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Role of Receptor and Non-Receptor Protein Tyrosine Kinases in Vasoactive Peptide-Induced Signaling

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Role of Receptor and Non-Receptor Protein Tyrosine Kinases in Vasoactive Peptide-Induced Signaling

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Résumé

L'endothéline-1 (ET-1) et l'angiotensine II (Ang II) jouent un rôle important dans le maintien de la pression artérielle et l'homéostasie vasculaire. Une activité accrue de ces peptides vasoactifs est présumée contribuer au développement de pathologies vasculaires, telles que l'hypertension, l'athérosclérose, l'hypertrophie et la resténose. Ceci est causé par une activation excessive de plusieurs voies de signalisation hypertrophiques et prolifératives, qui incluent des membres de la famille des Mitogen Activated Protein Kinases (MAPK), ainsi que la famille phosphatidylinositol 3-kinase (PI3-K) / protéine kinase B (PKB). Bien que l'activation de ces voies de signalisation soit bien élucidée, les éléments en amont responsables de l'activation des MAPK et de la PKB, induite par l'ET-1 et Ang II, demeurent mal compris. Durant les dernières années, le concept de la transactivation de récepteurs et/ou non-récepteurs protéines tyrosine kinases (PTK) dans le déclenchement des événements de signalisation induits par les peptides vasoactifs a gagné beaucoup de reconnaissance. Nous avons récemment démontré que la PTK Insulin-like Growth Factor type-1 Receptor (IGF-1R) joue un rôle dans la transduction des signaux induits par l'H_2O_2, menant à la phosphorylation de la PKB. Étant donné que les peptides vasoactifs génèrent des espèces réactives d'oxygène, telles que l'H_2O_2 lors de leur signalisation, nous avons examiné le rôle de d'IGF-1R dans la phosphorylation de la PKB et les réponses hypertrophiques dans les cellules muscle lisse vasculaires (CMLV) induites par l'ET-1 et Ang II. AG-1024, un inhibiteur spécifique de l'IGF-1R, a atténué la phosphorylation de la PKB induite à la fois par l'ET-1 et Ang II. Le traitement des CMLVs avec l'ET-1 et Ang II a également induit une phosphorylation des résidus tyrosine dans les sites d'autophosphorylation d'IGF-1R, celle-ci a été bloquée par l'AG-1024. En outre, l'ET-1 et l'Ang II on tous les deux provoqué la phosphorylation de c-Src, une PTK non-récepteur, bloqué par PP-2, inhibiteur spécifique de la famille Src. La PP-2 a également inhibé la phosphorylation de PKB et d'IGF-1R induite par l'ET-1 et l'Ang II. De plus, la synthèse de protéines ainsi que d'ADN, marqueurs de la prolifération cellulaire et de l'hypertrophie, ont également été atténuée par l'AG-1024 et le PP-2

Bien que ce travail démontre le rôle de c-Src dans la phosphorylation PKB induite par l'ET-1 et Ang II, son rôle dans l'activation des MAPK induit par l'ET-1 dans les CMLVs reste controversé. Par conséquent, nous avons examiné l'implication de c-Src dans l'activation de ERK 1/2, JNK et p38MAPK, par l'ET-1 et Ang II, ainsi que leur capacité à régulariser l'expression du facteur de transcription Early growth transcription factor-1 « Egr-1 ». ET-1 et Ang II ont induit la phosphorylation de ERK 1/2, JNK et p38MAPK, et ont amplifié l'expression d'Egr-1 dans les CMLVs. Cette augmentation de la phosphorylation des MAPK a été diminuée par la PP-2, qui a aussi atténué l'expression d'Egr-1 induite par l'ET-1 et l'Ang II. Une preuve supplémentaire du rôle de c-Src dans ce processus a été obtenue en utilisant des fibroblastes embryonnaires de souris déficientes en c-Src (Src -/- MEF). L'expression d'Egr-1, ainsi que l'activation des trois MAPKs par l'ET-1 ont été atténuées dans les cellules Src -/- par rapport au MEF exprimant des taux normaux Src. En résumé, ces données suggèrent que l'IGF-1R et c-Src PTK jouent un rôle essentiel dans la régulation de la phosphorylation de PKB et des MAPK dans l'expression d'Egr-1, ainsi que dans les réponses hypertrophiques et prolifératives induites par l'ET-1 et Ang II dans les CMLVs.

Mots-clés : Endotheline-1, Angiotensin II, PKB, MAPK, IGF-1R, c-Src, VSMC, Egr-1, prolifération, hypertrophie

Abstract

Endothelin-1 (ET-1) and angiotensin II (Ang II) play important roles in maintaining blood pressure and vascular homeostasis, and a heightened activity of these vasoactive peptides is thought to contribute to the development of vascular pathologies, such as hypertension, atherosclerosis, hypertrophy and restenosis. This is caused by an excessive activation of several growth and proliferative signaling pathways, which include members of the mitogen-activated protein kinase (MAPK) family, as well as the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) pathway. While the activation of these signaling pathways is well elucidated, the upstream elements responsible for ET-1 and Ang II-induced MAPK and PI3-K/PKB activation remain poorly understood. During the last several years, the concept of transactivation of receptor and/or non-receptor protein tyrosine kinases (PTK) in triggering vasoactive peptide-induced signaling events has gained much recognition. We have recently demonstrated that insulin-like growth factor-1 receptor (IGF-1R) plays a role in tranducing the effect of H_2O_2, leading to PKB phosphorylation. Since vasoactive peptides elicit their responses through generation of reactive oxygen species, including H_2O_2, we investigated whether IGF-1R transactivation plays a similar role in ET-1 and Ang II-induced PKB phosphorylation and hypertrophic responses in VSMC. AG-1024, a specific inhibitor of IGF-1R, attenuated both ET-1 and Ang II-induced PKB phosphorylation in a dose-dependent manner. ET-1 and Ang II treatment also induced the phosphorylation of tyrosine residues in the autophosphorylation sites of IGF-1R, which was blocked by AG-1024. In addition, both ET-1 and Ang II evoked tyrosine phosphorylation of c-Src, a non-receptor PTK, and pharmacological inhibition of c-Src PTK activity by PP-2, a specific inhibitor of Src-family tyrosine kinase, significantly reduced PKB phosphorylation as well as tyrosine phosphorylation of IGF-1R induced by the two vasoactive peptides. Furthermore, protein and DNA synthesis, markers of cell growth and proliferation, enhanced by ET-1 and Ang II were also attenuated by AG-1024 and PP-2.

While this work demonstrates the role of c-Src in ET-1 and Ang II-induced PKB phosphorylation, its role in ET-1-induced MAPK signaling and regulation of transcription factors, such as early growth response factor-1 (Egr-1), which was recently shown to be expressed in atherosclerotic plaque, remains controversial in VSMC. Therefore, we have also investigated the involvement of c-Src in ET-1 and Ang II-induced ERK 1/2, JNK and p38mapk activation, as well as Egr-1 regulation. ET-1 and Ang II-induced the phosphorylation of ERK 1/2, JNK and p38mapk, and enhanced the expression of Egr-1 in aortic VSMC. This increased phosphorylation was decreased by PP-2. Further proof for the role of c-Src in this process was obtained by using mouse embryonic fibroblasts (MEF) deficient in c-Src (Src -/- MEF). ET-1-induced Egr-1 expression, as well as MAPK activation, were found to be downregulated in Src -/- MEF, as compared to MEF expressing normal Src levels. In summary, these data demonstrate that IGF-1R and c-Src PTK play a critical role in mediating both PKB and MAPK phosphorylation and Egr-1 expression, as well as hypertrophic and proliferative responses induced by ET-1 and Ang II in VSMC.

Keywords: Endothelin-1, Angiotensin II, PKB, MAPK, IGF-1R, c-Src, VSMC, Egr-1, proliferation, hypertrophy

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List of Abbreviations

2K1C	Two kidney 1 clip rodent model of renal hypertension
ANF	Atrial natriuretic factor
Ang II	Angiotensin II
ANP	Atrial natriuretic peptide
AP-1	Activator protein 1
ApoE	Apolipoprotein E
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
BAD	Bcl-2 associated death promoter
Bcl-2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
BNP	Brain natriuretic peptide
BQ 123	Cyclo(D-Asp-Pro-D-Val-Leu-D-Trp, Na), a potent ET_A receptor antagonist
BQ 788	N-cis-2,6-Dimethylpiperidinocarbonyl-L-γ-MeLeu-D-Trp(MeOCO)-D-Nle-OH, Na, a potent ET_B receptor antagonist
Ca^{2+}	Calcium
cGMP	Cyclic guanosine monophosphate
CHF	Chronic heart failure
COX-2	Cyclooxygenase-2
CSF	Colony-stimulating factors
CVD	Cardiovascular disease
DAG	Diacyglycerol
DOCA	Deoxycorticosterone acetate
ECE	Endothelin converting enzyme
EGF	Epidermal growth factor

EGF-R	Epidermal growth factor receptor
Egr-1	Early growth response factor 1
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal-regulated kinase
ET	Endothelin
ET_A	Endothelin receptor type A
ET_B	Endothelin receptor type B
FGF	Fibroblast growth factor
G protein	Guanosine nucleotide binding protein
GDP	Guanosine diphosphate
GPCR	Guanosine nucleotide binding protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GSK-3	Glycogen synthase kinase 3
GSH	Glutathione
GST	Glutathione-s-transferase
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein-cholesterol
HIF	Hypoxia inducible factor
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor type 1 receptor
IL	interleukin
iNOS	inducible nitric oxide synthase
IR	insulin receptor
IRS	insulin receptor substrate
IP_3	inositol triphosphate
JNK	c-Jun N-terminal kinase
kDa	kiloDalton
LDL	low-density lipoprotein-cholesterol

MAPK	mitogen activated protein kinase
MEF2	myocyte enhancer factor 2
MEK	mitogen extracellular regulated kinase
MI	myocardial ischemia
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NADPH oxidase	nicotamide adenine dinucleotide phosphate oxidase
NF-1	nuclear factor 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NR-PTK	Non-receptor protein tyrosine kinase
($O_2^{\cdot -}$)	superoxide anion
($OH^{\cdot}$)	hydrogen radicals
($ONOO^{-}$)	peroxynitrite
$p70^{s6k}$	p70 ribosomal S6 kinase
$p90^{rsk}$	p90 ribosomal kinase
PDGF	platelet derived growth factor
PDGF-R	platelet derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PH	pleckstrin homology
PI3-K	phosphatidylinositol 3-kinase
PI	phosphatidylinositol
PIP	phosphatidylinositol 3 monophosphate
PIP2	phosphatidylinositol 4, 5 bisphosphate
PIP3	phosphatidylinositol 3, 4, 5 triphosphate
PLC β	phosphoinositide-specific phospholipase C β
PKB	protein kinase B
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate

PTK	protein tyrosine kinase
PTPase	Protein tyrosine phosphatase
PTP-1B	Protein tyrosine phosphatase 1B
PYK-2	proline-rich tyrosine kinase
R-PTK	receptor protein tyrosine kinase
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SH2	src homology 2
Shc	src homology collagen
SHR	spontaneously hypertensive rat
SHP-2	SH2 domain-containing tyrosine phosphatase-2
SOD	Superoxide dismutase
SOS	son of seven less
STAT	signal transducer and activator of transcription
TNF-α	tumour necrosis factor-α
TGF-β	transforming growth factor-β
VSMC	vascular smooth muscle cell
WKY	Wistar Kyoto rat

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Chapter 1

Introduction

1.1 Obesity, Diabetes and Hypertension: The Modern Epidemic

The insulin resistance syndrome, more commonly known as the metabolic syndrome, has been described as a cluster of multiple risk factors leading to the development of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD). Among these risk factors are obesity, irregular blood lipids (dyslipidemia), insulin resistance and hypertension [1]. These risk factors, beginning with insulin resistance, are caused by both genetic (where certain individuals or populations may be more likely to develop the metabolic syndrome due to their unique genetic background) and environmental/lifestyle factors, such as diet and exercise. Globally, the incidence of metabolic syndrome is rising at an alarming rate [2], yet the true prevalence of the disease is unknown, partly due to the lack of an accepted definition to define the metabolic syndrome. To understand insulin resistance and the metabolic syndrome, and how this dysregulation of hormonal signaling affects various aspects of normal physiology and pathophysiological states, it is important to define insulin, and to understand its functions in normal physiology.

Insulin is the primary hormone involved in blood glucose control. In response to increasing blood glucose levels, pancreatic β-cells secrete insulin, which stimulates glucose uptake in muscle and fat tissues via stimulation of glucose transporter 4 (GLUT4) from intracellular sites to the plasma membrane [3] and triggers inhibition of gluconeogenesis and glucose release by the liver by stimulating glycogen synthesis and inhibiting glycogenolysis and gluconeogenesis, respectively. In addition, insulin is an anabolic hormone, which promotes lipid synthesis and suppresses lipid degradation. These actions are all mediated by an intricate signaling cascade initiated by the binding of insulin to its receptor. An impaired insulin signaling and action results in a drastic decrease in glucose transport, glycogen synthesis, and lipid generation, leading to dysregulation of the physiological effects of insulin, which normally leads to insulin resistance, and T2DM [4]. When added to other environmental and genetic factors, this impaired signaling leads to symptoms of the metabolic syndrome, which can turn in to T2DM and CVD.

1.1.1 Insulin resistance and the metabolic syndrome

Simply put, insulin resistance is a condition in which the body produces insulin, but cannot use it properly. In a more scientific fashion, it is a condition in which insulin, exogenous or endogenous, produces an abnormally low biological response with respect to a decrease in blood glucose levels [5]. Insulin resistance is the earliest detectable metabolic trait of pre-diabetes, and possibly the most important risk factor in the development of Type 2 diabetes mellitus (T2DM). In the pre-diabetic state, or insulin resistance, serum insulin concentrations are elevated to compensate for insulin resistance in peripheral tissues, and to maintain a normal glucose balance. This is a compensatory mechanism, known as hyperinsulinemia.

