comparsion to the sham control. However, the ratio of lung wet to dry wt did not change in either treated or untreated AV shunt groups at this time point. Lung wet wt in AV shunt operated group was higher and this change was reversed by treatment with enalapril and/or losartan. However, no additive beneficial effect was noted after combination treatment. There was no significant change in lung wet to dry wt ratio at 16 weeks of AV shunt.

Table 2: General characteristics and hemodynamic changes in rats following the aortocaval shunt and treatment with or without enalapril, losartan or enalapril plus losartan treatments for 16 weeks

	SH	AV	AVE	AVL	AVEL
BW (g)	615±23	656±24	623±28	619±21	611±25
HW (mg)	1364±41	2209±117*	1671±97#	1526±105#	1491±91#‡
LVW (mg)	1067±29	1640±142*	1217±61#	1119±66#	1133±58#‡
RVW (mg)	300±13	554±42*	462±25#	394±36#	359±22#‡
HW/BW (mg/g)	2.23±0.13	3.37±0.14*	2.68±0.14#	2.47±0.17#	2.44±0.14#
HR (beats/min)	340±15	332±17	336±19	342±20	338±15
ASP (mmHg)	122±4	103±3*	104±3	100±11	102±6
ADP (mmHg)	84±6	53±9*	63±10	63±4	65±3
LVSP (mmHg)	115±5	85±2*	91±6#	93±7#	91±5#
LVEDP (mmHg)	5.2±1.4	28.0±1.9*	18.6±1.5#	13.8±0.6#	13.8±0.7#‡

Data are expressed as mean ±SEM from 8 rats in each group. BW: body weight; HW: heart weight; LVW: left ventricular weight; RVW: right ventricular weight; HW/BW ratio: heart weight to body weight ratio (mg/g); HR: heart rate; ASP: arterial systolic pressure; ADP: arterial diastolic pressure; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure; SH: sham control; AV: aortocaval shunt; AVE: aortocaval shunt with enalapril (10 mg/kg/day) treatment; AVL: aortocaval shunt with losartan (20 mg/kg/day) treatment; AVEL: aortocaval shunt with enalapril (10 mg/kg/day) and losartan (20 mg/kg/day) treatment. Enalapril and Lostartan were given daily by gastric gavage. *P<0.05 vs SH group; #P<0.05 vs AV group; ‡P<0.05 vs AVE group.

Table 3: Wet weight and wet to dry weight ratio of liver and lungs in rats following the aortocaval shunt and treatment with or without enalapril, losartan or enalapril plus losartan treatments for 4 weeks and 16 weeks

	SH	AV	AVE	AVL	AVEL
A. 4 Weeks					
Liver wet wt (g)	16.8±0.6	16.9±0.4	16.6±0.5	17.1±0.5	16.4±0.5
Liver wet/dry wt	3.30±0.25	3.56±0.19	3.58±0.18	3.51±0.21	3.65±0.15
Lung wet wt (g)	1.31±0.02	1.62±0.05*	1.55±0.04	1.53±0.05	1.54±0.04
Lung wet/dry wt	4.63±0.23	4.31±0.22	4.26±0.11	4.78±0.24	4.76±0.24
B. 16 Weeks					
Liver wet wt (g)	18.9±0.7	23.2±1.4*	19.7±0.7$^{\#}$	19.6±0.7$^{\#}$	18.6±0.5$^{\#}$
Liver wet/dry wt	3.28±0.16	3.79±0.19*	3.38±0.17$^{\#}$	3.37±0.19$^{\#}$	3.48±0.17$^{\#}$
Lung wet wt (g)	1.53±0.03	2.49±0.07*	1.59±0.04$^{\#}$	1.57±0.06$^{\#}$	1.55±0.04$^{\#}$
Lung wet/dry wt	4.59±0.23	4.69±0.25	4.36±0.22	4.61±0.23	4.61±0.27

Data are expressed as mean ± SEM from 8 rats in each group. Liver wet wt SH: sham control; AV: aortocaval shunt; AVE: aortocaval shunt with enalapril (10 mg/kg/day) treatment; AVL: aortocaval shunt with losartan (20 mg/kg/day) treatment; AVEL: aortocaval shunt with enalapril (10 mg/kg/day) and losartan (20 mg/kg/day) treatment. Enalapril and Lostartan were given daily by gastric gavage. *P<0.05 vs SH group; $^{\#}$P<0.05 vs AV group; ‡P<0.05 vs AVE group.

2. Hemodynamic changes at 4 and 16 weeks after surgery

The *in vivo* hemodynamic changes in AV shunt rats with or without enalapril, losartan or enalapril plus losartan treatments for 4 weeks are shown in Table 1. There was no significant change in the heart rate in each group. ASP was not changed in AV shunt groups as compared with the control group at this time point. A dramatic decrease in the ADP was noted in all AV shunt groups with or without medications, further demonstrating the shunted blood induced volume overload. LVSP was not decreased in the AV shunt group at 4 weeks as compared to the control group. However, due to the increased blood shunted from the abdominal aorta to inferior vena cava, a significant elevation in the LVEDP was noted at 4 weeks in AV shunt groups and this elevation was partially reversed essentially to the control level by treatment with either enalapril and/or losartan. Similarly at 16 weeks, there was no significant change in the heart rate in each group. The ASP dropped significantly in AV shunt groups as compared to the control group at this time point indicating the existence of congestive heart failure with decreased contractility of myocardium. Treatment of AV shunt groups with enelapril and/or losartan did not produce any reversible effect on this parameter. The LVSP was decreased in the AV shunt group at 16 weeks when compared to the control group; this decrease in LVSP was partially reversed by treatment with enelapril and/or losartan. At 16 weeks, there was a significant elevation in LVEDP and this was partially reversed in treatment groups. The combination treatment group showed greater improvement when compared with AVE group, but not AVL group. Both $+dP/dt$ and $-dP/dt$ were not altered at 4 weeks in AV shunt group (Figure 1, panel A), but these were greatly depressed at 16

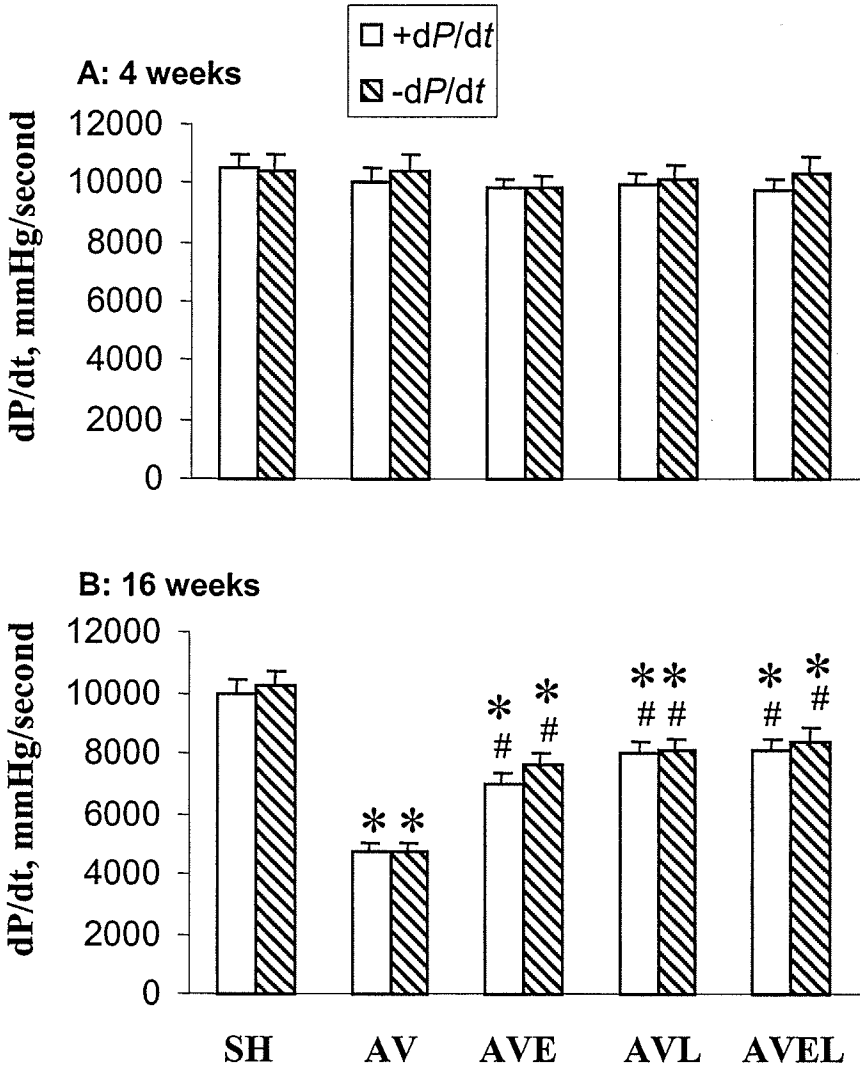

Figure 1: +d*P*/d*t* and -d*P*/d*t* (mmHg/second) in rats following the aortocaval shunt and treatment with or without enalapril, losartan or enalapril plus losartan treatments for 4 (panel A) and 16 weeks (panel B). * $p<0.05$ vs SH group; #$p<0.05$ vs AV group.

weeks indicating heart failure at this stage (AV vs SH, 48% or 46%, for +d*P*/d*t* or -d*P*/d*t* respectively; $p<0.05$). This depression in contractility was partially reversed by drug treatments (AVE, AVL, AVEL vs SH, for +d*P*/d*t* are 69.9%, 79.9% and 80.7%, respectively; for -d*P*/d*t* are 74.8%, 79.0% and 82.2%, respectively; $p<0.05$) (Figure 1, panel B). AEVL group showed better reversible effect compared with AVE group but not AVL group.