Hyperinsulinemia exists to maintain normal glucose levels, or normoglycemia, during the prediabetic/insulin resistant state. Even though normoglycemia may be maintained in some, it has been shown that other patients will have increased fasting glucose or impaired glucose tolerance (IGT) after a 2 hour oral glucose challenge, of 140 to 199 mg/dL [6]. Hyperinsulinemia is a stress on the pancreas, and may, at some point, not be able to handle this increased work load, and as a result its insulin secretory response may decrease. A decrease in insulin levels may cause a rise in blood glucose, leading to diabetes.

Today, it is almost impossible to speak of insulin resistance without talking about the metabolic syndrome. In fact, after having done an in depth review of the literature on the subject, I have come to realize that if not for the clinical definition of insulin resistance, the terms metabolic syndrome and insulin resistance have come to be almost interchangeable. Essentially, the metabolic syndrome is a constellation of abnormalities that is associated with increased risk for the development of T2DM and atherosclerotic vascular disease, such as heart disease and stroke [2].

As mentioned earlier, insulin levels may decrease after prolonged insulin resistance, yet in most of these insulin resistant individuals, large amounts of insulin continue to be secreted to overcome this defect in insulin action, thereby maintaining normal or near-normal glucose tolerance. While this compensatory hyperinsulinemia prevents the

development of hyperglycemia, these individuals run a risk of suffering from a certain degree of IGT, high plasma triglyceride (TG) and low high-density lipoprotein cholesterol (HDL-C), or "good cholesterol" levels, obesity, and most importantly, essential hypertension [7, 8]. These seem to constitute the cluster of risk factors leading to T2DM and CVD.

The first guide lines for the diagnosis of metabolic syndrome were presented in 1998 by the World Health Organization (WHO) [9]. This definition was later modified by the European Group for the Study of Insulin Resistance (EGIR) to focus more on glucose intolerance and insulin resistance [10]. In 2001, the National Cholesterol Education Programme Adult Treatment Panel III (ATPIII) again revised the metabolic syndrome diagnosis criteria. It is based more on plasma glucose levels and dyslipidemia, and omits insulin resistance as a criteria [11]. More recently, the American Heart Association (AHA) released its own criteria, which mirror the ATPIII, with the exception of stricter fasting glucose criteria [1]. The most recent definition of the metabolic syndrome criteria comes from the International Diabetes Federation, which names abdominal obesity and insulin resistance as the most important factors leading to metabolic syndrome [12]. Despite these more recent definitions, the most widely used definition and criteria to diagnose the metabolic syndrome remain those of the ATP III, and as such, we will concentrate on those criteria here. The ATPIII identifies 5 components of the metabolic syndrome that relate to CVD: abdominal obesity (waist circumference), atherogenic dyslipidemia, high density lipoprotein (HDL) cholesterol levels, glucose intolerance, and high blood pressure [11].

Visceral obesity is the form of obesity most strongly linked with insulin resistance and with the presence of related metabolic abnormalities of the metabolic syndrome. Clinically, it presents increased waist circumference. However, the mechanisms underlying the association between abdominal obesity and the metabolic syndrome are not fully understood. It has been assumed that abdominal adipose tissue releases an excess of fatty acids and cytokines that contribute to insulin resistance. For example, visceral adipose tissue secretes adipokines like adiponectin and inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor (TNF)-α, which contribute to the insulin resistant,

proinflammatory, and hypertensive states [13]. In fact, visceral adiposity and insulin resistance are connected by TNF-α at the molecular level. Studies have shown that in adipocytes, TNF-α causes serine phosphorylation of IR and IRS-1, impairing insulin signaling. This leads to increased lipolysis, as well as decreased adiponectin levels, and an increase in circulating free fatty acids (FFA) [14, 15]

Dyslipidemia and HDL levels present in routine lipoprotein analysis by high triglyceride levels, exceeding 200mg/dL and reduced concentrations of high density lipoproteins (HDL) cholesterol, usually below 40mg/dL [5]. In addition, it is characterized by other lipoprotein abnormalities, such as elevated apolipoprotein B, small low density lipoprotein (LDL) particles, caused by enriched triglycerides, and small HDL particles [5]. The combination of these factors increases the atherogenicity of LDL, even though LDL levels are usually not increased. Lipoprotein metabolism is regulated by genetic and environmental (diet, exercise or lack thereof) factors and both can aggravate dyslipidemia.

Glucose intolerance presents as impaired fasting glucose (levels of 110 to 125 mg/dL) or IGT (levels of 140 to 199 mg/dL) after a 2 hour oral glucose challenge, of 1.75 Kg of glucose per Kg of body weight for children and 75 g of glucose for adults [6]. These glucose levels are intermediates between normal values and overt diabetes. When glucose intolerance evolves into diabetes-level hyperglycemia due to the failure of the hyperinsulinemia mechanism described earlier, high glucose levels constitute a major, independent risk factor for CVD.

Blood pressure has been shown to be elevated in overweight/obese patients with insulin resistance or glucose intolerance. In fact, 50-60% of patients suffering from essential hypertension are insulin resistant to some degree [16]. Blood pressure regulation is complex and affected by dietary factors, physical activity and renal/adrenal organ function.

1.2 The metabolic Syndrome, Hypertension and Cardiovascular Disease

Obesity, diabetes, and the metabolic syndrome have clearly been shown to cause endothelial dysfunction [17]. Endothelial dysfunction is commonly characterised by reduced nitric oxide (NO) dependent activity, which leads to hypertension, coronary heart disease, and accelerated atherosclerosis [18]. Glucotoxicity (deleterious effects of hyperglycemia) and lipotoxicity (negative effects of increased plasma FFA and low HDL), as well as increased pro-inflammatory signaling are all adverse effects of the metabolic syndrome, which worsen insulin resistance, and contribute to endothelial dysfunction, hypertension and CVD.

1.3 Hypertension

Blood pressure is defined as the force that blood exerts on the vessel walls. Hypertension (HT), or high blood pressure, is a chronic medical condition characterized mainly by elevated arterial blood pressure [19]. The worldwide prevalence of hypertension is estimated to be at 1 billion people, and contributes to approximately 7.1 million deaths per year worldwide [20]. Persistent HT is a major risk factor contributing to pathophysiological events such as stroke, heart attack, heart failure and chronic renal failure [21]. In the United States, it is estimated that approximately 50 million people (almost 1 in 4 adults) suffer from hypertension [19], with proportions varying according to race, age, geographic location, gender and economic status. To add to these factors, not all people suffering from HT suffer from the same type of HT, as multiple types of HT exist. Furthermore, as blood pressure is not static, and varies from one minute to another in the day due to multiple external stimuli, it is difficult to define HT, in order to establish accepted treatment guidelines. Here, I will define blood pressure and discuss several of the proposed definitions of HT, as well as several different types of HT, and the differences between them.

1.3.1 Definition of hypertension

Blood pressure is calculated by measurements of systolic pressure (SBP) over diastolic blood pressure (DBP), measured in millimeters of mercury (mmHg). Systolic pressure is the peak pressure in the arteries, which occurs near the end of the cardiac cycle, during ventricular contraction. Diastolic pressure is the lowest pressure in the arteries, occurring at the start of the cardiac cycle, when the ventricles are filling with blood. It can be said that "normal" or healthy adult blood pressure, when taken at a resting state (normally how blood pressure is taken) is 115mmHg systolic over 75mmHg diastolic, read as 115/75 mmHg [22]. As mentioned earlier, blood pressure is not static, as it undergoes natural variations from one heart beat to another, in response to simple stimuli, such as movements, nutrients or metabolites in the circulation, exercise, disease and stress. High blood pressure (or hypertension (HT)) can be clinically diagnosed by either a SBP above 140mmHg or a DBP above 90 mmHg, measured at least twice, on at least 2 subsequent visits to have blood pressure taken [19]. Although these criteria help diagnose HT so that a patient can receive the proper treatment, recent studies show that a great deal of damage may have already occurred to the cardiovascular system by the time or before a patient's blood pressure reaches 140/90 mmHg. In fact, the Framingham Heart Study has shown that for every increase of 20/10 mmHg, from a base blood pressure of 120/80 mmHg, the risk of development of cardiovascular disease doubles [23]. Further to this data which highlights the increased risk of cardiovascular complications associated with blood pressure levels that used to be considered normal, the Joint National Committee on prevention, detection, evaluation and treatment of high blood pressure (JNC) convened to re-evaluate the definition of hypertension [22]. In this new definition, blood pressure is separated into 4 different categories (Table 1):

Normal: This category is composed of individuals with an SBP under 120 mmHg and a DBP under 80 mmHg.

- Prehypertension: This category is composed of individuals with an SBP between 120-139 mmHg and a DBP between 80-89 mmHg.

- Stage 1 hypertension: This category is composed of individuals with an SBP between 140-159 mmHg and a DBP between 90-99 mmHg.
- Stage 2 hypertension: This category is composed of individuals with an SBP over 160 mmHg and a DBP over 100 mmHg.

Longutidinal studies, such as the Framingham Heart Study, have shown the long-term effects of prolonged hypertension. Based on such studies and others, the new JNC definitions have made it easier to diagnose hypertension and the increased blood pressure conditions which precede HT. By adding a category to include "pre-hypertensive" patients (Table 1), individuals in whom early intervention could reduce blood pressure could easily be identified. These patients would be counseled to adopt lifestyle changes, which may not only lower blood pressure, but help prevent the progression of HT with age, and may prevent HT altogether [22]. It is important to note however, that the prehypertension category does not imply that an individual suffers from HT. This category was created to identify patients at risk of developing HT, such as individuals suffering from obesity, diabetes and kidney disease. While there is no pharmacological therapy suggested to treat the increase in blood pressure in the prehypertenvise group, patients in both stage 1 and stage 2 hypertension groups must receive pharmacological therapy, in addition to lifestyle changes, to decrease HT, and its associated risks, such as angina, myocardial infarction, stroke or chronic kidney disease [24]. Lifestyle changes include weight reduction through decreased caloric intake and exercise (Table 2), the adoption of an exercise/physical activity plan consisting of moderate exercise (at least 30 minute moderate walk 3 to 4 times per week), a reduction in dietary sodium intake (Table 2), as well as limiting the consumption of alcohol to no more than 2 drinks per day for men and 1 drink per day for women [24]. Adoption of lifestyle changes alone have been shown to reduce SBP by 2-20 mmHg [25-28], prevent/delay the incidence hypertension and increase the efficiency of antihypertensive pharmacological therapy [29]. When the adoption of lifestyle changes is not sufficient, pharmacological therapies must be included in treatment plans. These pharmacological therapies include diuretics, angiotensin converting enzyme (ACE) inhibitors, angiotensin II (Ang II) antagonists, and α- and β-blockers. Unfortunately, not all people suffering from HT suffer

from the same type of HT, as multiple forms of HT exist, and as such, treatments must be personalized.

Table 1: Categories of hypertension

BP classification	SBP* mmHg	DBP* mmHg	Lifestyle modification	Initial drug therapy	
				Without compelling indication	With compelling indications
Normal	<120	and <80	Encourage		
Prehypertension	120–139	or 80–89	Yes	No antihypertensive drug indicated.	Drug(s) for compelling indications.
Stage 1 Hypertension	140–159	or 90–99	Yes	Thiazide-type diuretics for most. May consider ACEI, ARB, BB, CCB, or combination.	Drug(s) for the compelling indications.
Stage 2 Hypertension	≥160	or ≥100	Yes	Two-drug combination for most (usually thiazide-type diuretic and ACEI or ARB or BB or CCB).	Other antihypertensive drugs (diuretics, ACEI, ARB, BB, CCB) as needed.

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin-receptor blocker; BB, beta blocker; BP, blood pressure; CCB, calcium channel blocker.

*Treatment determined by highest BP category

Chobanian AV et al. for JNC 7, Hypertension 2003, 42(6):1206-52.

Table 2: Lifestyle Modifications to Manage Hypertension

Modification	Recommendation	Approximate Systolic BP Reduction, Range
Weight reduction	Maintain normal body weight (BMI, 18.5-24.9)	5-20 mm Hg/10-kg weight loss
Adopt DASH eating plan	Consume a diet rich in fruits, vegetables, and low-fat dairy products with a reduced content of saturated and total fat	8-14 mm Hg
Dietary sodium reduction	Reduce dietary sodium intake to no more than 100 mEq/L (2.4 g sodium or 6 g sodium chloride)	2-8 mm Hg
Physical activity	Engage in regular aerobic physical activity such as brisk walking (at least 30 minutes per day, most days of the week)	4-9 mm Hg
Moderation of alcohol consumption	Limit consumption to no more than 2 drinks per day (1 oz or 30 mL ethanol [eg, 24 oz beer, 10 oz wine, or 3 oz 80-proof whiskey]) in most men and no more than 1 drink per day in women and lighter-weight persons	2-4 mm Hg

Abbreviations: BMI, body mass index calculated as weight in kilograms divided by the square of the height in meters; BP, blood pressure; DASH, Dietary Approaches to Stop Hypertension

*For overall cardiovascular risk reduction, stop smoking. The effects of implementing these modifications are dose and time dependent and could be higher for some individuals

Chobanian AV et al. for JNC 7, Hypertension 2003, 42(6):1206-52.

1.3.2 Varying forms of hypertension

As mentioned above, multiple forms of HT exist, and specific classifications have been defined to identify them. HT is classified under 2 main categories: essential, or idiopathic HT, and secondary HT.

Essential hypertension (EHT) is diagnosed when no clear medical cause can be identified to explain the increased blood pressure [30]. This is the most common type of hypertension, with approximately 95% of all HT being diagnosed at EHT [19, 30]. It is unassociated with secondary causes, such as renovascular disease, renal failure, pheochromocytoma, aldosteronism or monogenic HT [19]. Multiple pathophysiological factors have been suggested to contribute to the development of essential HT, among them, obesity, high sodium intake, high alcohol intake, and inadequate potassium and calcium intake [31-37]. It is for this reason that HT has been termed a heterogeneous disorder, as different patients have different causal factors leading to HT [19]. Insulin resistance (IR) and/or glucose intolerance has also been shown to play an important role in the development of EHT. Blood pressure has been shown to be elevated in overweight/obese patients with IR. In fact, 50-60% of patients suffering from EHT are insulin resistant to some degree [16]. Blood pressure regulation is complex and affected by dietary factors, physical activity and renal/adrenal organ function.

To complicate matters further, the presence of obesity, IR and EHT are hallmarks of the metabolic syndrome, a constellation of abnormalities that is associated with an increased risk of development of type 2 diabetes mellitus (T2DM) and atherosclerotic vascular disease, such as heart disease and stroke. Obesity, diabetes, and the metabolic syndrome have clearly been shown to cause endothelial dysfunction [17], commonly characterized by reduced nitric oxide production, leading to HT, coronary heart disease and accelerated atherosclerosis [18].

Secondary hypertension (SHT), unlike EHT, is caused by an identifiable secondary cause, usually a pre-existing medical condition, such as congestive heart failure,

liver damage, kidney damage, hormonal irregularities, certain types of cancers and different types of medications [38]. It is less frequent as well, affecting only about 5% of people with HT [38]. Here, I will briefly describe SHT associated to 1) chronic kidney disease and 2) gestational hypertension, which are two of the more common forms of SHT encountered.

Chronic kidney (renal) disease, also known as renal parenchymal disease, is the most common cause of SHT. HT may be an early sign of renal disease and may be present even before a decline in renal function, yet it is mostly caused by renal artery stenosis. Renal artery stenosis causes the kidney attached to the constricted arteries to become ischemic, leading to an increased activation of the renin-angiotensin system (RAS) [39]. An increased production of Ang II leads to augmented aldosterone secretion, causing sodium and water retention, leading to hypertension [39, 40]. Renal artery stenosis may be unilateral, affecting the artery going only to one kidney, or bilateral, affecting the arteries going to both kidneys. The latter is the most common cause of renal failure. In the case of unilateral stenosis, volume overload of the affected kidney is avoided, as the contralateral kidney responds to the rise in blood pressure through increased excretion of sodium through the urine, or natriuresis [41]. In the case of bilateral stenosis, or in patients with only one functional kidney, there is no compensatory diuresis, thus inhibiting sodium excretion.

Open surgical revascularization was at one point the only treatment available for renal artery stenosis. In fact, this method is still the primary treatment used in patients where the stenosis extends into segmental arteries, or involves multiple small arteries or the early branching primary renal artery [41]. Balloon angioplasty and especially renal artery stent insertion have also become popular “surgical” treatments, especially with the development of stents coated with different types of medications [42]. Pharmacological treatment options include angiotensin-converting enzyme inhibitors, angiotensin receptor blockers and calcium channel blockers, which are recommended for the treatment of unilateral renal artery stenosis-induced hypertension only. However, caution must be exercised with the use of these medications, and their administration must be carefully monitored by constant laboratory testing, as they may cause acute renal failure [41, 43]. These treatments are, for the most part, very successful in terms of restoring blood pressure to

normal levels, as well as preventing further renal, endocrine or cardiac complications, such as atherosclerosis, cardiovascular disease, heart failure and stroke. Unlike EHT, adoption of healthy lifestyle changes will not cure HT due to renal artery stenosis, but will certainly cause no harm!

Gestational hypertension (GHT) is another type of secondary hypertension. Gestational HT is defined as the onset of HT more than 20 weeks into the pregnancy [44]. To be considered GHT, blood pressure must return to normal within 12 weeks after the birth. Therefore, the diagnosis of GHT is often made in retrospect [45]. GHT is the most common medical complication of pregnancy [44]. Treatments for GHT are very limited, due to the fact that most antihypertensive medications have been found to be teratogenic to some degree.

It is also a temporary diagnosis, which may include the eventual development of preeclampsia [46]. Up to 50% of women diagnosed with GHT develop preeclampsia [47], which is defined as the development of hypertension and proteinuria after 20 weeks of gestation, even into the first week postpartum [48]. Preeclampsia is a condition endangering both the mother and the fetus, and is responsible for up to 20% of maternal mortality, as well as the majority of morbidities, prenatal deaths, preterm births and fetus growth restrictions [48]. Treatment options for GHT and preeclampsia are limited, as many pharmacological treatments may pose severe risks to the fetus [45]. The most common antihypertensive used in GHT is the sympathetic nervous system inhibitor α-methyldopa, which has shown few or no lasting side effects to the mother or foetus [45], yet in depth studies on methyldopa, as well as many other classes of antihypertensives, are lacking. For the most part, treatment of GHT includes bed rest and limiting movement, and if the pregnancy is close to term, labour is induced. If the development of preeclampsia occurs, the most common treatment is for labour to be induced, especially if gestation is close to or at 32 weeks [45, 48].

Cardiovascular Diseases

According to the World Health Organization (WHO), more people die of cardiovascular diseases (CVD) yearly than any other cause or pathological condition, with

approximately 17.3 million deaths due to CVDs in 2008, a number which is predicted to rise to 23.6 million by the year 2030 [49]. Alterations in vascular smooth muscle cell (VSMC) growth, migration, proliferation and plasticity is believed to contribute to abnormal vascular functions associated with or leading to CVDs, such as hypertension, atherosclerosis, and stenosis after angioplasty [50-52]. Under normal physiological conditions, vasoactive peptides, such as angiotensin II (Ang II) and endothelin-1 (ET-1), normalize blood pressure through the regulation of salt and/or water homeostasis, sympathetic nervous system modulation, as well as VSMC contraction and relaxation [53-56]. Increased levels of both ET-1 and Ang II, present in certain pathophysiological states, such as essential hypertension, obesity, or advanced stages of diabetes, have been suggested to contribute to the pathogenesis of CVDs, by activating signaling events intimately linked to migration and proliferation of VSMC [53, 57-59].

1.4 Endothelins

Originally identified in 1988 by Yanagisawa et al. from porcine aortic endothelial cell cultures [60], endothelin (ET) is the most potent vasoconstrictor peptide known. Today, ET has been characterized in almost all organs and physiological systems, and remains one of the most important regulators of blood pressure, sodium and water homeostasis in the body [61]. ET also exhibits important mitogenic and inotropic properties, can stimulate the renin-angiotensin-aldosterone system (RAAS) as well as the sympathetic nervous system (SNS) [54-56]. The general role of ET is to increase vascular tone and blood pressure in response to potential hypotensive states. However, through its mitogenic properties, ET is thought to play an important role in vascular remodeling associated with hypertension, which contributes to the development of various CVDs [62], by increasing cell proliferation, hypertrophy and cell migration, through the activation of several signal transduction pathways linked to these events in the cardiovascular system [63, 64].

1.4.1 Structure, Regulation and Biosynthesis of ET-1

Three members of the mammalian ET gene family exist and have been characterized: ET-1, ET-2 and ET-3 [61]. All three ET peptides are 21 amino acids long connected by two interchain disulfide bonds (Cys1-Cys15 and Cys3-Cys11) at the N-terminal end, with a cluster of three polar charged side chains on amino acid residues 8-10 and a hydrophobic C-terminus (residues 16-21) containing the aromatic indole side chain at Trp21, essential for its loop configuration and its bioactivity [61] (Figure 1). The ET-2 peptide shares a 90% sequence homology with ET-1, varies from it by only two amino acids (Trp6-Leu7), while ET-3 shares 71 % sequence homology with ET-1 and ET-2, and varies by six amino acids (Thr2, Phe4-Thr5-Tyr6-Lys7 and Tyr14). ET-1 is encoded by a gene on chromosome 6 in humans (chromosome 13 in mouse), while ET-2 and ET-3 are encoded by independent genes located on chromosomes 1 and 20, respectively [65]. The sequences for all ET family members are preserved in mammals, and are related to the sarafotoxin snake venom [66] (Figure 1). Among the three ET peptides, ET-1 is the most important isopeptide in the vasculature.

Multiple stimuli, including hypoxia, shear stress, lipoproteins, hormones and growth factors, free radicals and endotoxins, can induce and/or increase ET-1 generation, while others, such as nitric oxide (NO), natriuretic peptides, nitrovasodilators, heparin and prostaglandins, all of which increase intracellular cyclic guanosine monophosphate (cGMP) levels, inhibit ET-1 production [67]. Production of ET-1 is regulated by the preproET-1 gene, at the transcriptional level [68]. Binding sites for multiple regulatory elements, such activating protein 1 (AP-1), CAAT-binding nuclear factor 1 (NF-1), nuclear factor of activated T-cells (NFAT)-binding domains and GATA binding protein 2 are located in the 5' promoter region of the preproET-1 gene, which mediate ET-1 mRNA induction by several factors, including Ang II and transforming growth factor-β (TGF-β) [69]. The adenine-uracil-rich motifs present in the non-translated 3' region mediate selective destabilization of preproET-1 mRNA, contributing to the relatively short biological half-life of ET-1 (15-20 minutes) [65].

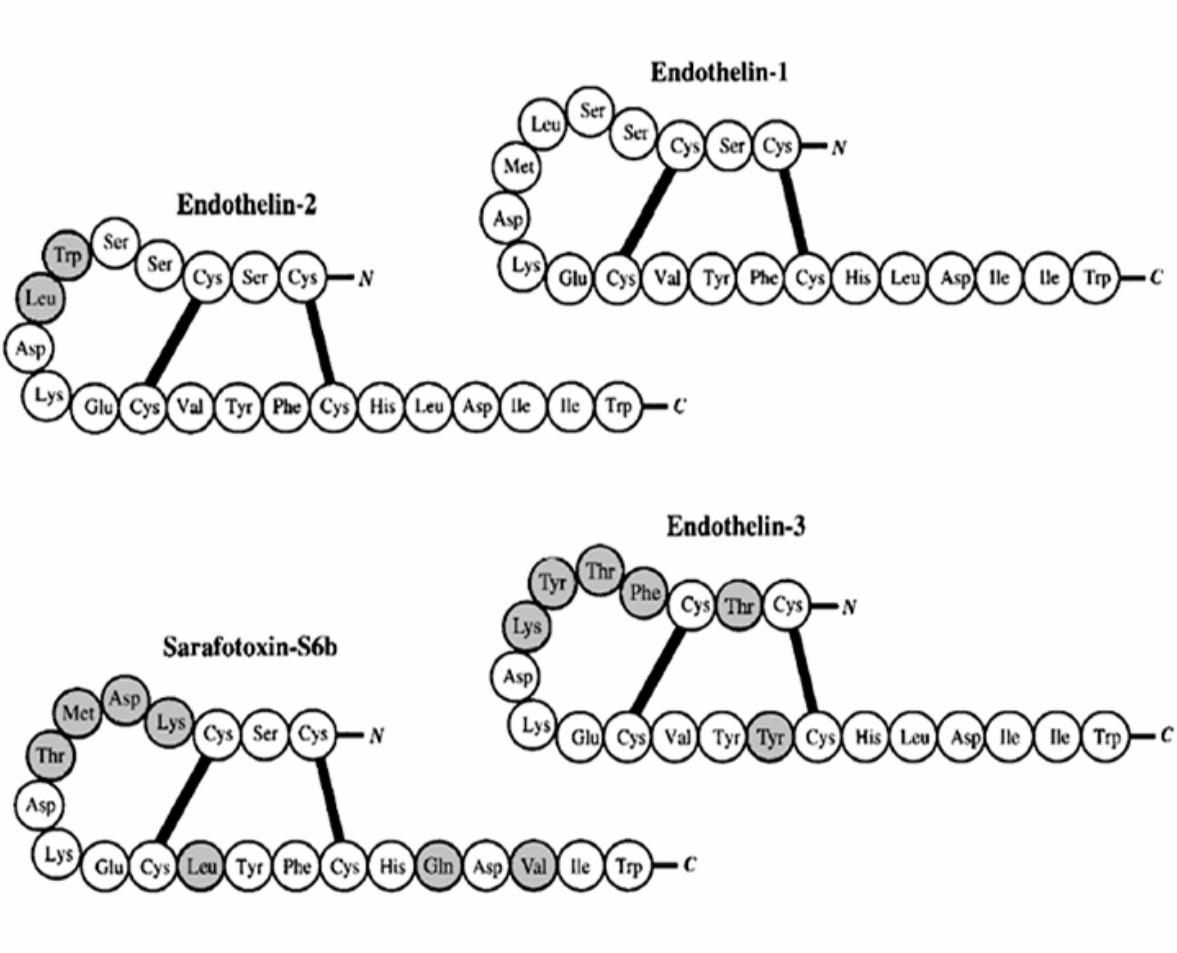

Figure 1: Structure of the Endothelins. Amino acid sequences of the three members of the endothelin family and of the structurally related snake venom toxin sarafotoxin S6b. All three ET peptides are 21 amino acids long connected by two interchain disulfide bonds (Cys1-Cys15 and Cys3-Cys11) at the N-terminal end ET-1 (top right) is a 21 amino acid cyclic peptide with two disulphide bridges joining the cysteine residues at positions 1-15 and 3-11. Grey circles indicate where amino acids differ from those of endothelin-1. (Haynes WG, J. Hypertens 1998, 16(8):1081-98)

Compared to all other cell types, ET-1 is primarily produced in vascular endothelial cells, which express high levels of ET-1 mRNA, preproET-1 mRNA and its converting enzyme [65]. ET-1 has also been shown to be produced by the heart, kidney, the posterior pituitary gland and the CNS [67]. ET-1 is also expressed in VSMC, yet its production in this cell type is 100 fold less than in endothelial cells [70]. While ET-2 is secreted by heart, kidney and endothelial cells (albeit in limited quantities), ET-3 does not seem to be present in endothelial cells, and is secreted mainly by the CNS, gastro-intestinal (GI) and endocrine systems [71, 72].

The formation of the mature ET-1 peptide is preceded by multiple steps, including the cleavage of precursor peptides. Human ET-1 mRNA encodes a 212 amino acid peptide named preproET-1 [61]. The signal sequence is then cleaved by a signal peptidase to form proET-1, which is further cleaved by dibasic-pair-specific endopeptidases, including furin and PC7, to from bigET-1, a 38 amino acid peptide whose vasoconstrictor efficacy is two orders of magnitude less than that of the mature ET-1 peptide [73]. BigET-1 is then cleaved between Trp21 and Val22 by one of several endothelin converting enzymes (ECE) to form the mature 21 amino acid ET-1 peptide (Figure 2).