3. Alterations of ERK1/2 protein content in cardiac hypertrophy and heart failure

a. Protein content of ERK1 and phosphorylated-ERK1

Results in Figure 2 show the typical Western blot bands and statistical data for ERK1 and phosphorylated ERK1 in cardiac hypertrophy and heart failure induced by the AV shunt. 4 weeks after the operation (Figure 2, panel A), no significant difference was found in protein content of ERK1, which represents the total amount of both non-phosphorylated and phosphorylated ERK1. However, the relative protein content of phosphorylated-ERK1 at this time point in AV shunt group was increased as compered to the SH group (AV vs SH, 185%, $p<0.05$). This increase was partially reversed by treatment with enalapril, losartan, or enalapril plus losartan (AVE, AVL, AVEL vs Sham, 124%, 118%, 118%, respectively; $p<0.05$). At 16 weeks, both the protein content of total ERK1 and phosphorylated-ERK1 were elevated in AV shunt non-treated group (AV vs SH, 122% and 547%, respectively; $p<0.05$)(Figure 2, panel B). This increase was partially reversed by treatment with enalapril, losartan, or enalapril plus losartan (AVE, AVL, AVEL vs SH for total ERK1 are 94%, 94%, and 101%, respectively; for phosphorylated-ERK1 are 309%, 124%, 121%, respectively, $P<0.05$). AVEL treatment

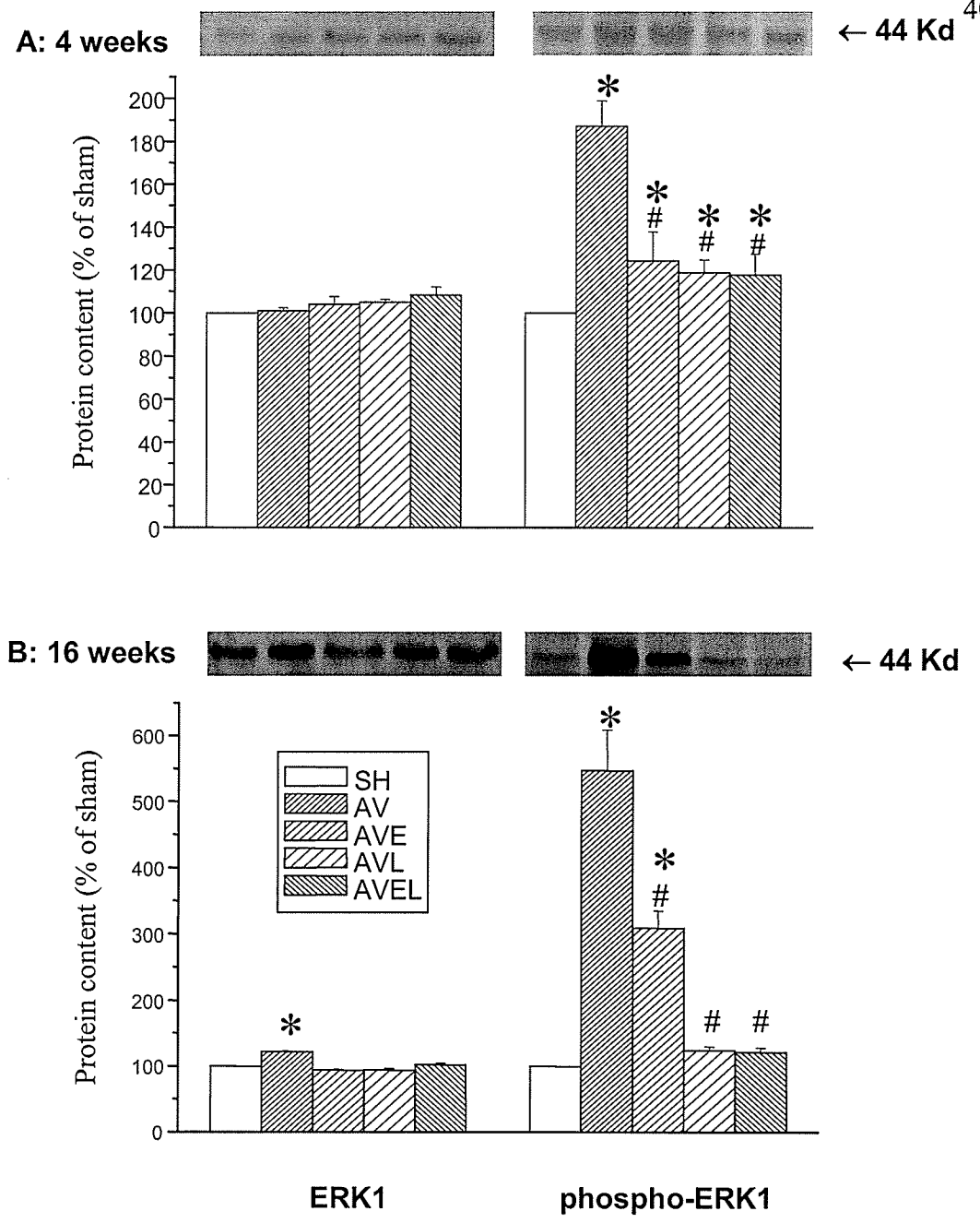

Figure 2: Western blotting analysis showing alteration in the protein content of ERK1 and phospho-ERK1 at 4 (panel A) and 16 weeks of aortocaval shunt rats with or without treatment with enalapril and/or losartan . * $p<0.05$ vs SH group; #$p<0.05$ vs AV group.

group showed more beneficial effect on reversing phosphorylated-ERK1 when compared to AVE group but not AVL group.

b. Protein content of ERK2 and phosphorylated-ERK2

Results of the protein content of ERK2 and phosphorylated-ERK2 in cardiac hypertrophy and heart failure induced by AV shunt are shown in Figure 3. At 4 weeks, the protein content of total ERK2 did not show any significant difference among each group (Figure 3, panel A). However, the protein content of phosphorylated-ERK2 at this time point was increased as compared to the sham control group (AV vs SH, 187%, $p<0.05$). Treatment of AV shunt groups with enalapril, losartan, or enalapril plus losartan partially reversed the increment in protein content (AVE, AVL, AVEL vs SH, 124%, 117%, 118%, respectively; $p<0.05$,). At 16 weeks, both the total ERK2 and phosphorylated-ERK2 were elevated in AV shunt group (AV vs SH, 120% and 554%, respectively; $p<0.05$) (Figure 3, panel B). This increase was partially reversed by treatment with enalapril, losartan, or enalapril plus losartan (AVE, AVL, AVEL vs SH for total ERK2 are 95%, 95%, and 101%, respectively; for phosphorylated-ERK1 are 313%, 125%, 122%, respectively. $P<0.05$). AVEL treatment group showed better reversal effect on phosphorylated-ERK2 as compared to AVE group, but not AVL group.

4. Alterations of ERK1/2 activities

Alterations in ERK1/2 activities during cardiac hypertrophy and heart failure induced by AV shunt were studied by measuring the protein content of phosphorylated-Elk1, which is the downstream substrate of phosphorylated ERK1 and ERK2. Elk1 is the transcription factor located in the nuclei and can be phosphorylated by the phosphorylated-ERK1/2, which is translocated from the cytosol into the nuclei. Typical

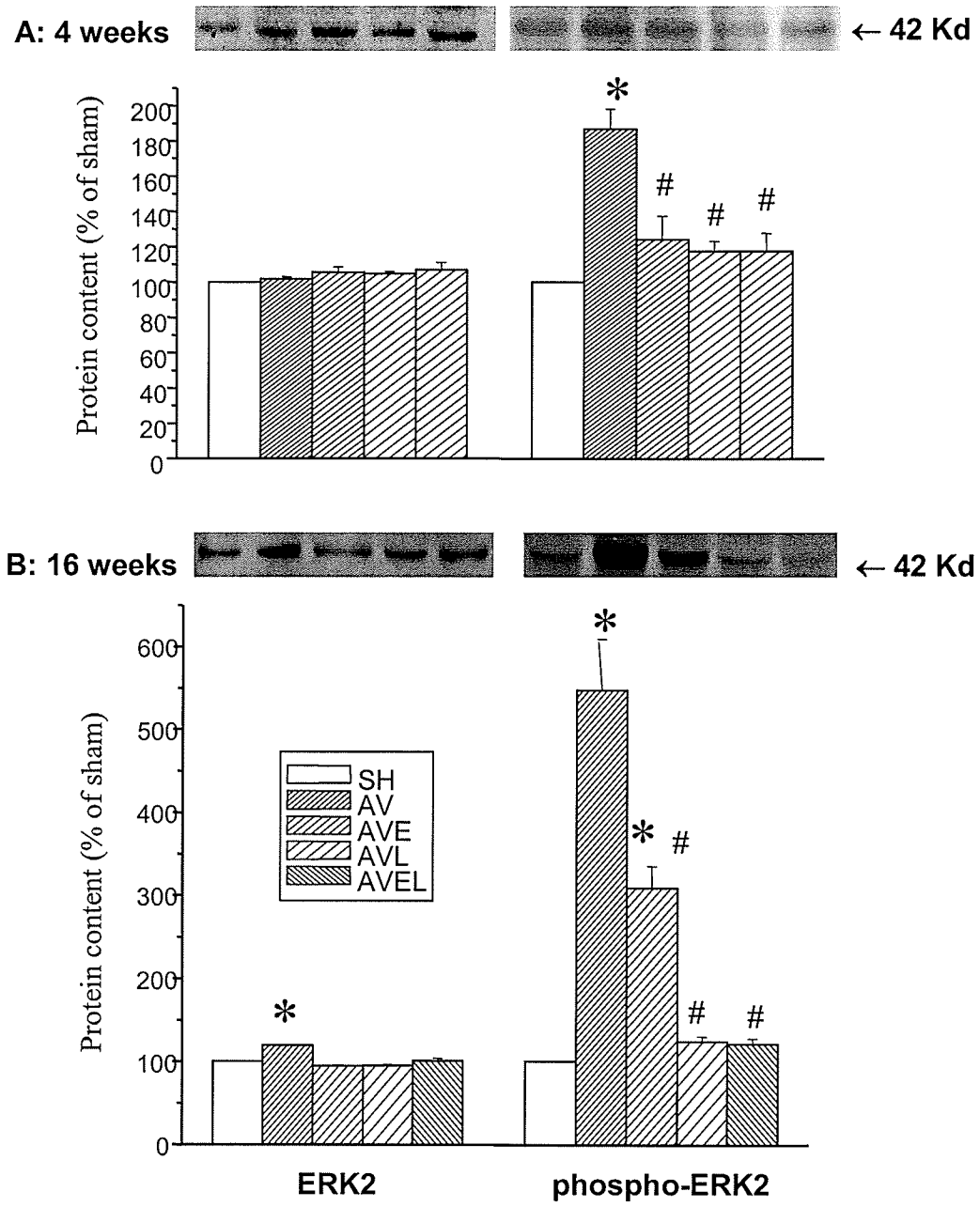

Figure 3:Western blotting analysis showing alteration in the protein content of ERK2 and phospho-ERK2 at 4 (panel A) and 16 weeks (panel B) of aortocaval shunt rats with or without treatment with enalapril and/or losartan . * $p<0.05$ vs SH group; #$p<0.05$ vs AV group.

Western blot band and analysis at 4 weeks after surgery are shown in Figure 4, panel A. The protein content of phosphorylated-Elk1 significantly increased in the AV shunt group as compared to the sham group (AV vs Sham, 335%, $p<0.05$). This increment was partially reduced by treatment with enalapril, losartan, or enalapril plus losartan (AVE, AVL, AVEL vs Sham, 188%, 201%, 153%, respectively; $p<0.05$, vs AV). At 16 weeks (Figure 4, panel B), the elevation in protein content of the phosphorylated-Elk1 was much greater than at 4 weeks. At this time point, as compared to the sham group, activities of phosphorylated-ERK1/2 increased 771% ($p<0.05$). This increment was partially decreased by enalapril and losartan or their combined treatment (AVE, AVL, AVEL vs SH, 192%, 112%, 114%, respectively; $p<0.05$, vs AV). AVEL treatment group showed more beneficial effect on reversing the phosphorylated-Elk1 as compared to AVE group but not AVL group.