Three isoforms (ECE-1, ECE-2 and ECE-3) of ECE have been identified, all belonging to a family of zinc peptidases and related to neutral endopeptidase-24.11 and Kell protein, but not to angiotensin converting enzyme (ACE). ECE-1 is the isoform predominantly found in endothelial cells, and has the greatest affinity for BigET-1, but has been found to proteolyze other peptides as well. Four isoforms of ECE-1 exist (ECE-1a, -1b, -1c and -1d) and all are derived from alternative splicing of the same gene [74, 75]. ECE-1a is primarily expressed in intracellular vesicles and on the cell surface of ET-1 "producer" cells, such as endothelial cells, while ECE-1b is located mostly near the trans-Golgi network, in the endosomal compartment of "responder" cells, such as VSMC. ECE-1b is then transported to the plasma membrane where it is responsible for the cleavage of extracellular BigET-1 [61, 76]. ECE-1c and ECE-1d are both located on the extracellular face of the plasma membrane [73].

ECE-2 hydrolyzes ET-1, and has a 60% homology with ECE-1. It also has four isoforms, which vary at the amino acid terminus level, possibly responsible for their different intracellular localization [77]. The optimal functional pH of ECE-2 is 5.5, and it has virtually no activity at pH 7.0, indicating that ECE-2 is involved in intracellular processes, particularly at the level of the trans-Golgi network [61, 77]. ECE-1 and ECE-2 are relatively selective for big ET-1, having much less activity in cleaving big ET-2 and big ET-3, yet ECE-1 and ECE-2 knockout mice still display significant levels of ET-1, indicating that ET-1 formation may also be regulated by other proteases and/or enzymes, yet their physiological relevance is not clear [78]. While ECE-3 has been identified, studies have demonstrated that it has a preferential activity for ET-3 [76].

In healthy subjects, circulating concentrations of ET-1 in venous plasma are in the range 0.1-10 pmol/l [79]. While these concentrations are lower than the concentrations able to induce vascular constriction in vivo and in vitro, ET-1 concentrations at the interface between VSMC and endothelial cells are many times higher [61, 70]. This is supported by studies demonstrating that ET-1 is secreted by cultured endothelial cells into the basolateral (towards VSMC) compartment and not apically [80]. In a similar fashion, renal tubule cells secrete ET-1 towards the interstitium, and only minimally into the urine [61]. As such, rather than acting as a circulating endocrine peptide, ET-1 acts primarily as a paracrine factor, affecting local cell and tissue metabolism. Consequently, ET-1 levels in circulation do not reflect its full physiological impact. This phenomenon has led to the use of venous plasma BigET-1 and inactive ET-1 C-terminal fragment concentrations as markers for endothelial ET-1 synthesis, as they better reflect the quantities of ET-1 generated, as opposed to measuring ET-1 generation. This is due to the efficient clearance mechanism of ET-1, owing to its short half life (approx. one minute, although its pressor effects are maintained for up to one hour) and the unusual binding characteristics of the ET receptors for their ligand, which is almost irreversible [81, 82].

Circulating ET-1 is eliminated mainly by the kidney, liver and lungs, the latter of which is responsible for more than 50% of the elimination of ET-1 in humans [81]. ET is degraded mainly by endopeptidases, such as neutral endopeptidase (NEP), found in the

proximal tubules of the kidney, and cathepsin G, generated in vascular endothelial and pulmonary epithelial cells [83]. ET-1-binding to its cell-surface receptors and subsequent lysosomal internalization and degradation are also important mechanisms in ET-1 clearance. This theory stems from studies showing that pulmonary clearance of labelled ET-1 can be blocked by pre-treatment of the cells with a large dose of unlabelled ET-1, suggesting that ET-1 clearance is receptor-dependent [84]. Investigations have also shown an increase in plasma ET-1 levels following a pharmacological blockade of the ET_B receptor [85], yet in most studies the pharmacological blockade of the ET_A receptor had little impact on plasma ET-1 levels [61]. However, studies using specific disease models in which there is thought to be some degree of ET_B dysfunction, such as the ET_B-deficient rat, or the DOCA-salt hypertensive rat model, pharmacological blockade of the ET_A receptor led to an increase in circulating ET-1 levels [84, 86]. As such, it cannot be concluded that the ET_A receptor does not play a role in ET clearance. Moreover, plasma ET levels are increased within 15 minutes of ET receptor blockade, without any effect on C-terminal fragment and Big ET-1 concentrations, confirming that the increase in ET levels is not entirely due to peptide synthesis, but is mediated by ET-1 receptor displacement [87, 88].

1.4.2 ET-1 Receptors

All mammalian ET receptors are coded from two separate genes [61]. ET_A and ET_B are the two main ET-1 receptor subtypes through which ET-1 exerts its biological effects in a paracrine/autocrine fashion. Both of these receptors have been cloned in humans [89, 90], and belong to the rhodopsin class A of seven transmembrane guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs), which signal through activation of G proteins. GPCRs have an approximate 400 amino acid sequence with an N-terminal extracellular region and C-terminal intracellular region, and contain seven 22-27 hydrophobic amino acid transmembrane domains.

The ET_A receptor contains 427 amino acids, and its gene is located on chromosome 4. ET_A has the strongest affinity for ET-1, yet binds ET-2 to a lesser or equal extent, and can also bind ET-3, albeit to a much lessor extent that ET-2 and ET-1 [91]. Studies have

recently reported the existence of splice variants of the ET_A receptor in the rat anterior pituitary gland, of which one variant was found to have reduced efficacy in stimulating adenylyl cyclise activity and mobilizing intracellular calcium (Ca^{2+}) [92]. Nevertheless, it remains to be seen if these splice variants exist in other tissues and have a significant physiological contribution.

The ET_B receptor, whose gene is located on chromosome 13, contains 442 amino acids, and has the capacity to bind all ET peptides with equal affinity [89, 93]. Several splice variants of the ET_B receptor have been identified. One in particular contains an additional 10 amino acids, is found only in humans, and appears not to present any differences in cellular signalling events [94]. A second ET_B receptor variant was found to have important differences in the cytoplasm domain and the 3'-untranslated domain, yet this splice variant is thought to function primarily as a clearance receptor, as it has shown little to no cellular signalling activity [95]. An ET_B receptor splice variant has also been discovered in the rat brain and possibly other tissues, yet its functional characteristics have yet to be identified [96]. ET_B receptors are found predominantly in endothelial and renal tubule cells, yet are expressed in smaller quantities in VSMC, cardiomyocytes, hepatocytes, osteoblasts, neurons, epithelial cells and fibroblasts [61, 90].

ET receptors couple to the G_i, G_q, G_s and $G_{\alpha 12/13}$ members of the G protein family, regulating various signalling cascades, including adenylyl cyclases, nitric oxide synthase (NOS), serine/threonine kinases, tyrosine kinases, cyclooxygenases, amongst others [61, 69]. More often than not, ET_A and ET_B receptors have opposite actions, depending on their localization, even in the same organ, yet many exceptions exist. For example, in the vasculature, both ET_A and ET_B receptors located on VSMC induce ET-1-induced vasoconstriction, cell adhesion and cell growth (Figure 2). In opposition, ET_B receptor binding of ET-1 in endothelial cells induces vasorelaxation, through the release of nitric oxide (NO) and prostacyclin on to VSMC [97], as well as contributing to the prevention of endothelial cell apoptosis, inhibition of ECE-1 expression and increase in ET-1 clearance [98] (Figure 2). Many pharmacological ET receptor agonists and antagonists have been

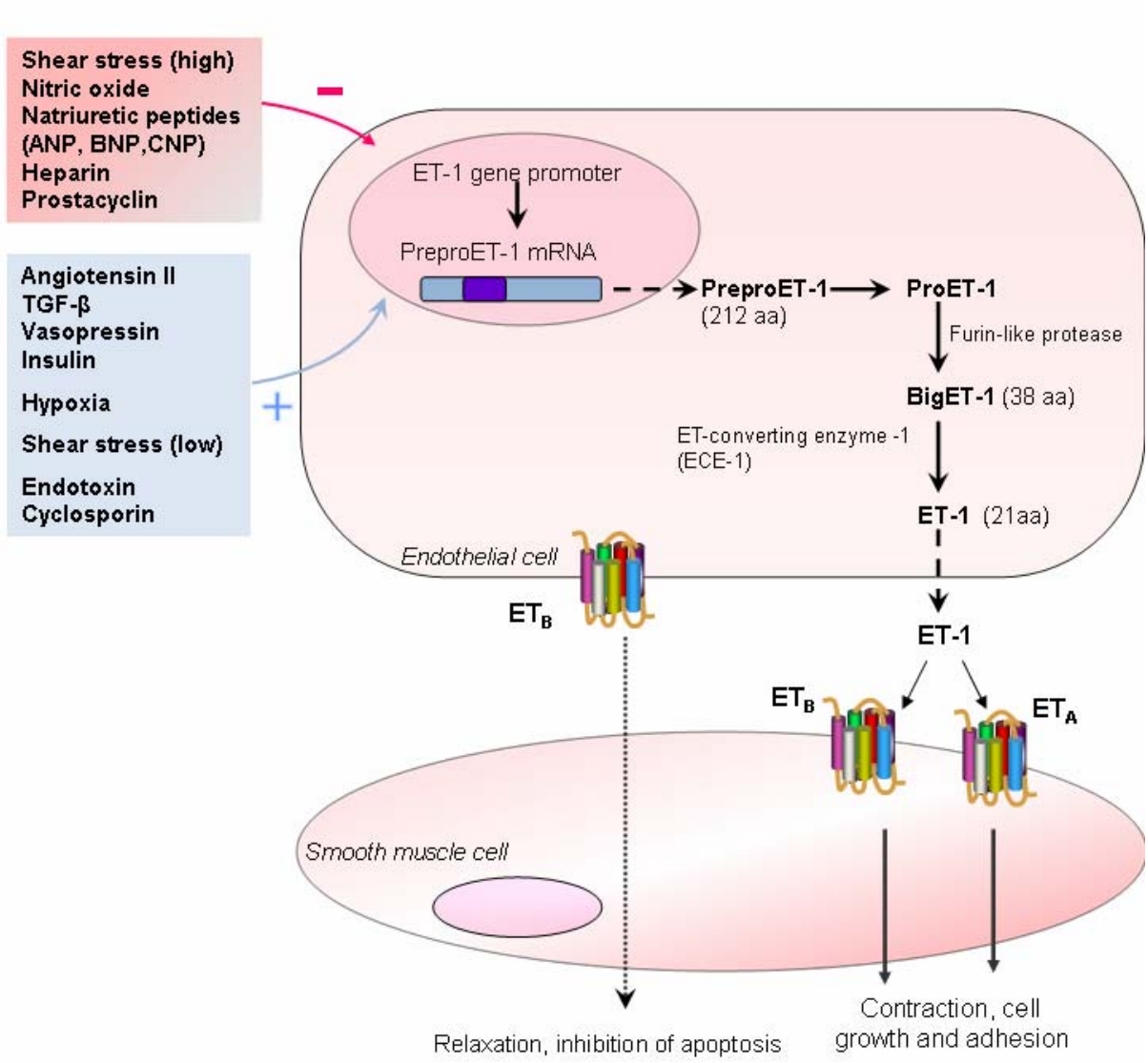

Figure 2: Factors affecting regulation of ET-1 synthesis and its subsequent ET receptor mediated actions on vascular smooth muscle cells. The generation and secretion of ET-1 is regulated by multiple factors, including hypoxia, shear stress and various growth factors and peptides. The formation of the mature ET-1 peptide is preceded by multiple steps. In humans, ET-1 mRNA encodes a 212 amino acid peptide named preproET-1, which is cleaved by a signal peptidase to form proET-1. ProET-1 is again cleaved by dibasic-pair-specific endopeptidases, including furin and PC7, to from bigET-1, a 38 amino acid peptide. BigET-1 is then cleaved between Trp21 and Val22 by one of several endothelin converting enzymes (ECE) to form the mature 21 amino acid ET-1 peptide. In the vasculature, binding of ET-1 to ET_A and ET_B receptors on VSMCs induces vasoconstriction, cell adhesion and cell growth. In opposition, binding of ET-1 to ET_B receptor on endothelial cells induces vasorelaxation, through the release of nitric oxide (NO) and prostacyclin on to VSMCs. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; TGF-β, transforming growth factor β.(Adapted from Remuzzi, G. et al., Nat.Rev.Drug. Disc 2002, 1(12):986-1001)

developed and used to better identify and understand the role of ET receptors in physiological states. For example, BQ123 and BQ788 are highly selective ET_A and ET_B receptor antagonists, respectively, while sarafotoxin 6c is a powerful and highly selective ET_B receptor agonist [99]. Orally active ET receptor antagonists have also been developed and used clinically, such as Bosentan, an $ET_{A/B}$ receptor antagonist, as well as Sitaxsentan, a selective ET_A receptor inhibitor [99]. Use of such compounds has also helped to identify the phenomenon of ET_A and ET_B receptor heterodimerization, possibly affecting receptor functionality, since ET_A/ET_B heterodimers were shown to have delayed ET receptor internalization and a prolonged increase in intracellular Ca^{2+} in response to ET-1 [100, 101].

1.4.3 Biological actions of ET-1

1.4.3.1 ET-1 in the vasculature and heart

Hickey et al. were the first to demonstrate that media taken from cultured endothelial cells produced constriction in classic muscle bath preparations [102]. Yanagisawa et al. later isolated and purified the peptide and gave it the name endothelin, stemming from its endothelial cell origin [60] and demonstrated that a bolus injection of ET caused a transient hypotension immediately followed by a prolonged increase in blood pressure [60]. Later studies indicated that these effects are due to ET_B and ET_A receptor, respectively. However, further detailed studies have shown that ET_B receptor is also implicated in vasoconstriction and induction of hypertensive effects of ET. In VSMC, ET_A and ET_B activation by ET leads to the vasoconstrictor response of ET (Figure 2). Both receptor types were shown to be present on the plasma membrane, cytosol, nuclear envelope membrane and the nucleplasm of human VSMC [103].

On the other hand, ET_B activation by ET in the endothelium leads to the transient vasodilatation response described earlier, usually followed by vasoconstriction (Figure 2). Studies have attributed this vasodilation to the generation of NO via the activation of endothelial NOS (eNOS). Activation of eNOS is thought to occur through ET-1-induced protein kinase B (PKB) phosphorylation and activation in endothelial cells [104]. The

subsequent ET_B receptor-dependent vasoconstriction mediated by ET-1 varies, however, from one vascular bed to another [61]. Veins seem to have a more potent ET_B receptor-dependent vasoconstriction action than arteries, yet the functional implication of these findings remains to be determined [105]. Results from several investigations have suggested that NO release and vasodilatation are mediated by ET_{B1} receptor isotype, while the subsequent vasoconstriction requires ET_{B2} receptor isotype [106]. It is interesting to note that hypotension does not occur when ET concentrations rise slowly, as opposed to a bolus dose [107]. As mentioned earlier, ET response varies from one vascular bed to another. Renal and coronary vascular beds are the most sentitive to the systemic vasoconstrictor effects of ET-1, however, hindquarter skeletal muscle beds display only a minimal ET-1-induced constriction [108-110]. Mesenteric vascular beds, like their coronary counter parts, have a potent ET-1-induced vasoconstrictor response [110] (Figure 3). The concentration and localisation of the different ET receptors in these vascular beds may be the cause of this varying degree of ET-1-induced response. In VSMC, ET-1 is known to also act as a mitogenic factor, as long-term treatment of VSMC resulted in an increased growth and proliferation of this cell type, which was shown to be mediated through ET-1-induced ET_A receptors activation [111, 112] (Figure 3). As well as being a mitogenic peptide in VSMC, ET-1 also displays co-mitogenic properties, potentiating the effects of such growth factors as epidermal growth factor (EGF), platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) [112].

In the heart, the regulation of cardiac function regulated by the ET system has been fairly well studied, particularly the role of ET in cardiac pathophysiological conditions, such as cardiac hypertrophy, ischemia/reperfusion injury, arrhythmias and congestive heart failure [61] (Figure 3). In terms of production, there is some controversy as to whether or not cardiomyocytes produce ET-1. Several studies have reported ET-1 mRNA, mature peptide and/or production in rat and chick neonatal cardiomyocytes, as well as adult porcine cardiomyocytes [113-116]. In contrast, others have demonstrated the absence of ET-1 mRNA in cardiomyocytes from adult pig and rat hearts [117, 118]. This has led to the speculation that

neonatal or embryonic hearts may produce larger amounts of ET-1 than healthy adult, which may produce small amounts [61].

While it may only be produced in small amounts in the heart, ET-1 secreted from VSMC and/or endothelial cells has an impact on cardiomyocyte function [119]. In general, ET has positive inotropic effects on the heart, which is to say an increase in cardiac contractility, yet this varies from species to species, ET dose and duration of exposure, sympathetic nerve activity, underlying cardiac pathology and other contributing factors (reviewed in [120]). Several studies have shown an increase in cardiomyocyte contractility by ET-1 in several species, including mouse, rat, dog and human, yet other studies have detected little or no ET-1-enhanced cardiomyocyte contractility, possibly due to the aforementioned factors [121-125]. Despite the fact that the inotropic effects of exogenous ET-1 were examined in these studies, they did not look at the inotropic effect of endogenous ET-1. In studies where the ET-1 gene was knocked out specifically in mouse cardiomyocytes, the mice displayed an increase in cardiac cell apoptosis associated with the development of a dilated cardiomyopathy as of seven months of age. Until then, no difference in left ventricular function was observed. These studies suggest that while ET-1 production by cardiomyocytes in required for their survival, it may not be crucial for a normal heart function for a large part of the animals' life [126]. In a similar fashion, mice in which the ET_A receptor gene was deleted, specifically in cardiomyocytes, displayed normal baseline and Ang II-induced cardiac contractility, suggesting that ET-1-induced cardiomyocyte contractility is not of physiological relevance [127]. These conclusions are based on the hypothesis that the ET_A receptor is responsible for the inotropic effects of ET (reviewed in [61, 128]). However, diffusion of BQ123, a selective pharmacological inhibitor of ET_A receptor subtype, into left coronary artery of patients presenting with atypical chest pain, reduced contractility. These results confirm that ET exerts a positive inotropic effect via ET_A receptor [129]. Cardiac contractility in response to endogenous ET was also examined in vitro, in studies examining the response of cardiac muscle to stretch test. An increase in both rapid and slow cardiac muscle shortening and/or developed force, due to the Frank-Starling mechanism and slow force response, respectively, was observed after myocardial stretch,

with the study concluding that ET may be involved in slow force response [130]. This was confirmed by studies showing that ET_A receptor antagonism inhibited slow force response in cat cardiac papillary muscle after mechanical stretch [131], yet had no effect on the Frank-Starling response in intact hearts of normal rats [132], substantiating the notion that cardiomyocyte-derived endogenous ET is important to cardiac adjustments, and plays a physiological role [61].

1.4.3.2 ET-1 in the nervous system

ET is known to play an important role in the development of the neural crest and of enteric neurons, as well as of peripheral sympathetic ganglia which modulate systemic hemodynamics [133, 134], where both ET-1 and ET-3 production has been demonstrated in neurons cultured from superior cervical ganglion (SCG) [135], as well as in the dorsal root ganglia [136]. These results demonstrate a clear presence of ET in neurons within the sympathetic ganglia. Spontaneously hypertensive rats (SHR) have been shown to have increased SCG ET-1 levels, as compared to Wistar Kyoto rats (WKY), yet it is not clear as to whether or not this is a significant contributing factor to the increased hypertensive state of SHR [137].

ET mRNA and mature peptides are also present in regions of the hypothalamus and brain stem known to regulate brain function, such as the paraventricular nuclei, dorsal motor nucleus of the vagus nerve, medulla oblongata, choroid plexus and rostral brain regions [136, 138]. Intracerebroventricular (ICV) administration of ET-1 or ET-3 caused an increase in blood pressure and a decrease in heart rate in conscious rats, effects which were reversed by paraventricular nuclei produced NO, suggesting that the hypertensive effect of centrally administered ET may involve baroreflex activity, yet direct ET-induced baroreflex modulation is not clearly demonstrated [139, 140]. ET-1 ICV injection was also shown to produce a pressor response in SHR and WKY [141]. Furthermore, ET_A receptor blockade decreased blood pressure in SHR, but not in WKY, suggesting that endogenous ET-1 from the central nervous system (CNS) causes a tonic hypertensive effect through the ET_A

receptor in SHR [141]. Earlier, Yamamoto et al. reported that ICV ET-1 injections stimulate vasopressin secretion leading to increased blood pressure with a reduction in renal water and electrolyte excretion [142] (Figure 3). It has been also reported that ET, via both sympathetic nervous system and the hypothalamo-pituitary-adrenal axis, and through its interaction with brain natriuretic peptide (BNP) in the CNS, may regulate cardiovascular and hormonal functions [143].

Studies have also demonstrated that ET peptides have the capacity to modulate the release of classical neurotransmitters and influence action potential generation (Figure 3), through the implication of the ET_B receptor, which plays a role in modulation of ATP release, and the ET_A receptor, which is implicated in catecholamine biosynthesis [144, 145]. It may be concluded from these investigations that ET-1, through the activation of ET_B receptor G_i proteins, can inhibit ATP release by decreasing Ca^{2+} influx through L-type Ca^{2+} channels [61]. In summary, the ET system is present in sympathetic ganglia, and is involved in cardiac and vascular innervations, release of neurotransmitters and action potential generation, and as such, participates both directly and indirectly in the regulation of blood pressure.

ET-1 can also affect baroreceptor activity (Figure 3), yet this mechanism is not fully understood [61]. In the dog, baroreceptor activity is inhibited by direct exposure to ET-1 [146]; however, perfusion of low concentrations (1 nM) of ET-1 in isolated rat carotid sinus increased baroreceptor activity, while perfusion with high concentration (10-100 nM) decreased it [147]. These results suggest that the paracrine actions of ET-1 on baroreceptors modulate baroreflex activity and alter blood pressure [61]. In summary, the CNS can synthesize and bind ET through its baroreceptors, leading to a modulation in systemic hemodynamics, yet the mechanisms through which these events take place are still vague.

1.4.3.3 ET-1 in the kidney

Early studies following the discovery of ET-1 in endothelial cells found that ET-1 was produced in large quantities in the kidney, an organ which was identified as a major player in the ET system [148]. In fact, the inner medulla of the kidney was shown to contain

the greatest concentration of immunoreactive ET-1 in the whole body, using the pig as a model [148]. Later studies showed that every cell type in the kidney had the capacity to synthesize ET-1 and also contained ET receptors, which were predominantly abundant in the vasculature and the medullar regions [61], and that the kidney was up to 10-fold more sensitive to the vascular effects of ET-1 than other organ beds [149, 150]. As such, it is not unexpected that the ET system is capable of regulating kidney function and multiple renal functional parameters, such as total and regional blood flow, sodium (Na) and water excretion, drug transporters and acid/base control, cell proliferation, inflammation and glomerular filtration rate (GFR). The renal ET system has also been shown to play an important role in regulating renal injury and disease progression in several pathological conditions [69] (Figure 3).

Exogenously administered high doses of ET-1 have been shown to reduce renal blood flow and GFR in both animals and humans [151-154]. These effects have been linked to decreased Na and water excretion [155]. In studies where ET receptor agonists were administered prior to intravenous administration of BigET-1, ET-1 or ET-3, there was an increase in Na excretion [156-159], but others shown the opposite [160-162]. Concentrations of ET seem to dictate the type of response elicited, yet it should be noted that the control of urinary Na excretion by the renal ET system cannot be fully identified by experiments which have a generalized effect on the kidney.

Evidence from studies using systemic administration of ET seems to be more concrete. These studies have shown an increase in urinary water excretion [160-162], a natriuretic, diuretic and hypotensive effect of ET in the renal medulla. Also, by using transgenic mice exhibiting collecting duct-specific knockout of ET-1 that were given either a normal or high-sodium diet, or given regular water intake, and whose blood pressure and metabolites were examined, Kohan et al. confirmed that ET-1 promotes sodium and water excretion [163, 164]. The potential mechanisms by which ET-1 may increase Na and water excretion are the inhibition of tubular Na^+/K^+-ATPase activity in the proximal tubule and collecting duct [165], and the effects of anti-diuretic hormone (ADH) on tubular osmotic permeability, leading to a decrease in water re-absorption in the collecting duct [166].

Similarly to other tissues, the vascular effects in the kidney are mediated by the activation of either ET_B and/or ET_A receptors. Many investigations have shown that ET receptor subtype varies in the kidney in a species-specific manner, and as such, one species may contain more of one receptor subtype than another. In humans, it has been suggested that approximately 70% of the ET receptors in the renal cortex and the medulla are of the ET_B receptor subtype [167]. In terms of response, the natriuretic and diuretic effects of ET-1 appear to occur via ET_B receptor activation, since these effects were blocked solely by ET_B receptor antagonism, and not by ET_A receptor antagonist treatment [168]. Furthermore, ET_B receptor knockout mice have been shown to be hypertensive, with an increased renal retention of sodium [169]. In summary, the majority of studies seem to conclude that an increase in blood volume is associated with an increase in renal ET-1 production, suggesting that endogenous ET-1 produced by the kidney has an overall natriuretic effect.

1.4.3.4 ET-1 and the endocrine system

While certain corticosteroids, such as cortisol, and thyroid hormones can affect blood pressure, it is not their primary role to do so and as such, do not interact as much with the ET system. On the other hand, hormones such as aldosterone, catecholamines, natriuretic peptides and Ang II directly impact on blood pressure regulation and interact with the ET system, which has been shown to stimulate their secretion. The adrenal gland is one such example. Both human and animal adrenal glands have been shown to produce ET-1, with the discovery of ET-1 mRNA present in these glands [170-173]. ET-1 stimulates both cortical and medullo adrenal hormones, enhances the release of aldosterone from isolated cortical zona glomerulosa cells and stimulates adrenaline release from medullary chromaffin cells [174, 175].

ET can also stimulate production and release of atrial natriuretic peptide (ANP) by the myocardium [61] (Figure 3). In rat studies, ET-3 infusion was able to cause an increase in plasma ANP levels [176]. ET-1 was also able to cause ANP plasma level increases, along with natriuresis, which was blocked by administration of an anti-ANP antibody [177]. ET-1 can also increase ANP peptide and mRNA production in isolated rat adult and neonatal atrial

myocytes [178], as well as in ventricular myocytes [179]. In fact, ET-1 is the most potent secretagogue known to date [180]. It can also lead to increases in brain natriuretic peptide (BNP) mRNA and peptide in cultured rat atrial and ventricular myocytes [152, 181]. While ET-1 may potently increase natriuretic peptides, their biological effects are often opposite. For instance, ET-induced renal vasoconstriction is prevented by ANP, while ANP-induced vasorelaxation is inhibited ET-1 in rat aortic SMC, through a decrease in cGMP levels [182, 183]. In cardiac myocytes, ET-1 was able to inhibit cardiac natriuretic peptide (CNP)-induced increases in cGMP and decreases in contractility [184]. ANP was also able to decrease basal, ANG II- and thrombin-induced ET-1 synthesis in human endothelial cells [185]. In summary, while local cardiac ET-1 can generate natriuretic peptide production, ANP can then in turn downregulate the cardiac and vascular effects of ET-1.

Adipose tissue is another important player in the endocrine system which may be affected by ET-1 actions. ET-1 was shown to inhibit adiponectin secretion through a phosphatidylinositol 4, 5-bisphosphate (PIP2)/actin-dependent mechanism in basal and insulin-stimulated 3T3-L1 adipocytes [186]. A decrease in adiponectin expression and secretion by white adipose tissue has been positively correlated with a decrease in insulin sensitivity and observed in insulin-resistant states such as obesity, type 2 diabetes and CVD [186] (Figure 3). A recent study has demonstrated that stimulation of adipocytes with ET-1 leads to an increased secretion of Interleukin-6 (IL-6), a pro-inflammatory cytokine. Increased levels of IL-6 are also thought to contribute to insulin-resistance and CVD (Figure 3). These studies imply that increased levels of IL-6 mediate ET-1-induced insulin resistance [187].