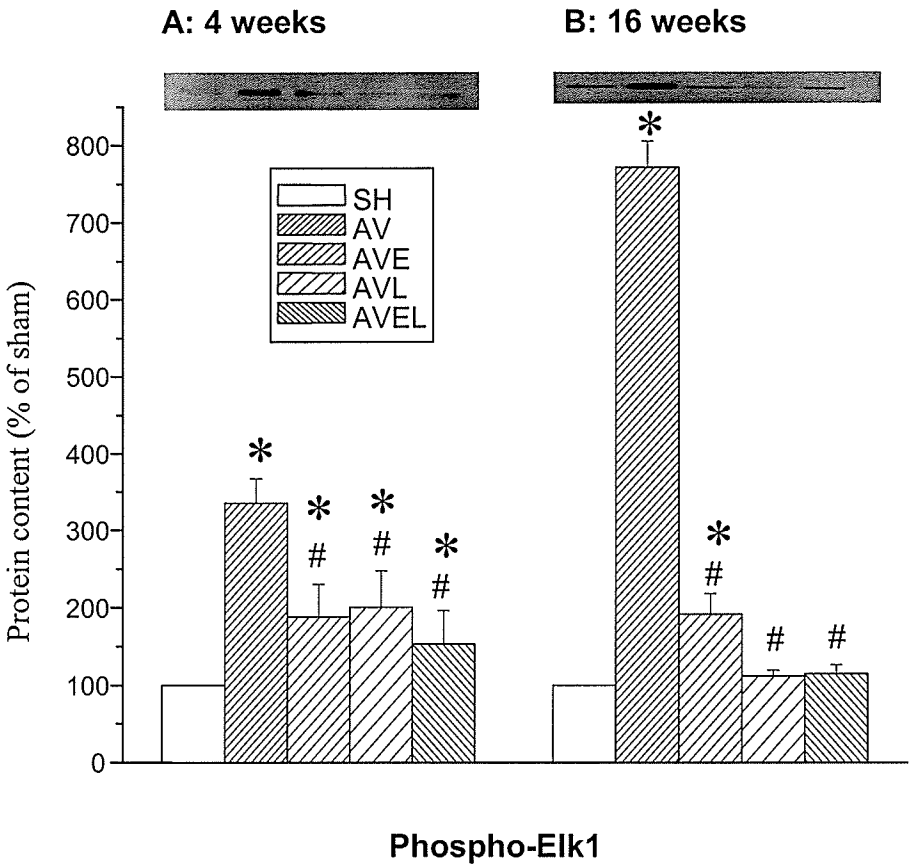

Figure 4: Western blotting analysis showing alteration in the total ERK activity at 4 (panel A) and 16 weeks (panel B) of aortocaval shunt rats with or without treatment with enalapril and/or losartan * $p<0.05$ vs SH group; #$p<0.05$ vs AV group.

DISCUSSION

1. Development of cardiac hypertrophy and heart failure in rat due to AV shunt.

In this study, we examined the cardiac mass, physical characteristics and symptoms of circulatory congestion in a volume overload model induced by the aortocaval shunt in rats at 4 and 16 weeks. It has been estabolished in our laboratory that rat at 4 weeks showed compensated cardiac hypertrophy and at 16 weeks showed heart failure (21). At 4 weeks, the HW, HW/BW ratio, LV wt and RV wt were increased in AV shunt groups when compared to the sham control group. These increments were attenuated when the animals were treated with enalapril, losartan or enalapril plus losartan. At this time point, the increase in lung wt in AV shunt group was most probably due to pulmonary edema evoked by increased blood volume in the right atrium as a result of the shunted vena cava. In fact, by using radioactive microsphere technique, many studies have shown dramatic increase in the cardiac output after inducing an arteriovenous fistula (180-182) and up to 75% of the cardiac output was shunted through the fistula (183). Although treatment with enalapril plus losartan showed more benefical effect than enalapril alone, this effect was not better than losartan treatment alone. Clinically, losartan may be a better option in patients who cannot tolerate the side effects of enalapril such as cough, first-dose hypotension, angioedema and renal dysfunction. At 16 weeks of AV shunt, there was an increase in the HW, HW/BW ratio, LV wt and RV wt as compard with the sham control group. Treatment of the AV shunt animals with enalapril and/or losartan prevented the increase in these parameters, showing the beneficial effects of using these drugs. These drugs also produce anti-remodeling (132), antisodium and water retention (16), and anti-apoptosis effects (120, 119), which will further assist in alleviating some of the syndromes of heart failure. Furthermore, the ability of these

drugs to prevent changes in heart function following the AV shunt shows their strong potential for use in treatment of impaired heart function in high-output heart failure.

Lung wet wt was elevated in both 4 and 16 weeks of AV shunt operated animals and this elevation was attenuated by treatment with enalapril and/or losartan. However, there was no additive effect when treated with these two drugs simultaneously. There was no significant change in the ratio of lung wet to dry wt. Apart from the vasodialative effect of these drugs, at 16 weeks time point, the anti-sodium and water retention effect (16) and their anti-fibrosis effect (119, 120) on lung tissue may contribute to the restoration of heart function. The increased lung wet wt was probably due to the accumulation of blood at the end stage of heart failure, which induces severe lung edema. Previous histological studies in our laboratory have shown that the chronic lung edema may progressively result in thickening of the pulmonary interstitial tissue with dilated capillaries and fine fibrosis (184, 185), therefore increasing the dry wt of the lungs. Several reports have indicated that Ang II participates in the proliferation of lung fibroblast, and that the ACE inhibitors or AT_1 blocker showed protective effect in preventing lung fibrosis in either human lung fibroblast (186) or in animal models (187, 188). There was no significant change in the lung wet/dry wt ratio. These data may not reflect negative results because the fluid-dependent (edema) change most likely contributes to the increment of lung wet wt, whereas the fluid-independent lung fibrosis change will contribute to both the dry and wet lung wt. The ability of Ang II blockers to reverse these changes was not only due to alleviation of the congested circulation by these drugs but might also be due to their anti-fibrosis effect on the lung. The suppresssed lung fibrosis will result in decreased dry lung wt. Therefore, similar decresses in dry and wet lung wt would result in no change in lung wet/dry wt ratio. The increased liver wet wt at 16 weeks is possibly due to congested circulation at the end stage heart failure. Also, a previous study in our laboratory has demonstrated histological changes in rat liver in AV shunt model at 16 weeks after surgery, which involves distension of the central

veins, and fibrosis of the vessel wall in the liver. These findings are constistent with those noted in the long-term congestion of the liver (184) and may explain the increase in liver wet to dry wt ratio. These results indicate the existence of congestive circulation due to heart failure at 16 weeks AV shunt. Ability of ACEI and AT_1 receptor blocker to prevent these effects on liver probably includes alleviation of congested circulation, but may also due to the inhibition of fibrosis in liver. Other investigators have reported similar results (189, 190). For example, it has been reported that losartan has beneficial effects on the splanchnic hemodynamics and liver fibrosis, and may reflect the decreased hepatic resistances in fibrotic liver (189). Some investigators have also indicated that angiotensin II may play an important role in the fibrogenic response to injury in the liver (188, 190, 191).

2. Hemodynamic changes at cardiac hypertrophic stage and heart failure stage

Our hemodynamic findings show a lack of changes in the ASP at 4 weeks in AV shunt animals as compared to the sham control. These findings are consistent with those reported by Huang and Flaim and represent the compensatory growth response to AV shunt as well as a significant flow of cardiac output through the AV shunt (181, 182). The dramatic decrease in the ADP observed in AV shunt group further confirms the volume overload induced by the shunted blood. There was an elevation in the LVEDP at 4 weeks after the AV shunt; Ang II blockade partially prevented the elevation in LVEDP. At 16 weeks AV shunt, the treatment with enalapril, losartan or enalapril plus losartan could not alleviate the drop in ASP. The diastolic dysfunction presented at severe stage of heart failure has been shown to be due to a drop in ADP observed in the AV shunt group with or without treatment with enalapril and/or losartan. Both the systolic and diastolic function at this stage of heart failure could not be maintained by the increased heart mass. These abnormalities therefore lead to circulatory congestion, and are accompanied by

biochemical alterations in the heart. The lack of changes in +d*P*/d*t* and –d*P*/d*t* at 4 weeks may represent the compensated cardiac hypertrophy, while the greatly depressed +d*P*/d*t* and –d*P*/d*t* at 16 weeks may represent the decompensated stage of the failing heart. The depressed +d*P*/d*t* and –d*P*/d*t* at 16 weeks was partially prevented by the treatment with enalapril and/or losartan. These findings are consistent with reports from several other laboratories indicating the anti-remodeling effects of and favourable alteration in the left ventricular loading condition of Ang II blockers in postinfarction models (128-130, 192, 193). The depressed LVSP and elevated LVEDP (122, 194, 195) in AV shunt groups at 4 and 16 weeks were partially attenuated by treatment with enalapril and/or losartan. This is consistent with several reports from other laboratories that ACEIs improve long-term prognosis in heart failure. For example, it has been reported that in addition to the venodilation effect (14), enalapril improved ventricular relaxation (15). Furthermore, some investigators found that ACEI, captopril or losartan, significantly improved hemodynamic conditions, diminished water retention and prevented cardiac hypertrophy in AV shunt model (16). Because enalapril has only minimal effects on the stroke volume, heart rate, and blood pressure in AV shunt rats (47), improvement in LV diastolic function by enalapril likely contributes to the attenuation of the increase in LVEDP (196).

3. ERK1/2 in cardiac hypertrophy and heart failure

MAP kinases are important mediators of the signal transduction pathways responsible for cell growth, proliferation, and apoptosis (2-4, 197, 198). Since phosphorylated-ERK1/2 were increased at 4 and 16 weeks and these changes were partially reversed by treatement with enalapril, losartan or both, indicating the activation of MAP kinase signaling pathways by the AV shunt induced volume-overload and its interaction with the renin-angiotensin system. It is noted that at 16 weeks, the total ERK1/2 were also elevated and this could be a marker for heart failure. Treatment with enalapril and/or losartan reversed these changes. Other investigators have reported that activation of ERK1 was greater than that of ERK2 after the Ang II infusion (55).

Results of the present study show similar activation of both ERK1 and ERK2. Because both effects were partially reversed by treatment with enalapril and/or losartan, we suggest that the renin-angiotensin system is involved in the regulation of cardiac hypertrophy and heart failure at least partially via the ERK1/2 signaling pathway in this model.

4. Alterations of ERK1/2 activity in cardiac hypertrophy and heart failure

Alteration in ERK1/2 activities during cardiac hypertrophy and heart failure induced by aortocaval shunt were further studied by measuring the protein content of phosphorylated-Elk1 which is the downstream substrate of phosphorylated ERK1/2. The results are consistent with the phosphorylation of ERK1 and ERK2 at 4 and 16 weeks post surgery. There was a significant elevation in the protein content of phosphorylated-Elk1 at 4 and 16 weeks of AV shunt. Further, the increase in phospho-Elk1 was much higher at 16 weeks as compared to 4 weeks which is consist with the increased production of ERK1/2 at 16 weeks. These data demonstrate the involvement of ERK signaling pathway in the development of cardiac hypertrophy and heart failure (80, 81). Treatment of AV shunt animals with enalapril, losartan, or enalapril plus losartan partially reversed the increment of phosphorylated-ERK1/2 activities. These results further suggested that the increased activation of MAP kinase signaling pathway in decompensated cardiac hypertrophy may occur via an interaction with the renin-angiotensin system in AV shunt induced volume-overload model.

5. Effects of enalapril and losartan on ERK1/2 signaling in cardiac hypertrophy and heart failure

As noted by many investigators, the RAS is considered to be critically involved in the development of cardiac hypertrophy and heart failure (133, 134, 199) Ang II appears to mediate the hypertrophic response of the heart by binding to AT_1 receptors and this interaction further

activates different downstream intracellular signaling transduction pathways. In addition, it is now recognized that activation of RAS is one of the important upstream signaling pathways that which are involved in the regulation of MAP kinase signaling transduction pathways. Infusion of angiotensin II produced differential activation of ERKs between the left and right ventricles, and thus providing further evidence that ERKs play an important role in the cardiac function through RAS cascade (55). In a study using hypertrophic neonatal rat cardiac myocytes, Aoki and colleagues (62) noted that Ang II activated ERKs, while PD98059, a specific inhibitor of MAPK/ERK kinase (MEK) inhibited Ang II-induced expression of atrial natriuretic factor (ANF) at both the mRNA and polypeptide levels, thus comfirming that activation of ERK was possibility mediated by Ang II. These studies show that ERK activation and ANF up-regulation were regulated by Ang II. Yamazaki (173) also reported that CV-11974, an antagonist of AT_1 receptor, completely blocked activation of MAP kinases in mechanical stretched neonatal rat cardiomyocytes cultured in the stretch-conditioned medium. These data show that Ang II was involved in the activation of Raf-1-MKK-MAPK signaling pathway. In another study carried out on the neonatal rat cardiac myocytes, Zou (66) reported that PKC and Raf-1, but not tyrosine kinases or Ras, are critical for Ang II-stimulated ERKs activities. Although a number of different models have been used to study cardiac hypertrophy and heart failure, it should be noted that different experimental animal models of heart failure without fibrosis provide a powerful tool to examine changes in the activation of MAP kinases in relation to the RAS. The AV shunt in rats has been well established as the high-output volume overload model without fibrosis in our laboratory. We have now used this model to determine whether volume overload-induced cardiac hypertrophy and heart failure is mediated through the intracellular MAPK pathway, and treatment of the experimental animals with ACE inhibitor and/or AT_1 antagonist results in the attenuation of the MAPK activation. Results of the present study show that enalapril and losartan appear to interact with the MAP kinase signaling pathways by partially preventing changes in

ERK1/2 protein content and activities. Thus, it seems that ERK1/2 are the direct or indirect targets for these drugs. However, simultaneous treatment of AV shunt animals with enalapril and losartan did not produce additive effect on any of the parameters examined in the present study. In views of these findings, it is concluded that treatment with enalapril or losartan individually produced beneficial effects, but the combined therapy did not produce additional beneficial effects.

It is clear that treatment with either ACEI or AT_1 antagonist could not completely block activation of the ERK signaling pathway. The combined treatment did not produce additive effect in preventing the activation of ERK1 and ERK2 by Ang II. Therefore, we consider that there might be some other mediators participating in the activation of this signaling cascade. First, RAS is only one of the systems involved in the development of cardiac hypertrophy and heart failure. Other systems like adrenergic system is also reported to be involved(184). Second, it is also possible losartan cannot completely block the Ang II binding to its receptor. Third, although Ang II is a very important mediator that stimulates the hypertrophic response through activation of ERK signaling pathway, other signalling pathways such as cardiac stretch (185) must also be involved in the whole process of cardiac hypertrophy and heart failure. Apart from Ang II, other mitogenic agents are known to participate in regulating the activation of ERK signaling cascade, indicates that blockade of the Ang II cannot completely prevent activation of the ERK signaling pathway. Furthermore, Ang II can be generated from many other pathways (27, 153); for example, Ang II can be formed from Ang I via chymase, a chymotrypsin-like proteinase, which is not affected by ACE inhibition (27). In addition, Ang II can be directly synthesized from angiotensinogen by the proteolytic actions of kallikrein and catheosin G (27, 153). These information may explain why ACEI and AT_1 receptor antagonist could not completely block activation of the ERK pathways. Nonetheless, the results of this study provide

evidence regarding the role of renin-angiotensin system in cardiac hypertrophy induced by the volume overload and its interaction with MAP kinase signal transduction pathway.

SUMMARY AND CONCLUSIONS

In order to understand the significance of MAPK in cardiac hypertrophy and heart failure and to examine their relationship to the renin-angiotensin system, rats with AV shunt were treated with enalapril and/or losartan and protein content and activity of ERK1 and ERK2 were studied. Volume–overload was induced by the AV shunt in rat, the sham-operated animals served as control. The LV dysfunction, as reflected by the decreased LVDP, $+dP/dt$, $-dP/dt$, and elevated LVEDP in the volume-overload rats, was improved by treatment with enalapril, losartan or their combination. Cardiac hypertrophy at 4 weeks and heart failure at 16 weeks in AV shunted rats were attenuated by treatment with enalapril and/or losartan. Protein content of ERK1 and ERK2 as well as the activity of ERKs as measured by phospho-Elk1 were increased in hypertrophied and failing heart. These alterations were partially prevented by the treatment with enalapril and losartan, but the combination of enalapril and losartan did not show any additive beneficial effect. Our findings indicate that the volume overload-induced myocardial hypertrophy and heart failure may be mediated by the angiotensin receptor activated MAPK signal transduction pathways.

Based on these results, the following conclusions can be drawn:

1. Compensated cardiac hypertrophy and decompensated heart failure become evident at 4 weeks and 16 weeks follwong the AV shunt, respectively.
2. Blockade of the RAS by using enalapril and/or losartan was found to partially prevent alterations in general characteristics and hemodynamic parameters in cardiac hypertrophy and heart failure induced by volume overload.
3. Both ERK1 and ERK2 may be involved in the development of cardiac hypertrophy and heart failure.

4. Blockade of RAS may attenuate the development of cardiac hypertrophy and heart failure partially through ERK1 and ERK2 signaling transduction pathway.
5. The combined treatment with enalapril and losartan did not show additive beneficial effect in cardiac hypertrophy and heart failure induced by AV shunt.

REFERENCES

1. Schluter, K. D. and H. M. Piper. Regulation of growth in the adult cardiomyocytes. *FASEB J.* 13 Suppl. : S17-S22, 1999.
2. Ma, X. L., S. Kumar, F. Gao, C. S. Louden, B. L. Lopez, T. A. Christopher, C. Wang, J. C. Lee, G. Z. Feuerstein, and T. L. Yue. Inhibition of p38 mitogen-activated protein kinase decreases cardiomyocyte apoptosis and improves cardiac function after myocardial ischemia and reperfusion. *Circulation* 99: 1685-1691, 1999.
3. Gottlieb, R. A., K. O. Burleson, R. A. Kloner, B. M. Babior, and R. L. Engler. Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J.Clin.Invest.* 94: 1621-1628, 1994.
4. Roulston, A., C. Reinhard, P. Amiri, and L. T. Williams. Early activation of c-Jun N-terminal kinase and p38 kinase regulate cell survival in response to tumor necrosis factor alpha. *J.Biol.Chem.* 273: 10232-10239, 1998.
5. Bogoyevitch, M. A., M. B. Andersson, J. Gillespie-Brown, A. Clerk, P. E. Glennon, S. J. Fuller, and P. H. Sugden. Adrenergic receptor stimulation of the mitogen-activated protein kinase cascade and cardiac hypertrophy. *Biochem.J.* 314: 115-121, 1996.
6. Hines, W. A. and A. Thorburn. Ras and rho are required for galphaq-induced hypertrophic gene expression in neonatal rat cardiac myocytes. *J.Mol.Cell. Cardiol.* 30: 485-494, 1998.
7. Gerdes, A. M. Remodeling of ventricular myocytes during cardiac hypertrophy and heart failure. *J. Fla.Med. Assoc.* 79: 253-255, 1992.
8. Katz A.M. The Hypertrophic Response: Programmed Cell Death. Heart Failure Pathophysiology, Molecular Biology, and Clinical Management. Philadelphia.Baltimore.

New York. London. Buenos Aires. Hong Kong.Sydney. Tokyo, LIPPNCOTT WILLIAMS & WILKINS. 2000, 173-226.

9. Lowe T.E. and Bate E.W. The diameter of cardiac muscle fibres: a study of the diameter of muscle fibres in the left ventricle in normal hearts and in the left ventricular enlargement of simple hypertension. *Med. J. Austi*: 467-469, 1948.
10. Linzbach A.J. Heart failure from the point of view of quantitative anatomy. *Am. J.Cardiol.* 5: 370-382, 1960.
11. Sugden, P. H. Signalling pathways in cardiac myocyte hypertrophy. *Ann.Med.* 33: 611-622, 2001.
12. Gerdes, A. M. and J. M. Capasso. Structural remodeling and mechanical dysfunction of cardiac myocytes in heart failure. *J. Mol.Cell. Cardiol.* 27: 849-856, 1995.
13. Beltrami, C. A., N. Finato, M. Rocco, G. A. Feruglio, C. Puricelli, E. Cigola, F. Quaini, E. H. Sonnenblick, G. Olivetti, and P. Anversa. Structural basis of end-stage failure in ischemic cardiomyopathy in humans. *Circulation* 89: 151-163, 1994.
14. Raya, T. E., S. J. Fonken, R. W. Lee, S. Daugherty, S. Goldman, P. C. Wong, P. B. Timmermans, and E. Morkin. Hemodynamic effects of direct angiotensin II blockade compared to converting enzyme inhibition in rat model of heart failure. *Am. J. Hypertens.* 4: 334S-340S, 1991.
15. Schunkert, H., V. J. Dzau, S. S. Tang, A. T. Hirsch, C. S. Apstein, and B. H. Lorell. Increased rat cardiac angiotensin converting enzyme activity and mRNA expression in pressure overload left ventricular hypertrophy. Effects on coronary resistance, contractility, and relaxation. *J. Clin. Invest.* 86: 1913-1920, 1990.
16. Qing, G. and R. Garcia. Chronic captopril and losartan (DuP 753) administration in rats with high-output heart failure. *Am. J. Physiol.* 263: H833-H840, 1992.

17. Tokioka-Akagi, T., A. Fujimori, M. Shibasaki, O. Inagaki, and I. Yanagisawa. Comparison of the angiotensin II type 1-receptor antagonist YM358 and the angiotensin-converting enzyme inhibitor enalapril in rats with cardiac volume overload. *Jpn.J. Pharmacol.* 86: 79-85, 2001.

18. Gealekman, O., Z. Abassi, I. Rubinstein, J. Winaver, and O. Binah. Role of myocardial inducible nitric oxide synthase in contractile dysfunction and beta-adrenergic hyporesponsiveness in rats with experimental volume-overload heart failure. *Circulation* 105: 236-243, 2002.

19. Liu, H., A. Ma, C. Wang, Y. Liu, H. Tian, and L. Bai. Variation and significance of microtubules in rat volume overload cardiac hypertrophy. *Chin. Med. J. (Engl.)* 116: 337-340, 2003.

20. Petretta, M., L. Spinelli, F. Marciano, C. Apicella, M. L. Vicario, G. Testa, M. Volpe, and D. Bonaduce. Effects of losartan treatment on cardiac autonomic control during volume loading in patients with DCM. *Am. J. Physiol. Heart Circ.Physiol.* 279: H86-H92, 2000.

21. Wang, X. β-Adrenoceptor Signal Transduction in High-Output Heart Failure Due to Aortocaval Shunt in Rat. 2000. Department of Physiology and Institute of Cardiovascular Sciences St. Boniface General Hospital Research Centre Faculty of Medicine. Ref Type: Thesis. University of Manitoba.

22. Katz A.M. Maladaptive Hypertrophy and the Caridomyopathy of Overload: Familial Cardiomyopathies. Heart Failure Pathophysiology, Molecular Biology, and Clinical Management. Philadelphia.Baltimore. New York. London. Buenos Aires. Hong Kong.Sydney. Tokyo, LIPPNCOTT WILLIAMS & WILKINS. 2000, 277-307.