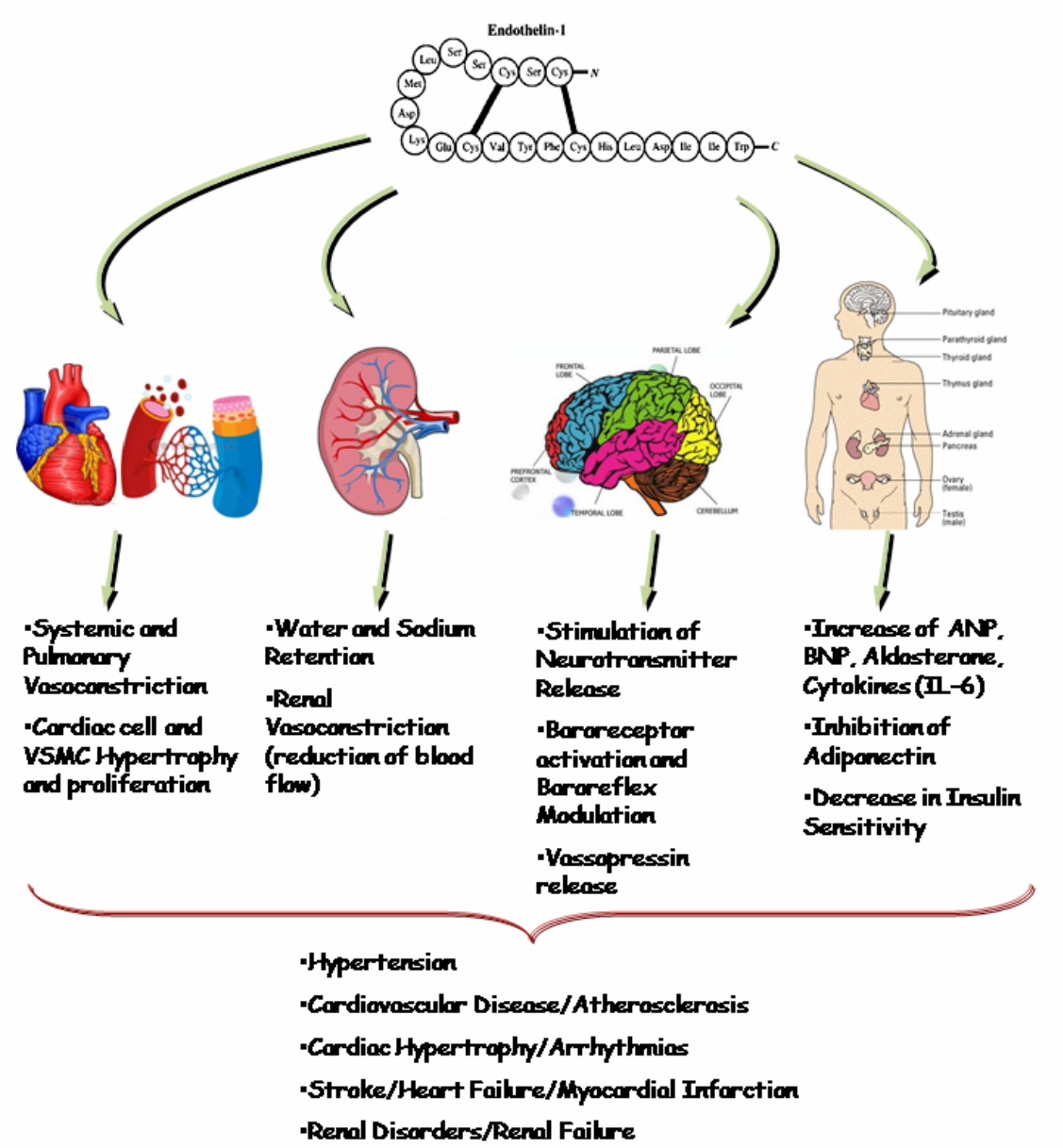

Figure 3: Biological actions of ET-1 on various systems leading to multiple pathophysiological states. ET-1 plays an important role in maintaining blood pressure and vascular homeostasis, yet a heightened ET-1 activity can lead to increases in systemic and pulmonary vasoconstriction, cardiac cell hypertrophy and proliferation, water and sodium retention, modulations in baroreceptor activity and modulation of various hormones. These physiological changes are thought to contribute to the development of vascular pathologies, such as hypertension, atherosclerosis, restenosis, arrhythmias, congestive heart failure, kidney disease, glucose intolerance and diabetes.

1.5 Role of ET-1 in cardiovascular disease

The significant implication of ET-1 in multiple cellular functions and in many physiological systems signifies that a dysregulation of the ET system should lead to the development of multiple cardiovascular pathophysiological states, such as hypertension, atherosclerosis, cardiac hypertrophy, congestive heart failure and coronary artery disease, as well as CVDs linked to diabetes, pulmonary hypertension, kidney failure and other important processes (reviewed in [61]).

1.5.1 Role of ET-1 in essential and experimental hypertension

Studies investigating the role of ET in essential hypertension have revealed no change in plasma concentrations of ET-1 in hypertensive patients as compared to plasma concentrations of normotensive patients [188], most probably due to the fact that ET-1 is generally produced and acts in an autocrine and/or paracrine fashion. As such, plasma concentrations of ET-1 are not an accurate indicator of essential hypertension. However, other factors, such as age, smoking and renal dysfunction, seem to lead to more significant increases in ET-1 levels than does essential hypertension [189]. Ethnicity also seems to play a significant role in the measurement of ET-1 levels in hypertensive patients, as increased plasma ET-1 levels were observed in hypertensive African Americans [190]. While ET-1 levels may not be a clear indicator of hypertension, it is interesting to note that increased levels of preproET-1 are regularly detectable in the endothelium of small arteries of patients with moderate or severe hypertension [191], correlating with the enhanced role of hypertrophic signalling caused by increased ET-1 levels. In VSMC, increased levels of ET-1 protein and mRNA have been shown to lead to the formation of larger elastic and muscular arteries in hypertensive patients [192]. Despite the difficulty in detecting ET-1 due to its low levels in plasma, increased ET-1 signaling is thought to contribute to hypertensive states, based on studies using ET_A or ET_B receptor antagonism (Figure 3). ET_A receptor antagonism led to a greater vasodilation response in forearm vessels of patients with essential hypertension, as compared with normotensive subjects, in whom

vasodilation was also observed [193]. In contrast, ET_B antagonism induced forearm resistance artery vasoconstriction in normotensive subjects, yet led to vasodilation and increased forearm circulation of hypertensive subjects [194, 195], indicating that a vasoconstrictor effect of ET_B receptors are found in hypertensive but not normotensive individuals. An increased expression of vasoconstricting ET_B receptors on VSMC of African Americans may explain, in part, the important role of the ET system in these hypertensive patients [190]. Furthermore, selective, as well as dual-acting ET receptor blockers can reduce systemic blood pressure, as described in an Australian study of 293 patients with mild-to-moderate essential hypertension which were treated with Bosentan, an ET_A/ET_B receptor antagonist [196]. The reduction in blood pressure by Bosentan was similar to that observed in patients treated with the angiotensin converting enzyme (ACE) inhibitor enalapril [196]. Darusentan, a selective ET_A receptor antagonist, also significantly reduced blood pressure in hypertensive human patients [197], without elevation of liver enzymes, a side-effect of Bosentan.

While pharmacotherapy may be one means to help combat hypertension, physical activity and an active lifestyle have been long lauded for its beneficial effects. In fact, recent data indicates that moderate aerobic exercise reduces ET-1-mediated vasoconstrictor tone [198] (Table 2). These reductions in the activation of the ET-1 system by aerobic exercise could contribute to the known beneficial effects of exercise in the treatment and/or prevention of hypertension and CVDs.

The pressor actions of ET-1 are also thought to contribute to the pathogenesis of CVDs in animal and experimental models of hypertension [60]. Significant increases in ET-1 plasma concentrations are observed in certain models of hypertension, such as deoxycorticosterone acetate (DOCA) salt-hypertensive rats, DOCA salt-treated spontaneously hypertensive rats (SHR), Dahl salt-sensitive rats, Ang II-induced hypertension, 1-kidney 1-clip Goldblatt hypertensive rats, and stroke-prone SHR (reviewed in [199]). All are experimental models of hypertension that exhibit an increase in systemic levels of ET-1. This increase in ET-1 production and signalling has been associated with the remodelling and hypertrophy of the vasculature, including resistance arteries [200].

Pharmacological antagonism of either one or both ET receptors led to regression of growth and hypertrophy, as well as a decrease in blood pressure.

Proliferation of VSMC in blood vessels of the aforementioned models of experimental models of hypertention, induced by ET-1, may be mediated partly by increased ROS production. Several studies have reported that ET-1 activates NADPH oxidase in VSMC and in blood vessels, leading to an increased ROS production [201-203]. Similar results were seen in studies using an aldosterone-induced hypertensive model, where systolic blood pressure, plasma ET, systemic oxidative stress, and vascular NADPH activity were all increased and were associated with an increase in small artery hypertrophic remodeling [204].

1.5.2 Role of ET-1 in atherosclerosis and heart failure

The main characteristics of atherosclerosis vary and include a wide range of events, such as endothelial cell injury, vessel inflammation, infiltration of monocytes in the vessel wall, release of growth factors and cytokines, lipid accumulation in foam cells and migration of VSMCs to the intima region of the artery (reviewed in [205]) (Figure 4). Due to its hypertrophic and pro-inflammatory effects, ET-1 plays a major role in the development of atherosclerosis, in addition to its role as a blood pressure modulator [206], through elevation of ET-1 protein and receptor levels in experimental models of atherosclerosis and in human coronary artery atherosclerotic plaques [207-209]. ECE-1 has also been shown to be significantly increased in apolipoprotein E-deficient (apoE) atherosclerotic mice [210], adding yet another arm of the ET system to the development of atherosclerosis. ET_A receptor antagonism was able to reduce atheroma formation in these atherosclerotic apoE mice [211]. The same study also revealed that chronic ET_A receptor blockade normalized NO-mediated endothelial dysfunction, an important component in the development of atherosclerosis [211]. Furthermore, through infusion of BQ123, an ET_A receptor antagonist, Bohm et al. observed an improvement in coronary vascular function in patients with coronary artery disease, suggesting that ET receptor blockade may be a possible therapeutic strategy to improve coronary vascular function in patients with atherosclerosis [212].

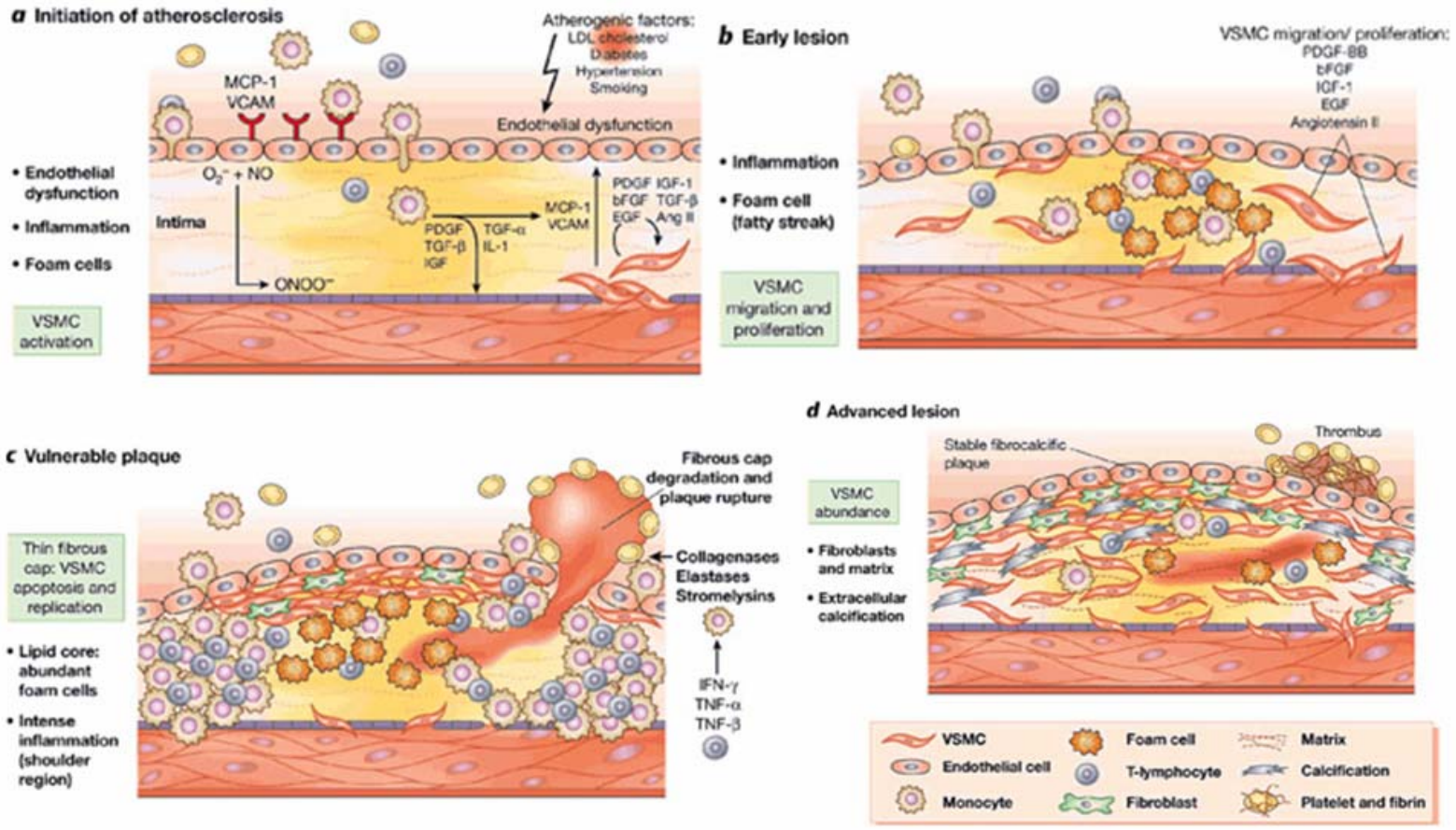

Figure 4: Events leading to the dysfunction of endothelial cells and VSMCs leading to the development of atherosclerosis. The main characteristics of atherosclerosis vary and include endothelial cell injury, vessel inflammation, infiltration of monocytes in the vessel wall, release of growth factors and cytokines, lipid accumulation in foam cells, proliferation and migration of VSMCs to the intima region of the artery. LDL, low-density lipoprotein; MCP, monocyte chemoattractant protein; VCAM, vascular cell adhesion molecule; PDGF-BB, platelet-derived growth factor (BB, -chain homodimer); TNF, tumor necrosis factor; TGF, transforming growth factor; IL, interleukin 1; IGF, insulin-like growth factor; bFGF, basic fibroblast growth factor; Ang II, angiotensin II; EGF, epidermal growth factor; IFN, interferon. (Dzau VJ et al., Nat Med. 2002, 8(11):1249-56.)