23. Sealey, J. H. and J. H. Laragh. The renin-angiotensin-aklosterone system for normal regulation of blood pressure and sodium and potassium homeostasis. In Laragh, J. H. and

B. M. Brenner, eds. Hypertension: Pathophysiology, Diagnosis and Management. New York, Raven Press. 1990, 1287.

24. Weir, M. R. and V. J. Dzau. The renin-angiotensin-aldosterone system: a specific target for hypertension management. *Am. J. Hypertens.* 12: 205S-213S, 1999.

25. Braun-Menendez E, Fasciolo E, Leloir JC, and Munoz JM. The substance causing renal hypertension. *J.Physiol. (Lond)* 98: 283-298, 1940.

26. Dzau, V. J., N. K. Hollenberg, and G. H. Williams. Neurohumoral mechanisms in heart failure: role in pathogenesis, therapy, and drug tolerance. *Fed.Proc.* 42: 3162-3169, 1983.

27. Urata, H., B. Healy, R. W. Stewart, F. M. Bumpus, and A. Husain. Angiotensin II-forming pathways in normal and failing human hearts. *Circ.Res.* 66: 883-890, 1990.

28. Pfeffer, J. M., T. A. Fischer, and M. A. Pfeffer. Angiotensin-converting enzyme inhibition and ventricular remodeling after myocardial infarction. *Annu.Rev.Physiol.* 57.: 805-826, 1995.

29. Antus, B., I. Mucsi, and L. Rosivall. Apoptosis induction and inhibition of cellular proliferation by angiotensin II: possible implication and perspectives. *Acta.Physiol. Hung.* 87: 5-24, 2000.

30. Timmermans, P. B., P. C. Wong, A. T. Chiu, W. F. Herblin, P. Benfield, D. J. Carini, R. J. Lee, R. R. Wexler, J. A. Saye, and R. D. Smith. Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol.Rev.* 45: 205-251, 1993.

31. Kobayashi, M., Y. Furukawa, and S. Chiba. Positive chronotropic and inotropic effects of angiotensin II in the dog heart. *Eur.J. Pharmacol.* 50: 17-25, 1978.

32. Ikenouchi, H., W. H. Barry, J. H. Bridge, E. O. Weinberg, C. S. Apstein, and B. H. Lorell. Effects of angiotensin II on intracellular Ca2+ and pH in isolated beating rabbit hearts and myocytes loaded with the indicator indo-1. *J. Physiol* 480: 203-215, 1994.

33. Sadoshima, J. Cytokine actions of angiotensin II. *Circ.Res.* 86: 1187-1189, 2000.

34. Timmermans, P. B. and R. D. Smith. Angiotensin II receptor subtypes: selective antogonists and functional correlates. *Eur.Heart J.* 15: 79-87, 1994.

35. Zemel, S., M. A. Millan, P. Feuillan, and G. Aguilera. Characterization and distribution of angiotensin-II receptors in the primate fetus. *J. Clin.Endocrinol.Metab.* 71: 1003-1007, 1990.

36. Stoll M, Steckelings U.M., Paul M, Bottari S.P., Metzger R, and Unger T. The angiotensin AT2-receptor mediates inhibition of cell proliferation in coronary endothelial cells. *J. Clin. Invest.* 95: 651-657, 1995.

37. Ichiki, T., P. A. Labosky, C. Shiota, S. Okuyama, Y. Imagawa, A. Fogo, F. Niimura, I. Ichikawa, B. L. Hogan, and T. Inagami. Effects on blood pressure and exploratory behaviour of mice lacking angiotensin II type-2 receptor. *Nature* 377: 748-750, 1995.

38. Hein, L., G. S. Barsh, R. E. Pratt, V. J. Dzau, and B. K. Kobilka. Behavioural and cardiovascular effects of disrupting the angiotensin II type-2 receptor in mice. *Nature* 377: 744-747, 1995.

39. Dzau, V. J. Evolving concepts of the renin-angiotensin system. Focus on renal and vascular mechanisms. *Am. J. Hypertens.* 1: 334S-337S, 1988.

40. Gibbons, G. H. Autocrine-paracrine factors and vascular remodeling in hypertension. *Curr.Opin.Nephrol.Hypertens.* 2: 291-298, 1993.

41. Griendling, K. K., T. Tsuda, B. C. Berk, and R. W. Alexander. Angiotensin II stimulation of vascular smooth muscle. *J. Cardiovasc. Pharmacol.* 14 Suppl.6 : S27-S33, 1989.

42. Katz A.M. The Hemodynamic Defense Reaction. Heart Failure Pathophysiology, Molecular Biology, and Clinical Management. Philadelphia.Baltimore. New York. London. Buenos Aires. Hong Kong.Sydney. Tokyo, LIPPNCOTT WILLIAMS & WILKINS. 2000, 109-152.

43. Boer, P. H., M. Ruzicka, W. Lear, E. Harmsen, J. Rosenthal, and F. H. Leenen. Stretch-mediated activation of cardiac renin gene. *Am. J. Physiol.* 267: H1630-H1636, 1994.

44. Pieruzzi, F., Z. A. Abassi, and H. R. Keiser. Expression of renin-angiotensin system components in the heart, kidneys, and lungs of rats with experimental heart failure. *Circulation* 92: 3105-3112, 1995.

45. Iwai, N., H. Shimoike, and M. Kinoshita. Cardiac renin-angiotensin system in the hypertrophied heart. *Circulation* 92: 2690-2696, 1995.

46. Iijima, K., E. Geshi, A. Nomizo, Y. Arata, and T. Katagiri. Alterations in sarcoplasmic reticulum and angiotensin II type 1 receptor gene expression after myocardial infarction in rats. *Jpn.Circ.J.* 62: 449-454, 1998.

47. Ruzicka, M., B. Yuan, E. Harmsen, and F. H. Leenen. The renin-angiotensin system and volume overload-induced cardiac hypertrophy in rats. Effects of angiotensin converting enzyme inhibitor versus angiotensin II receptor blocker. *Circulation* 87: 921-930, 1993.

48. Sadoshima, J., Y. Xu, H. S. Slayter, and S. Izumo. Autocrine release of angiotensin II mediates stretch-induced hypertrophy of cardiac myocytes in vitro. *Cell* 75: 977-984, 1993.

49. Pan, J., K. Fukuda, H. Kodama, S. Makino, T. Takahashi, M. Sano, S. Hori, and S. Ogawa. Role of angiotensin II in activation of the JAK/STAT pathway induced by acute pressure overload in the rat heart. *Circ.Res.* 81: 611-617, 1997.

50. Marrero, M. B., B. Schieffer, W. G. Paxton, L. Heerdt, B. C. Berk, P. Delafontaine, and K. E. Bernstein. Direct stumulation of Jak/STAT by the angiotensin II AT1 receptor. *Nature* 375: 247-249, 1997.

51. Yamazaki, T., I. Komuro, S. Kudoh, Y. Zou, I. Shiojima, T. Mizuno, H. Takano, Y. Hiroi, K. Ueki, K. Tobe, and . Angiotensin II partly mediates mechanical stress-induced cardiac hypertrophy. *Circ.Res.* 77: 258-265, 1995.

52. Linz, W., B. A. Scholkens, and D. Ganten. Converting enzyme inhibition specifically prevents the development and induces regression of cardiac hypertrophy in rats. *Clin. Exp. Hypertens.A.* 11: 1325-1350, 1989.

53. Crabos M and Erne P. Angiotensin II receptors in cultured rat cardiac fibroblasts: coupling to signalling systems and gene expression. *Circulation* 6-6, 1992.

54. Fiordaliso, F., B. Li, R. Latini, E. H. Sonnenblick, P. Anversa, A. Leri, and J. Kajstura. Myocyte death in streptozotocin-induced diabetes in rats in angiotensin II- dependent. *Lab. Invest.* 80: 513-527, 2000.

55. Yano, M., S. Kim, Y. Izumi, S. Yamanaka, and H. Iwao. Differential activation of cardiac c-jun amino-terminal kinase and extracellular signal-regulated kinase in angiotensin II-mediated hypertension. *Circ.Res.* 83: 752-760, 1998.

56. Malarkey, K., C. M. Belham, A. Paul, A. Graham, A. McLees, P. H. Scott, and R. Plevin. The regulation of tyrosine kinase signalling pathways by growth factor and G-protein-coupled receptors. *Biochem.J.* 309: 361-375, 1995.

57. Huang, w. and R. L. Erikson. MAP kinases in multiple signaling pathways. In Heldin, C. H. and M. Purton, eds. Signal Transduction. London, CHAPMAN & HALL, 159-172, 1996.

58. Chesley, A., M. S. Lundberg, T. Asai, R. P. Xiao, S. Ohtani, E. G. Lakatta, and M. T. Crow. The beta(2)-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G(i)-dependent coupling to phosphatidylinositol 3'-kinase. *Circ.Res.* 87: 1172-1179, 2000.

59. Yamauchi-Takihara, K., H. Hirota, K. Kunisada, H. Matsui, Y. Fujio, T. Taga, and T. Kishimoto. Roles of gp130 signaling pathways in cardiac myocytes: recent advances and implications for cardiovascular disease. *J.Card. Fail.* 2: S63-S68, 1996.

60. Tsutsumi, Y., H. Matsubara, N. Ohkubo, Y. Mori, Y. Nozawa, S. Murasawa, K. Kijima, K. Maruyama, H. Masaki, Y. Moriguchi, Y. Shibasaki, H. Kamihata, M. Inada, and T. Iwasaka. Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. *Circ.Res.* 83: 1035-1046, 1998.

61. Pellieux, C., T. Sauthier, J. F. Aubert, H. R. Brunner, and T. Pedrazzini. Angiotensin II-induced cardiac hypertrophy is associated with different mitogen-activated protein kinase activation in normotensive and hypertensive mice. *J.Hypertens.* 18: 1307-1317, 2000.

62. Aoki, H., M. Richmond, S. Izumo, and J. Sadoshima. Specific role of the extracellular signal-regulated kinase pathway in angiotensin II-induced cardiac hypertrophy in vitro. *Biochem.J.* 347 Pt 1: 275-284, 2000.

63. Aikawa, R., I. Komuro, T. Yamazaki, Y. Zou, S. Kudoh, W. Zhu, T. Kadowaki, and Y. Yazaki. Rho family small G proteins play critical roles in mechanical stress-induced hypertrophic responses in cardiac myocytes. *Circ.Res.* 84: 458-466, 1999.

64. Clerk, A. and P. H. Sugden. Activation of protein kinase cascades in the heart by hypertrophic G protein-coupled receptor agonists. *Am J.Cardiol.* 83: 64H-69H, 1999.

65. Sadoshima, J., Z. Qiu, J. P. Morgan, and S. Izumo. Angiotensin II and other hypertrophic stimuli mediated by G protein-coupled receptors activate tyrosine kinase, mitogen-activated protein kinase, and 90-kD S6 kinase in cardiac myocytes. The critical role of Ca(2+)-dependent signaling. *Circ.Res.* 76: 1-15, 1995.

66. Zou, Y., I. Komuro, T. Yamazaki, R. Aikawa, S. Kudoh, I. Shiojima, Y. Hiroi, T. Mizuno, and Y. Yazaki. Protein kinase C, but not tyrosine kinases or Ras, plays a critical role in angiotensin II-induced activation of Raf-1 kinase and extracellular signal-regulated protein kinases in cardiac myocytes. *J.Biol.Chem.* 271: 33592-33597, 1996.