Ballinger et al. have also recently contributed to the notion of development of atherosclerosis by ET-1 through the ETA receptor in studies showing that ETA receptor activation by ET-1 led to an increase in the size of glycosaminoglycans (GAG) on chondroitin/dermatan sulfate proteoglycans synthesized by VSMC [213]. This increase in GAG size lead to an increase in low density lipoprotein (LDL) binding, and increased vessel lipid binding is a known early event in human coronary artery atherosclerosis [213].

In addition to atherosclerosis, increased ET-1 levels have also been associated with animal and human heart failure [214, 215] (Figure 3), and studies have established a positive correlation between ET-1 plasma levels and cardiac hemodynamics and function in patients with congestive heart failure [216]. Low cardiac output, a common symptom of heart failure, leads to an increase in ET-1 production. This phenomenon has been demonstrated in models of low cardiac output and in models of low ventricular filling pressures, produced by constriction of the thoracic inferior vena cava [217]. The increased production of ET-1 can in turn stimulate the secretion of multiple neurohormones, which can cause long-term effects on the heart, and contribute to the development and progression of heart failure [218]. A possible treatment option may be the blockade of ET_A and/or ET_B receptors (reviewed in [219]). Initial studies in patients with symptomatic heart failure, such as the REACH study (Research on Endothelin Antagonism in Chronic Heart Failure) and the ENABLE (Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure) trial showed no improvements in clinical status of patients upon treatment with ET antagonists, and were prematurely stopped due to unexpected increases of adverse events, such as hypotension and abnormal liver function [220, 221]. However, others have demonstrated the beneficial effects of ET_A and/or ET_B receptor blockade in limiting the complications of heart failure [222, 223]. Amongst several pharmacological compounds, Bosentan was able to improve and pulmonary hemodynamics in heart failure patients [222]. Similar results were observed in a 3-year clinical study of end-stage heart failure patients with pulmonary hypertension, on a waiting list for cardiac transplantation [223]. Bosentan was able to significantly improve hemodynamic parameters in these patients, and increased their one-year survival rates on the cardiac transplantation waiting list by 20% [223]. Despite these and

other positive results, more research is needed in this area to adjust treatment dosages with the goal of reducing negative side effects or to develop more potent, less toxic molecules to help improve the quality of life of heart failure patients through ET-1 cascade interactions.

1.6 Angiotensin II and the Renin-Angiotensin-system (RAS)

The RAS is one of the principal hormonal systems regulating blood pressure, blood flow, fluid volume and electrolyte balance in the body [224]. As well as being part of the endocrine system, it also acts in both a paracrine and autocrine fashion on local tissues and organs. Angiotensin II (Ang II) is the primary effector of this system and has been found to play a role in most, if not all, organs, including the heart and vasculature, brain and kidneys, having both necessary beneficial functions and pathological effects (reviewed in [53]). Short term stimulation leads to vasoconstriction and salt/water homeostasis, which leads to the regulation of blood pressure. Long term exposure, or chronic stimulation, has been shown to stimulate hypertrophic effects in VSMCs, as well as being involved in cardiac hypertrophy and remodeling, in-stent restenosis, renal fibrosis and a host of other mechanisms leading to other cardiopathophysiological states [53].

1.6.1 Structure, Regulation and Biosynthesis of Angiotensin II through the RAS

To understand the full extent of its structure and regulation, the biosynthesis of Ang II by the RAS must first be described in detail.

Classically, the substrate of the RAS system is angiotensinogen (AGT), a plasma α-glycoprotein, part of the serpin serine-protease inhibitor family, synthesized by the liver and always present in the plasma in high concentration. AGT mRNA has also been recently discovered in brain, male and female reproductive systems, heart, vasculature and skin (reviewed in [225]). AGT is cleaved in the circulation by the aspartyl protease renin, an enzyme secreted from the granular cells of the juxtaglomerular apparatus of the kidney, usually in response to a decreased NaCl sensing by the macula densa of the distal tubules in the kidney, extracellular fluid volume or blood pressure (Figure 5).

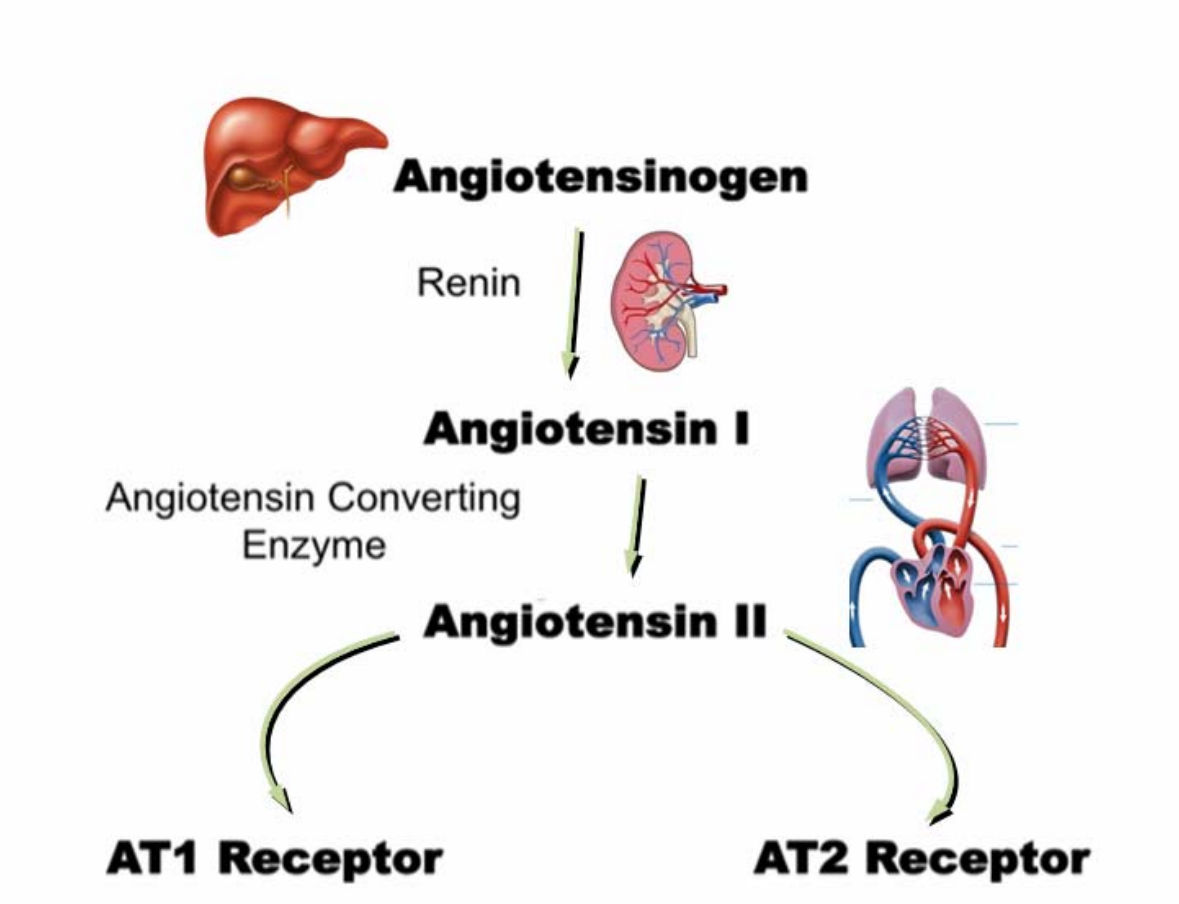

Figure 5: Synthesis of Angiotensin II (Ang II) by the renin-angiotensin system (RAS). Angiotensinogen, a plasma α-glycoprotein, synthesized by the liver and always present in the plasma in high concentration, is cleaved in the circulation by the aspartyl protease renin, an enzyme secreted from the granular cells of the juxtaglomerular apparatus of the kidney, usually in response to a decreased NaCl, extracellular fluid or blood pressure, to form the decapeptide angiotensin I. During passage through the lungs via pulmonary circulation, Ang I is again cleaved to form the octapeptide angiotensin II (Ang II) by the dipeptidyl carboxypeptidase angiotensin I converting enzyme (ACE), a membrane-bound metalloproteinase predominantly expressed in high concentrations on endothelial cell surface in the pulmonary circulation. Ang II can then lead to multiple physiological effects through activation of its two main membrane bound receptors, Ang II type 1 receptor (AT_1R) and Ang II type 2 receptor (AT_2R). (Adapted from Angus PW et al., J Gastroenterol Hepatol 2008, 23(9):1327-38)

Once secreted into the blood, renin will cleave a leucine-valine bond (in humans, or a leucine-leucine bond in other species) of AGT to form the decapeptide angiotensin I (Ang I). During passage through the lungs via pulmonary circulation, Ang I is again cleaved to form the octapeptide angiotensin II (Ang II) (Figure 5). The dipeptidyl carboxypeptidase angiotensin I converting enzyme (ACE), a membrane-bound metalloproteinase predominantly expressed in high concentrations on endothelial cell surface in the pulmonary circulation, is responsible for the cleavage of Ang I to form Ang II, by cleavage of the C-terminal histidine and leucine amino acids of Ang I [226].

Ang II, also known as Ang (1-8) (Figure 6), is an 8 amino acid peptide (free amino group-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-free carboxyl group) [227]. Free N-terminal and C-terminal groups allow for further cleavage of Ang II to generate smaller peptides, such as the formation of Ang (2-8) (Figure 6), also known as Ang III, formed by the removal of the N-terminus amino acid aspartic acid (Asp) by Aminopeptidase A (APA). The Ang III peptide consists of seven amino acids starting from the second amino acid of Ang II [228]. Ang III is biologically active, with approximately 40% of the vasocontricting properties and 100% of the aldosterone stimulating properties of Ang II. Ang III can be further cleaved at its Arg-Val N-terminal bond by aminopeptidase N or B to form Ang IV (known as Ang (3-8)) [229] (Figure 6). Ang IV has been shown to affect the central nervous system, and has been shown to signal through its own distinct receptor (AT_4R), with some of its actions opposing those of Ang II [229]. Another angiotensin peptide, Ang (1-7), is formed from the cleavage of either Ang I by APA, ACE, neutral endopeptidase (NEP) and prolylendopeptidase (PEP) on both N- and C-terminal amino acid bonds, or by cleavage of Ang II by APA and ACE2, an ACE homologue that acts as a carboxypeptidase by selectively removing the C-terminal Phe amino acid from Ang II [230]. Both Ang (1-7) and ACE2 have recently been demonstrated to have biological activity leading to vasodilation and many other actions, opposing the vasoconstrictor actions of Ang II [230].

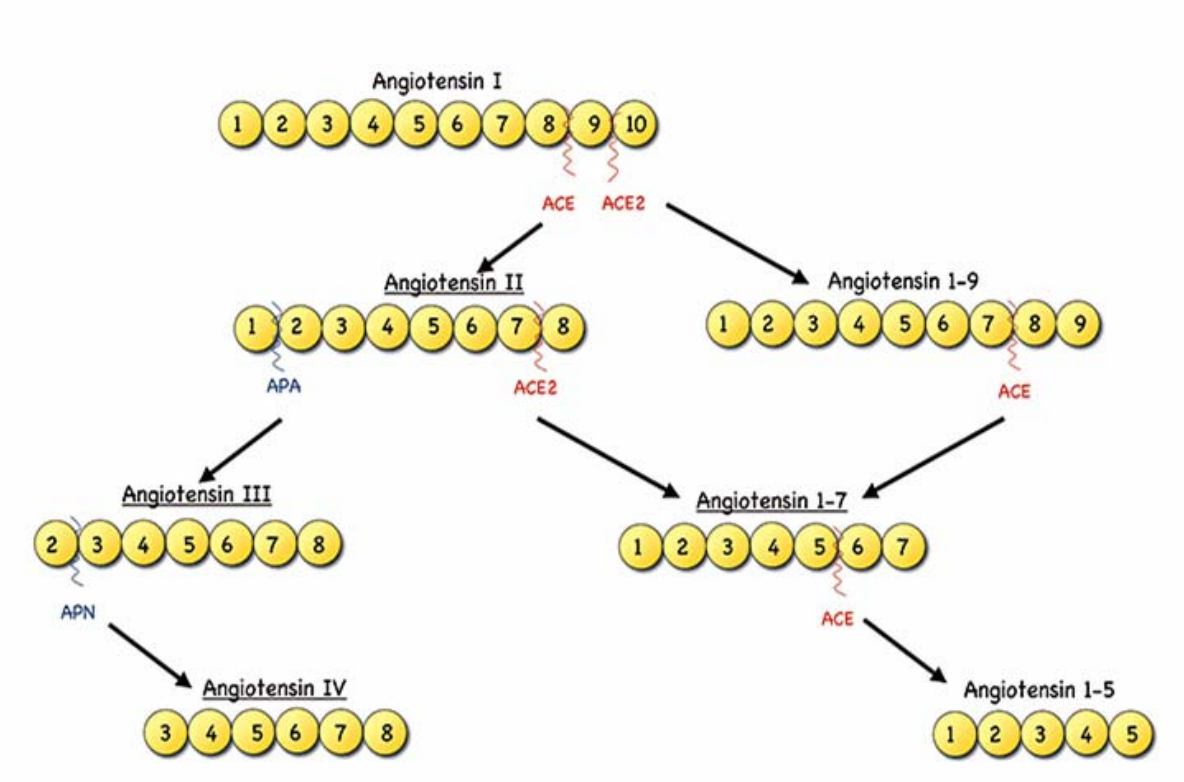

Figure 6: Structure of Angiotensins. Angiotensin I (Ang I) is a decapeptide (Ang-(1-10)) formed by the cleavage of angiotensinogen by the aspartyl protease renin. This peptide can be fragmented by several different enzymes, such as Angiotensin I converting enzyme 1 or 2 (ACE1 or ACE2) to form other biologically active angiotensin peptides, such as Angiotensin II (Ang II). Ang II, also known as Ang (1-8), is an 8 amino acid peptide. Free N-terminal and C-terminal groups allow for further cleavage of Ang II to generate smaller peptides, such as the formation of Ang III (2-8) formed by the removal of the N-terminus amino acid aspartic acid (Asp) by Aminopeptidase A (APA). Ang III is also biologically active, with approximately 40% of the vasocontricting properties and 100% of the aldosterone stimulating properties of Ang II. Ang III can be further cleaved at its Arg-Val N-terminal bond by aminopeptidase N (APN) to form Ang IV (known as Ang (3-8)). Another angiotensin peptide, Ang (1-7), is formed from the cleavage of either Ang I by APA, ACE, neutral endopeptidase (NEP) and prolylendopeptidase (PEP) on both N- and C-terminal amino acid bonds, or by cleavage of Ang II by APA and ACE2, an ACE homologue that acts as a carboxypeptidase by selectively removing the C-terminal Phe amino acid from Ang II. Amino acids are given numerical values, where 1, aspartic acid; 2, arginine; 3, valine; 4, tyrosine; 5, isoleucine; 6, histidine; 7, proline; 8, phenylalanine; 9, histidine; 10, leucine. (Angus PW et al., J Gastroenterol Hepatol 2008, 23(9):1327-38).