67. Cano, E., C. A. Hazzalin, and L. C. Mahadevan. Anisomycin-activated protein kinases p45 and p55 but not mitogen-activated protein kinases ERK-1 and -2 are implicated in the induction of c-fos and c-jun. *Mol.Cell Biol.* 14: 7352-7362, 1994.

68. Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.* 7: 2135-2148, 1993.

69. Gille, H., A. D. Sharrocks, and P. E. Shaw. Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. *Nature* 358: 414-417, 1992.

70. Sturgill, T. W. and J. Wu. Recent progress in characterization of protein kinase cascades for phosphorylation of ribosomal protein S6. *Biochim.Biophys.Acta* 1092: 350-357, 1991.

71. Taylor, S. J., H. Z. Chae, S. G. Rhee, and J. H. Exton. Activation of the beta 1 isozyme of phospholipase C by alpha subunits of the Gq class of G proteins. *Nature* 350: 516-518, 1991.

72. Crespo, P., N. Xu, W. F. Simonds, and J. S. Gutkind. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. *Nature* 369: 418-420, 1994.

73. Pelech, S. L. and D. L. Charest. MAP kinase-dependent pathways in cell cycle control. *Prog.Cell Cycle. Res.* 1:33-52.: 33-52, 1995.

74. Steinberg, S. F., M. Goldberg, and V. O. Rybin. Protein kinase C isoform diversity in the heart. *J. Mol.Cell. Cardiol.* 27: 141-153, 1995.

75. Farnsworth, C. L., N. W. Freshney, L. B. Rosen, A. Ghosh, M. E. Greenberg, and L. A. Feig. Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. *Nature* 376: 524-527, 1995.

76. Cook, S. J. and F. McCormick. Inhibition by cAMP of Ras-dependent activation of Raf. *Science* 262: 1069-1072, 1993.

77. Liu, M. and M. I. Simon. Regulation by cAMP-dependent protein kinase of a G-protein-mediated phospholipase C. *Nature* 382: 83-87, 1996.

78. Sevetson, B. R., X. Kong, and J. C. Lawrence, Jr. Increasing cAMP attenuates activation of mitogen-activated protein kinase. *Proc.Natl.Acad.Sci.U.S.A* 90: 10305-10309, 1993.

79. Gonzalez, F. A., A. Seth, D. L. Raden, D. S. Bowman, F. S. Fay, and R. J. Davis. Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. *J.Cell. Biol.* 122: 1089-1101, 1993.

80. Gille, H., M. Kortenjann, O. Thomae, C. Moomaw, C. Slaughter, M. H. Cobb, and P. E. Shaw. ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. *EMBO J.* 14: 951-962, 1995.

81. Marais, R., J. Wynne, and R. Treisman. The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* 73: 381-393, 1993.

82. Laderoute, K. R. and K. A. Webster. Hypoxia/reoxygenation stimulates Jun kinase activity through redox signaling in cardiac myocytes. *Circ.Res.* 80: 336-344, 1997.

83. Whitmarsh, A. J., P. Shore, A. D. Sharrocks, and R. J. Davis. Integration of MAP kinase signal transduction pathways at the serum response element. *Science* 269: 403-407, 1995.

84. Livingstone, C., G. Patel, and N. Jones. ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO J.* 14: 1785-1797, 1995.

85. van Dam, H., D. Wilhelm, I. Herr, A. Steffen, P. Herrlich, and P. Angel. ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *EMBO J.* 14: 1798-1811, 1995.

86. Cook, S. A., P. H. Sugden, and A. Clerk. Activation of c-Jun N-terminal kinases and p38-mitogen-activated protein kinases in human heart failure secondary to ischaemic heart disease. *J.Mol.Cell. Cardiol.* 31: 1429-1434, 1999.

87. Takeishi, Y., A. Bhagwat, N. A. Ball, D. L. Kirkpatrick, M. Periasamy, and R. A. Walsh. Effect of angiotensin-converting enzyme inhibition on protein kinase C and SR proteins in heart failure. *Am.J.Physiol.* 276: H53-H62, 1999.

88. Hefti, M. A., B. A. Harder, H. M. Eppenberger, and M. C. Schaub. Signaling pathways in cardiac myocyte hypertrophy. *J.Mol.Cell. Cardiol.* 29: 2873-2892, 1997.

89. Bogoyevitch, M. A., P. E. Glennon, M. B. Andersson, A. Clerk, A. Lazou, C. J. Marshall, P. J. Parker, and P. H. Sugden. Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J.Biol.Chem.* 269: 1110-1119, 1994.

90. Bogoyevitch, M. A. and P. H. Sugden. The role of protein kinases in adaptational growth of the heart. *Int.J.Biochem.Cell. Biol.* 28: 1-12, 1996.

91. Yoshida, K., M. Yoshiyama, T. Omura, Y. Nakamura, S. Kim, K. Takeuchi, H. Iwao, and J. Yoshikawa. Activation of mitogen-activated protein kinases in the non-ischemic myocardium of an acute myocardial infarction in rats. *Jpn.Circ.J.* 65: 808-814, 2001.

92. Bogoyevitch, M. A., J. Gillespie-Brown, A. J. Ketterman, S. J. Fuller, R. Ben Levy, A. Ashworth, C. J. Marshall, and P. H. Sugden. Stimulation of the stress-activated mitogen-activated protein kinase subfamilies in perfused heart. p38/RK mitogen-activated protein kinases and c-Jun N-terminal kinases are activated by ischemia/reperfusion. *Circ.Res.* 79: 162-173, 1996.

93. Kyriakis, J. M., D. L. Brautigan, T. S. Ingebritsen, and J. Avruch. pp54 microtubule-associated protein-2 kinase requires both tyrosine and serine/threonine phosphorylation for activity. *J.Biol.Chem.* 266: 10043-10046, 1991.

94. Davis, R. J. Transcriptional regulation by MAP kinases. *Mol.Reprod.Dev.* 42: 459-467, 1995.

95. Gupta, S., D. Campbell, B. Derijard, and R. J. Davis. Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 20;267: 389-393, 1995.

96. Bird, T. A., J. M. Kyriakis, L. Tyshler, M. Gayle, A. Milne, and G. D. Virca. Interleukin-1 activates p54 mitogen-activated protein (MAP) kinase/stress-activated protein kinase by a pathway that is independent of p21ras, Raf-1, and MAP kinase kinase. *J.Biol.Chem.* 269: 31836-31844, 1994.

97. Kyriakis, J. M., P. Banerjee, E. Nikolakaki, T. Dai, E. A. Rubie, M. F. Ahmad, J. Avruch, and J. R. Woodgett. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369: 156-160, 1994.

98. Sluss, H. K., T. Barrett, B. Derijard, and R. J. Davis. Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol.Cell. Biol.* 14: 8376-8384, 1994.

99. Sprenkle, A. B., S. F. Murray, and C. C. Glembotski. Involvement of multiple cis elements in basal- and alpha-adrenergic agonist-inducible atrial natriuretic factor transcription. Roles for serum response elements and an SP-1-like element. *Circ.Res.* 77: 1060-1069, 1995.

100. Yue, T. L., C. Wang, J. L. Gu, X. L. Ma, S. Kumar, J. C. Lee, G. Z. Feuerstein, H. Thomas, B. Maleeff, and E. H. Ohlstein. Inhibition of extracellular signal-regulated kinase enhances Ischemia/Reoxygenation-induced apoptosis in cultured cardiac myocytes and exaggerates reperfusion injury in isolated perfused heart. *Circ.Res.* 86: 692-699, 2000.

101. Xia, Z., M. Dickens, J. Raingeaud, R. J. Davis, and M. E. Greenberg. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* 270: 1326-1331, 1995.

102. Tournier, C., P. Hess, D. D. Yang, J. Xu, T. K. Turner, A. Nimnual, D. Bar-Sagi, S. N. Jones, R. A. Flavell, and R. J. Davis. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 288: 870-874, 2000.

103. Andreka, P., J. Zang, C. Dougherty, T. I. Slepak, K. A. Webster, and N. H. Bishopric. Cytoprotection by Jun kinase during nitric oxide-induced cardiac myocyte apoptosis. *Circ.Res.* 88: 305-312, 2001.

104. Kunapuli, P., J. A. Lawson, J. A. Rokach, J. L. Meinkoth, and G. A. FitzGerald. Prostaglandin F2alpha (PGF2alpha) and the isoprostane, 8, 12-iso-isoprostane F2alpha-III, induce cardiomyocyte hypertrophy. Differential activation of downstream signaling pathways. *J.Biol.Chem.* 273: 22442-22452, 1998.

105. Adams, J. W., Y. Sakata, M. G. Davis, V. P. Sah, Y. Wang, S. B. Liggett, K. R. Chien, J. H. Brown, and G. W. Dorn. Enhanced Galphaq signaling: a common pathway mediates cardiac hypertrophy and apoptotic heart failure. *Proc.Natl.Acad.Sci.U.S.A* 95: 10140-10145, 1998.

106. Minamino, T., T. Yujiri, P. J. Papst, E. D. Chan, G. L. Johnson, and N. Terada. MEKK1 suppresses oxidative stress-induced apoptosis of embryonic stem cell-derived cardiac myocytes. *Proc.Natl.Acad.Sci.U.S.A* 96: 15127-15132, 1999.

107. Han, J., J. D. Lee, L. Bibbs, and R. J. Ulevitch. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265: 808-811, 1994.

108. Rouse, J., P. Cohen, S. Trigon, M. Morange, A. Alonso-Llamazares, D. Zamanillo, T. Hunt, and A. R. Nebreda. A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* 78: 1027-1037, 1994.

109. Mackay, K. and D. Mochly-Rosen. Involvement of a p38 mitogen-activated protein kinase phosphatase in protecting neonatal rat cardiac myocytes from ischemia. *J.Mol.Cell. Cardiol.* 32: 1585-1588, 2000.

110. Zhu, W., Y. Zou, R. Aikawa, K. Harada, S. Kudoh, H. Uozumi, D. Hayashi, Y. Gu, T. Yamazaki, R. Nagai, Y. Yazaki, and I. Komuro. MAPK superfamily plays an important

role in daunomycin-induced apoptosis of cardiac myocytes. *Circulation* 100: 2100-2107, 1999.

111. Kang, Y. J., Z. X. Zhou, G. W. Wang, A. Buridi, and J. B. Klein. Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. *J.Biol.Chem.* 275: 13690-13698, 2000.

112. Craig, R., A. Larkin, A. M. Mingo, D. J. Thuerauf, C. Andrews, P. M. McDonough, and C. C. Glembotski. p38 MAPK and NF-kappa B collaborate to induce interleukin-6 gene expression and release. Evidence for a cytoprotective autocrine signaling pathway in a cardiac myocyte model system. *J.Biol.Chem.* 275: 23814-23824, 2000.

113. Hoover, H. E., D. J. Thuerauf, J. J. Martindale, and C. C. Glembotski. alpha B-crystallin gene induction and phosphorylation by MKK6-activated p38. A potential role for alpha B-crystallin as a target of the p38 branch of the cardiac stress response. *J.Biol.Chem.* 275: 23825-23833, 2000.

114. Katz A.M. Signal Transduction Within Cells of the Failing Heart. Heart Failure Pathophysiology, Molecular Biology, and Clinical Management. Philadelphia.Baltimore. New York. London. Buenos Aires. Hong Kong.Sydney. Tokyo, LIPPNCOTT WILLIAMS & WILKINS. 2000, 237-276.

115. Eichhorn, E. J. and M. R. Bristow. Medical therapy can improve the biological properties of the chronically failing heart. A new era in the treatment of heart failure. *Circulation* 94: 2285-2296, 1996.

116. Katz A.M. Therapeutic Strategies for Managing Heart Failure. Heart Failure Pathophysiology, Molecular Biology, and Clinical Management. Philadelphia.Baltimore. New York. London. Buenos Aires. Hong Kong.Sydney. Tokyo, LIPPNCOTT WILLIAMS & WILKINS. 2000, 309-339.

117. Mooser, V., J. Nussberger, L. Juillerat, M. Burnier, B. Waeber, J. Bidiville, N. Pauly, and H. R. Brunner. Reactive hyperreninemia is a major determinant of plasma angiotensin II during ACE inhibition. *J. Cardiovasc. Pharmacol.* 15: 276-282, 1990.

118. Gainer, J. V., J. D. Morrow, A. Loveland, D. J. King, and N. J. Brown. Effect of bradykinin-receptor blockade on the response to angiotensin-converting-enzyme inhibitor in normotensive and hypertensive subjects. *N.Engl..J. Med.* 339: 1285-1292, 1998.

119. Yamada, T., M. Horiuchi, and V. J. Dzau. Angiotensin II type 2 receptor mediates programmed cell death. *Proc.Natl.Acad.Sci.U.S.A* 93: 156-160, 1996.

120. Kajstura, J., E. Cigola, A. Malhotra, P. Li, W. Cheng, L. G. Meggs, and P. Anversa. Angiotensin II induces apoptosis of adult ventricular myocytes in vitro. *J. Mol.Cell. Cardiol.* 29: 859-870, 1997.

121. Shao Q, V. Panagia, Beamish R.E., and Dhalla N.S. Role of renin-angiotensin system in cardiac hypertrophy and failure. In Dhalla, N. S., Zahradka P, Dixon I, and Beamish R.E., eds. Angiotensin II Receptor Blockade: Physiological and Clinincal Implications. Boston, Kluwer Academic. 1998, 283-310.

122. Shao Q, N. Takeda, Temsah R, and N. S. Dhalla. *Cardiovasc.Pathobiol.* 1: 1-7, 1996.

123. Sanbe, A., K. Tanonaka, R. Kobayasi, and S. Takeo. Effects of long-term therapy with ACE inhibitors, captopril, enalapril and trandolapril, on myocardial energy metabolism in rats with heart failure following myocardial infarction. *J.Mol.Cell. Cardiol.*. 27: 2209-2222, 1995.

124. Effects of enalapril on mortality in severe congestive heart failure. Results of the Cooperative North Scandinavian Enalapril Survival Study (CONSENSUS). The CONSENSUS Trial Study Group. *N.Engl.J. Med.* 316: 1429-1435, 1987.

125. Kober, L., C. Torp-Pedersen, J. E. Carlsen, H. Bagger, P. Eliasen, K. Lyngborg, J. Videbaek, D. S. Cole, L. Auclert, and N. C. Pauly. A clinical trial of the angiotensin-

converting-enzyme inhibitor trandolapril in patients with left ventricular dysfunction after myocardial infarction. Trandolapril Cardiac Evaluation (TRACE) Study Group. *N.Engl..J. Med.* 333: 1670-1676, 1995.

126. Effect of ramipril on mortality and morbidity of survivors of acute myocardial infarction with clinical evidence of heart failure. The Acute Infarction Ramipril Efficacy (AIRE) Study Investigators. *Lancet* 342: 821-828, 1993.

127. The SOLVD Investigators. Effect of enalapril on mortality and the development of heart failure in asymtomatic patients with reduced left ventricular ejection fraction. *N.Engl.J. Med.*685-691, 1992.

128. Cody, R. J. ACE inhibitors: myocardial infarction and congestive heart failure. *Am Fam.Physician* 52: 1801-1806, 1995.

129. Mulder, P., B. Devaux, V. Richard, J. P. Henry, M. C. Wimart, E. Thibout, B. Mace, and C. Thuillez. Early versus delayed angiotensin-converting enzyme inhibition in experimental chronic heart failure. Effects on survival, hemodynamics, and cardiovascular remodeling. *Circulation* 95: 1314-1319, 1997.

130. Nelson, K. M. and B. F. Yeager. What is the role of angiotensin-converting enzyme inhibitors in congestive heart failure and after myocardial infarction? *Ann.Pharmacother.* 30: 986-993, 1996.

131. Sigurdsson, A. and K. Swedberg. The role of neurohormonal activation in chronic heart failure and postmyocardial infarction. *Am.Heart J.*132: 229-234, 1996.

132. Wang, J., X. Liu, B. Ren, H. Rupp, N. Takeda, and N. S. Dhalla. Modification of myosin gene expression by imidapril in failing heart due to myocardial infarction. *J.Mol.Cell. Cardiol.* 34: 847-857, 2002.

133. Dhalla, N. S., Shao Q, and V. Panagia. *Heart Fail.Rev.* 2: 261-272, 1998.

134. McAlpine, H. M., J. J. Morton, B. Leckie, A. Rumley, G. Gillen, and H. J. Dargie. Neuroendocrine activation after acute myocardial infarction. *Br.Heart J.* 60: 117-124, 1988.

135. Sadoshima, J. and S. Izumo. Molecular characterization of angiotensin II--induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts. Critical role of the AT1 receptor subtype. *Circ.Res.* 73: 413-423, 1993.

136. Katz, A. M. Angiotensin II: hemodynamic regulator or growth factor? *J. Mol.Cell. Cardiol.* 22: 739-747, 1990.

137. Dostal, D. E., R. A. Hunt, C. E. Kule, G. J. Bhat, V. Karoor, C. D. McWhinney, and K. M. Baker. Molecular mechanisms of angiotensin II in modulating cardiac function: intracardiac effects and signal transduction pathways. *J. Mol.Cell. Cardiol.* 29: 2893-2902, 1997.

138. Katz, A. M. The cardiomyopathy of overload: an unnatural growth response in the hypertrophied heart. *Ann.Intern.Med.* 121: 363-371, 1994.

139. Baker, K. M., M. I. Chernin, S. K. Wixson, and J. F. Aceto. Renin-angiotensin system involvement in pressure-overload cardiac hypertrophy in rats. *Am. J. Physiol.* 259: H324-H332, 1990.

140. Kromer, E. P. and G. A. Riegger. Effects of long-term angiotensin converting enzyme inhibition on myocardial hypertrophy in experimental aortic stenosis in the rat. *Am. J. Cardiol.* 62: 161-163, 1988.

141. Litwin, S. E., S. E. Katz, E. O. Weinberg, B. H. Lorell, G. P. Aurigemma, and P. S. Douglas. Serial echocardiographic-Doppler assessment of left ventricular geometry and function in rats with pressure-overload hypertrophy. Chronic angiotensin-converting enzyme inhibition attenuates the transition to heart failure. *Circulation* 91: 2642-2654, 1995.

142. Mohabir, R., S. D. Young, and A. M. Strosberg. Role of angiotensin in pressure overload-induced hypertrophy in rats: effects of angiotensin-converting enzyme inhibitors, an AT1 receptor antagonist, and surgical reversal. *J Cardiovasc Pharmacol* 23: 291-299, 1994.

143. Luttrell, L. M., T. van Biesen, B. E. Hawes, W. J. Koch, K. M. Krueger, K. Touhara, and R. J. Lefkowitz. G-protein-coupled receptors and their regulation: activation of the MAP kinase signaling pathway by G-protein-coupled receptors. *Adv.Second Messenger Phosphoprotein Res.* 31: 263-277, 1997.

144. Ruzicka, M., B. Yuan, and F. H. Leenen. Effects of enalapril versus losartan on regression of volume overload-induced cardiac hypertrophy in rats. *Circulation* 90: 484-491, 1994.

145. Garcia, R., M. C. Bonhomme, and S. Diebold. Captopril treatment does not restore either the renal or the ANF release response during volume expansion in moderate to severe high output heart failure. *Cardiovasc. Res.* 28: 1533-1539, 1994.

146. Ruzicka, M., F. W. Keeley, and F. H. Leenen. The renin-angiotensin system and volume overload-induced changes in cardiac collagen and elastin. *Circulation* 90: 1989-1996, 1994.

147. Ruilope, L. Improving prognosis in hypertension: exploring the benefits of angiotensin II type 1 receptor blockade. *Blood Press. Suppl.* 1: 31-35, 2000.

148. Lacourciere, Y. and R. Asmar. A comparison of the efficacy and duration of action of candesartan cilexetil and losartan as assessed by clinic and ambulatory blood pressure after a missed dose, in truly hypertensive patients: a placebo-controlled, forced titration study. Candesartan/Losartan study investigators. *Am. J. Hypertens.* 12: 1181-1187, 1999.

149. Elmfeldt, D., M. George, R. Hubner, and B. Olofsson. Candesartan cilexetil, a new generation angiotensin II antagonist, provides dose dependent antihypertensive effect. *J. Hum.Hypertens.* 11 Suppl. 2:S49-53, 1997.

150. Heuer, H. J., G. Schondorfer, and A. M. Hogemann. Twenty-four hour blood pressure profile of different doses of candesartan cilexetil in patients with mild to moderate hypertension. *J. Hum.Hypertens.* 11 Suppl. 2:S55-6, 1997.

151. Liu, Y. H., X. P. Yang, V. G. Sharov, O. Nass, H. N. Sabbah, E. Peterson, and O. A. Carretero. Effects of angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists in rats with heart failure. Role of kinins and angiotensin II type 2 receptors. *J. Clin. Invest.* 99: 1926-1935, 1997.

152. Carey, R. M., Z. Q. Wang, and H. M. Siragy. Role of the angiotensin type 2 receptor in the regulation of blood pressure and renal function. *Hypertension* 35: 155-163, 2000.

153. Naveri, L. The role of angiotensin receptor subtypes in cerebrovascular regulation in the rat. *Acta. Physiol. Scand.Suppl.* 630: 1-48, 1995.

154. Tsutsumi, Y., H. Matsubara, and H. Masaki. Vascular smooth muscle-targeted over-expression of angiotensin II type 2 receptor causes endothelium-dependent depressor and vasodilative effects via activation of the vascular kinin system. *J. Clin. Invest.* 855-884, 1999.

155. Sudhir, K., J. S. MacGregor, M. Gupta, S. D. Barbant, R. Redberg, P. G. Yock, and K. Chatterjee. Effect of selective angiotensin II receptor antagonism and angiotensin converting enzyme inhibition on the coronary vasculature in vivo. Intravascular two-dimensional and Doppler ultrasound studies. *Circulation* 87: 931-938, 1993.

156. Sunman, W. and P. S. Sever. Non-angiotensin effects of angiotensin-converting enzyme inhibitors. *Clin. Sci.(Lond)* 85: 661-670, 1993.

157. Israili, Z. H. and W. D. Hall. Cough and angioneurotic edema associated with angiotensin-converting enzyme inhibitor therapy. A review of the literature and pathophysiology. *Ann.Intern.Med.* 117: 234-242, 1992.

158. Klahr, S., S. Ishidoya, and J. Morrissey. Role of angiotensin II in the tubulointerstitial fibrosis of obstructive nephropathy. *Am. J. Kidney Dis.* 26: 141-146, 1995.

159. Burdmann, E. A., T. F. Andoh, C. C. Nast, A. Evan, B. A. Connors, T. M. Coffman, J. Lindsley, and W. M. Bennett. Prevention of experimental cyclosporin-induced interstitial fibrosis by losartan and enalapril. *Am. J. Physiol.* 269: F491-F499, 1995.

160. Wu, L. L., A. Cox, C. J. Roe, M. Dziadek, M. E. Cooper, and R. E. Gilbert. Transforming growth factor beta 1 and renal injury following subtotal nephrectomy in the rat: role of the renin-angiotensin system. *Kidney Int.* 51: 1553-1567, 1997.

161. Zoja, C., M. Abbate, D. Corna, M. Capitanio, R. Donadelli, I. Bruzzi, S. Oldroyd, A. Benigni, and G. Remuzzi. Pharmacologic control of angiotensin II ameliorates renal disease while reducing renal TGF-beta in experimental mesangioproliferative glomerulonephritis. *Am. J. Kidney Dis.* 31: 453-463, 1998.

162. Schieffer, B., A. Wirger, M. Meybrunn, S. Seitz, J. Holtz, U. N. Riede, and H. Drexler. Comparative effects of chronic angiotensin-converting enzyme inhibition and angiotensin II type 1 receptor blockade on cardiac remodeling after myocardial infarction in the rat. *Circulation* 89: 2273-2282, 1994.

163. Vescovo, G., L. L. Dalla, F. Serafini, C. Leprotti, L. Facchin, M. Volterrani, C. Ceconi, and G. B. Ambrosio. Improved exercise tolerance after losartan and enalapril in heart failure: correlation with changes in skeletal muscle myosin heavy chain composition. *Circulation* 98: 1742-1749, 1998.

164. Linz, W., R. Henning, and B. A. Scholkens. Role of angiotensin II receptor antagonism and converting enzyme inhibition in the progression and regression of cardiac hypertrophy in rats. *J. Hypertens.Suppl.* 9: S400-S401, 1991.

165. Russo, D., A. Pisani, M. M. Balletta, L. De Nicola, F. A. Savino, M. Andreucci, and R. Minutolo. Additive antiproteinuric effect of converting enzyme inhibitor and losartan in normotensive patients with IgA nephropathy. *Am. J. Kidney Dis.* 33: 851-856, 1999.

166. Spinale, F. G., R. Mukherjee, J. P. Iannini, S. Whitebread, L. Hebbar, M. J. Clair, D. M. Melton, M. H. Cox, P. B. Thomas, and M. de Gasparo. Modulation of the renin-angiotensin pathway through enzyme inhibition and specific receptor blockade in pacing-induced heart failure: II. Effects on myocyte contractile processes. *Circulation* 96: 2397-2406, 1997.

167. Willenheimer, R., B. Dahlof, E. Rydberg, and L. Erhardt. AT1-receptor blockers in hypertension and heart failure: clinical experience and future directions. *Eur.Heart J.* 20: 997-1008, 1999.

168. Tonkon, M., N. Awan, and I. Niaxi. Irbesartan combined with conventional therapy, including angiotensin converting enzyme inhibitors, in heart failure. *J. Am. Cell. Cardiol.* 188A, 1990.

169. Hamroff, G., S. D. Katz, D. Mancini, I. Blaufarb, R. Bijou, R. Patel, G. Jondeau, M. T. Olivari, S. Thomas, and T. H. Le Jemtel. Addition of angiotensin II receptor blockade to maximal angiotensin-converting enzyme inhibition improves exercise capacity in patients with severe congestive heart failure. *Circulation* 99: 990-992, 1999.

170. McKelvie, R. S., S. Yusuf, D. Pericak, A. Avezum, R. J. Burns, J. Probstfield, R. T. Tsuyuki, M. White, J. Rouleau, R. Latini, A. Maggioni, J. Young, and J. Pogue. Comparison of candesartan, enalapril, and their combination in congestive heart failure:

randomized evaluation of strategies for left ventricular dysfunction (RESOLVD) pilot study. The RESOLVD Pilot Study Investigators. *Circulation* 100: 1056-1064, 1999.

171. Ots, M., H. S. Mackenzie, J. L. Troy, H. G. Rennke, and B. M. Brenner. Effects of combination therapy with enalapril and losartan on the rate of progression of renal injury in rats with 5/6 renal mass ablation. *J. Am. Soc.Nephrol.* 9: 224-230, 1998.

172. Hebert, L. A., M. E. Falkenhain, N. S. Nahman, Jr., F. G. Cosio, and T. M. O'Dorisio. Combination ACE inhibitor and angiotensin II receptor antagonist therapy in diabetic nephropathy. *Am. J. Nephrol.* 19: 1-6, 1999.

173. Yamazaki, T., I. Komuro, I. Shiojima, and Y. Yazaki. Angiotensin II mediates mechanical stress-induced cardiac hypertrophy. *Diabetes Res.Clin.Pract.* 30 Suppl.: 107-111, 1996.

174. Komuro, I., S. Kudo, T. Yamazaki, Y. Zou, I. Shiojima, and Y. Yazaki. Mechanical stretch activates the stress-activated protein kinases in cardiac myocytes. *FASEB J.* 10: 631-636, 1996.

175. Izumi, Y., S. Kim, T. Murakami, S. Yamanaka, and H. Iwao. Cardiac mitogen-activated protein kinase activities are chronically increased in stroke-prone hypertensive rats. *Hypertension* 31: 50-56, 1998.

176. Harada, K., I. Komuro, Y. Zou, S. Kudoh, K. Kijima, H. Matsubara, T. Sugaya, K. Murakami, and Y. Yazaki. Acute pressure overload could induce hypertrophic responses in the heart of angiotensin II type 1a knockout mice. *Circ.Res.*20;82: 779-785, 1998.

177. Takemoto, Y., M. Yoshiyama, K. Takeuchi, T. Omura, R. Komatsu, Y. Izumi, S. Kim, and J. Yoshikawa. Increased JNK, AP-1 and NF-kappa B DNA binding activities in isoproterenol-induced cardiac remodeling. *J. Mol.Cell. Cardiol.* 31: 2017-2030, 1999.

178. Nijjar M. and Dhalla N.S. Biochemical Basis of Calcium-Handling in Developing Myocardium. The Developing Heart. Philadelphia, Lippincott-Raven. 1997, 189-217.

179. Garcia, R. and S. Diebold. Simple, rapid, and effective method of producing aortocaval shunts in the rat. *Cardiovasc. Res.* 24: 430-432, 1990.

180. Liu, Z., D. R. Hilbelink, W. B. Crockett, and A. M. Gerdes. Regional changes in hemodynamics and cardiac myocyte size in rats with aortocaval fistulas. 1. Developing and established hypertrophy. *Circ.Res.* 69: 52-58, 1991.

181. Huang, M., R. L. Hester, and A. C. Guyton. Hemodynamic changes in rats after opening an arteriovenous fistula. *Am. J. Physiol.* 262: H846-H851, 1992.

182. Flaim, S. F., W. J. Minteer, and R. Zelis. Acute effects of arterio-venous shunt on cardiovascular hemodynamics in rat. *Pflugers. Arch.* 385: 203-209, 1980.

183. Huang, M., M. H. LeBlanc, and R. L. Hester. Evaluation of the needle technique for producing an arteriovenous fistula. *J. Appl.Physiol.* 77: 2907-2911, 1994.

184. Wang, X., B. Ren, S. Liu, E. Sentex, P. S. Tappia, and N. S. Dhalla. Characterization of cardiac hypertrophy and heart failure due to volume overload in the rat. *J.Appl.Physiol.* 94: 752-763, 2003.

185. Domingos, P. P., P. M. Fonseca, W. Nadruz, Jr., and K. G. Franchini. Load-induced focal adhesion kinase activation in the myocardium: role of stretch and contractile activity. *Am.J.Physiol. Heart Circ.Physiol.* 282: H556-H564, 2002.

186. Marshall, R. P., R. J. McAnulty, and G. J. Laurent. Angiotensin II is mitogenic for human lung fibroblasts via activation of the type 1 receptor. *Am. J. Respir.Crit. Care. Med.* 161: 1999-2004, 2000.

187. Molteni, A., J. E. Moulder, E. F. Cohen, W. F. Ward, B. L. Fish, J. M. Taylor, L. F. Wolfe, L. Brizio-Molteni, and P. Veno. Control of radiation-induced pneumopathy and lung fibrosis by angiotensin-converting enzyme inhibitors and an angiotensin II type 1 receptor blocker. *Int.J Radiat.Biol.* 76: 523-532, 2000.

188. Border, W. A. and N. Noble. Maximizing hemodynamic-independent effects of angiotensin II antagonists in fibrotic diseases. *Semin.Nephrol.* 21: 563-572, 2001.

189. Croquet, V., F. Moal, N. Veal, J. Wang, F. Oberti, J. Roux, E. Vuillemin, Y. Gallois, O. Douay, D. Chappard, and P. Cales. Hemodynamic and antifibrotic effects of losartan in rats with liver fibrosis and/or portal hypertension. *J. Hepatol.* 37: 773-780, 2002.

190. Paizis, G., R. E. Gilbert, M. E. Cooper, P. Murthi, J. M. Schembri, L. L. Wu, J. R. Rumble, D. J. Kelly, C. Tikellis, A. Cox, R. A. Smallwood, and P. W. Angus. Effect of angiotensin II type 1 receptor blockade on experimental hepatic fibrogenesis. *J. Hepatol.* 35: 376-385, 2001.

191. Weber, K. T. Fibrosis, a common pathway to organ failure: angiotensin II and tissue repair. *Semin.Nephrol.* 17: 467-491, 1997.

192. Pfeffer, M. A. and E. Braunwald. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. *Circulation* 81: 1161-1172, 1990.

193. Pfeffer, J. M. Progressive ventricular dilation in experimental myocardial infarction and its attenuation by angiotensin-converting enzyme inhibition. *Am. J. Cardiol.* 68: 17D-25D, 1991.

194. Weinberg E.D, Schoen F.J, George D, Kagaya T, Douglas P.S, litwin S.E, Schunkert H, Benedict C.R, and Lorell B.H. *Circulation* 50: 1410-1422, 1994.

195. Pfeffer, J. M., M. A. Pfeffer, and E. Braunwald. Influence of chronic captopril therapy on the infarcted left ventricle of the rat. *Circ.Res.* 57: 84-95, 1985.

196. Ruzicka, M. and F. H. Leenen. Relevance of blockade of cardiac and circulatory angiotensin-converting enzyme for the prevention of volume overload-induced cardiac hypertrophy. *Circulation* 91: 16-19, 1995.

197. Shimizu, N., M. Yoshiyama, T. Omura, A. Hanatani, S. Kim, K. Takeuchi, H. Iwao, and J. Yoshikawa. Activation of mitogen-activated protein kinases and activator protein-1 in myocardial infarction in rats. *Cardiovasc. Res.* 38: 116-124, 1998.

198. Kim, S. and H. Iwao. Activation of mitogen-activated protein kinases in cardiovascular hypertrophy and remodeling. *Jpn..J. Pharmacol.* 80: 97-102, 1999.

199. Dargie, H. J., H. M. McAlpine, and J. J. Morton. Neuroendocrine activation in acute myocardial infarction. *J. Cardiovasc. Pharmacol.* 9 Suppl .2: S21-S24, 1987.