1.6.2 Ang II Receptors

The effects of Ang II are mediated by two main membrane bound receptors, Ang II type 1 receptor (AT_1R) and Ang II type 2 receptor (AT_2R), both of which have been cloned and characterized [231, 232]. AT_3R and AT_4R subtypes have also been described [233, 234], yet these subtypes have not been fully characterized and do not account for the main vasoactive effects of Ang II [235].

The AT_1R is a seven transmembrane-domain G protein-coupled receptor, activating PLC through the heterotrimeric Gq protein, although it may also signal through G_i, $G_{11/13}$, and G_s [236]. The AT_1R has an approximate molecular weight of 50 kDa and is composed of 359 amino acids. It has four extracellular (including the N-terminus), four intracellular (including the C-terminus) and seven α-helical transmembrane domains. The N-terminus, first and third extracellular loops contain the epitopes for peptide binding [53]. AGTR1, the gene which codes for AT_1R, is located on chromosome 3q21-25, spanning approximately 60kb and includes five exons and four introns [237]. Multiple factors have been shown to regulate AGTR1 expression, such as LDL, insulin, IGF-1, hyperglycemia, hypoxia and sodium chloride, only to name a few [53].

The AT_1R is widely distributed throughout the cardiovascular systems and is also abundantly found in the renal, endocrine and nervous systems in humans [237]. In the vasculature, VSMC present high levels of AT_1R, while low levels of AT_1R are detected in the adventitia [237]. Through myocardial biopsies, high levels of AT_1R were also discovered in atrial and ventricular myocytes, as well as fibroblasts, of the human heart [238]. As compared to humans, rodents have two functionally distinct subtypes of the AT_1R, the $AT_{1A}R$ and the $AT_{1B}R$. Both have more than 95% amino acid sequence homology between themselves [239]. While angiotensin receptors do not have an intrinsic kinase activity, they are phosphorylated on serine, threonine and tyrosine residues, which are important for receptor activation, regulation and desensitization responses [240].

Like the AT_1R, the AT_2R is also a GPCR consisting of 363 amino acide, with an approximate molecular weight of 44 kDa. The AT_2R only has a 30% homology with the AT_1R and the AGTR2 gene coding the AT_2R is located on chromosome X [241]. AT_2R is mainly expressed in fetal mesenchyme, uterine smooth muscle, brain, ovary, adrenal medulla and heart, and plays an important modulatory role during embryonic development [241]. AT_2R expression decreases rapidly, however, after birth. In adults, this receptor is expressed mainly in pancreas, heart, kidney, adrenal brain and vascular tissues (reviewed in [235]). Furthermore, unlike the AT_1R, the AT_2R does not undergo receptor internalization, and has been shown to counteract several of the vasoconstrictor effects of the AT_1R [242].

Most of the vascular effects of Ang II are mediated by the AT_1R [53]. This receptor is responsible for the vasoconstrictor effects of Ang II, as well as vascular cell hypertrophy and retention of sodium. AT_1R activation has also been shown to increase ROS production, as well as taking part in the induction of inflammatory and fibrotic processes in the vasculature, such as superoxide production, endothelin secretion, lipid peroxidation and adhesion molecule expression (reviewed in [243]). Ang II can mediate these effects either by direct AT_1R activation, or by indirectly causing the secretion of other growth factors, vasoactive peptides or inflammatory cytokines, through a cross talk with other intracellular signaling cascades. In general, the hypertrophic effects of Ang II are mediated by the activation of pathways that involving the phosphorylation of tyrosine residues of certain proteins, generally leading to enhanced gene expression (reviewed in [235]).

The exact physiological roles of the AT_2R have yet to be elucidated, yet it seems that as a general rule, the AT_2R antagonizes several of the Ang II-induced AT_1R activated events, inducing vasodilation, apoptosis and inhibiting cell growth and hypertrophy [244]. The physiological relevance of AT_2R blood pressure regulation was initially demonstrated with the overexpression of this receptor in VSMC of transgenic mice, in which Ang II infusion did not lead to an increase in blood pressure, but did so in wild type mice [245]. Aortic explants from the AT_2R overexpressing transgenic mice had an increased cGMP production, as well as a decreased Ang II-induced vascular constriction. The results of this study also showed that the AT_2R in VSMC is responsible for an increased production of

bradykinin, leading to a stimulation of the NO/cGMP cascade, which in turn promotes vasodilation [245]. Despite these results, the AT_2R has also been shown to be implicated in certain pathophysiological conditions. For example, the ratio of AT_2R to AT_1R increases dramatically in the heart of heart failure patients [246]. It has also been reported that the level of AT_2R increased in vascular inflammation and injury [247]. AT_2R activation has also been shown to have pro-inflammatory and hypertrophic effects, such as NF-κβ activation, leading to vascular and cardiac hypertrophy [248-250]. These contradictory results concerning the AT_2R emphasize our limited understanding of the signaling and physiological role of the AT_2R.

1.6.3 Biological actions of Ang II

1.6.3.1 Ang II and the RAS in the vasculature and heart

While renin in the vascular wall is quite difficult to detect due to very low concentrations, renin mRNA been detected in both human and rat vessels [225, 251]. ACE is abundant in both the vasculature and heart, and is localized predominantly on endothelial cell surface, with smaller quantities present in the adventitia and minute levels found in VSMC, yet ACE levels in VSMC have been shown to increase in certain pathophysiological states, such as neointimal formation [252, 253] (Figure 7). Both AT_1R and AT_2R are present in the vasculature, albeit in different cell types. Cultured VSMC were shown to express only the AT_1R, while cultured endothelial cells were shown to express both AT_1R and AT_2R [231, 254]. Nonetheless, AT_2R distribution in vascular cell types is still a matter of debate, as recent studies have suggested AT_2R expression in VSMC as well [255].

RAS in the vasculature contributes to the regulation of cardiovascular homeostasis, through the opposing effects of the two Ang II receptor subtypes and their impact on vascular function. While it is difficult to distinguish the effects of vessel wall generated Ang II to those of plasma generated Ang II in *in vivo* studies, several studies have been successful in demonstrating the conversion of Ang I to Ang II in isolated perfused rat

hindquarters, which was inhibited by ACE inhibition, demonstrating the presence of a functional RAS system in the vasculature contributing to the formation of local Ang II [256].

Similarly to the vasculature, difficulties in distinguishing between plasma-born and intra-cardiac produced Ang II in the heart have been encountered. Even so, the actions of RAS inhibitors, such as ACE inhibitors and Ang II receptor blockers, on the heart can be explained by modulation at the local cellular level. Such is the case of cardiac remodeling [225].

Several studies have demonstrated the presence, albeit low, of renin mRNA in cardiac tissue [251, 257, 258]. Transgenic rats carrying a genomic construct of the mouse Ren-2 gene under control of its own promoter expressed high levels of renin mRNA in the heart [259], providing further proof that at least in certain species, the heart is source of extrarenal renin production. Cardiac ACE production is also quite evident and can easily be measured in both rat and human heart tissue [251, 260]. As for cardiac Ang I and Ang II, studies using radiolabeled peptides have demonstrated that more than 90% of Ang I and more than 75% of Ang II are synthesized locally in the heart [261].

1.6.3.2 Ang II in the nervous system

The existence of the RAS system in the nervous system and the brain has been known since the early 1960's, through cross-circulation studies in dogs demonstrating interactions between Ang II and the central nervous system leading to an increase in blood pressure [262]. Shortly thereafter, renin activity was reported in the human brain, and renin mRNA was confirmed in synaptosomes, as well as in the rat and mouse brain [263, 264]. The hypothalamus, pituitary and pineal glands seem to be the regions of the brain exhibiting high renin-like activity [265, 266]. Renin protein and mRNA is also measurable in astrocytes, neurons and glia cells in human, rat and mouse samples [263, 266, 267]. More recently, renin promoter activity was detected in mouse astrocytes and neurons [268], leaving little room for doubt that brain renin truly exists.

ACE is also clearly expressed throughout multiple regions of the brain, including the choroid plexus, caudate putamen, cerebellum, brain stem and the hippocampus [269], and

seems to colocalize with renin in synaptosomal fractions of several brain regions, including the thalamus, hypothalamus and posterior pituitary gland [264]. The detection of Ang II in the brain is also quite clear and distinguishable, since the blood-brain barrier was shown to be impenetrable to Ang II [270]. The highest levels of brain Ang II are detected in the hypothalamus, pituitary gland and cerebral cortex, yet is also present in the cerebellum, hippocampus, olfactory bulb and brain stem [271].

Angiotensin receptors have easily been detected in brain tissue [272]. While both $AT_{1A}R$ and $AT_{1B}R$ mRNA have been detected in the hippocampus, cingulated cortex and choroid plexus of the rat brain, much higher levels of $AT_{1B}R$ than $AT_{1A}R$ were detected in the anterior pituitary gland [273]. It is widely accepted that the $AT_{1A}R$ seems to be involved in the control of central blood pressure, while the $AT_{1B}R$ controls drinking responses in rodents such as mice [274]. AT_2R was also detected in several brain regions, including nuclei of the thalamic, medial geniculate and optic tract regions [275]. Distribution of AT_1R and AT_2R in the mouse brain closely resembles that of the rat, yet in human brain tissue, only the AT_1R was detected in the forebrain, midbrain, pontine, medullary spinal cord and choroid plexus, while both AT_1R and AT_2R were detected in the molecular layer of the cerebellum, in contrast to results obtained from rat cerebellum [276]. Unlike other tissues, however, the brain and nervous system are also rich in AT_4R, which binds Ang IV, which seems to be implicated in stress, spatial learning and memory acquisition [277]. These Ang IV binding sites seem to be abundant in cortex, hippocampus, amygdale and thalamus of mice, while rat AT_4R were also present in the piriform cortex, habenulae, colliculi and the arcuate nucleus of the hypothalamus [278, 279]. Despite this plethora of knowledge, it still seems that brain Ang II receptors remain to be characterized. A study demonstrated that the majority of Ang II receptors in the gerbil brain differed from the AT_1R, AT_2R and the AT_4R [280]. As such, extensive research remains to be accomplished to fully characterize the RAS system in the brain and nervous system. The RAS functional impact on blood pressure regulation, drinking and food intake, effect on the blood-brain barrier, central actions on the reproductive system, as well as its actions on motor control, behavior and

emotions and the visual system are quite evident and well documented (reviewed in [225]) (Figure 7).

1.6.3.3 Ang II in the kidney

The intrarenal RAS system has been studied to an extensive length, yet difficulties in determining the contributions of plasma Ang delivered to regions of the kidney from locally produced renal Angiotensin still exist, as the RAS is known to act in an endocrine, paracrine, autocrine and intracrine fashion [281]. Nonetheless, the kidney is unique, as every component of the RAS has been detected and compartmentalized in the tubular and interstitial networks [282], with renal Ang II found in levels much greater than can be explained by delivery from arterial blood flow. These results substantiate the notion that the majority of the Ang II concentrations found in renal tissues are generated locally from angiontensinogen (either delivered to the kidney or locally produced in proximal tubular cells) [283, 284].

Renin is present in the kidney, secreted by cells in the juxtaglomerular apparatus, which is then channeled to the renal interstitium, leading to production of renal Ang I [285]. ACE is present in the kidney, and found in abundance in the proximal and distal tubules, as well as in the collecting ducts and in renal endothelial cells [286]. As such, all components required to produce Ang II are present in the nephron.

The activation of AT_1R and AT_2R, which are distributed in various regions of the kidney, are responsible for most of the actions of Ang II on renal function. The AT_1R is abundantly present in the proximal tubule, the ascending limb of the loop of Henle, the vasa recta, arcuate arteries, and juxtaglomerular cells [287]. In rodents, the $AT_{1A}R$ seems to be expressed on the renal microvasculature in both the cortex and medulla, afferent and efferent arterioles VSMC, epithelial cells of the thick ascending limb of Henle, the proximal tubular apical and basolateral membranes, as well as mesangial cells, distal tubules, collecting ducts, and cells of the macula densa (reviewed in [282]). It should be noted, however, that afferent arterioles express both the $AT_{1A}R$ and $AT_{1B}R$, while efferent arterioles express only the $AT_{1A}R$ [288]. Multiple studies have suggested the importance of

the intrarenal RAS sytem and the angiotensin receptors in the regulation of blood pressure. Amongst them, Coffman et al. clearly reported a 20mmHg decrease in blood pressure in a kidney specific $AT_{1A}R$ knock-out mouse displaying normal $AT_{1A}R$ expression in all other tissues [289]. Further evidence from both clinical and experimental kidney transplant studies have shown that kidney recipients had higher blood pressure post-transplantation if the kidney came from a hypertensive or high blood pressure prone donor [290], strengthening the role of the intrarenal RAS system in the pathobiology of hypertension (Figure 7). The endocrine role of the RAS system and Ang II was described earlier in the introduction of the RAS, and as such, will not be repeated here.

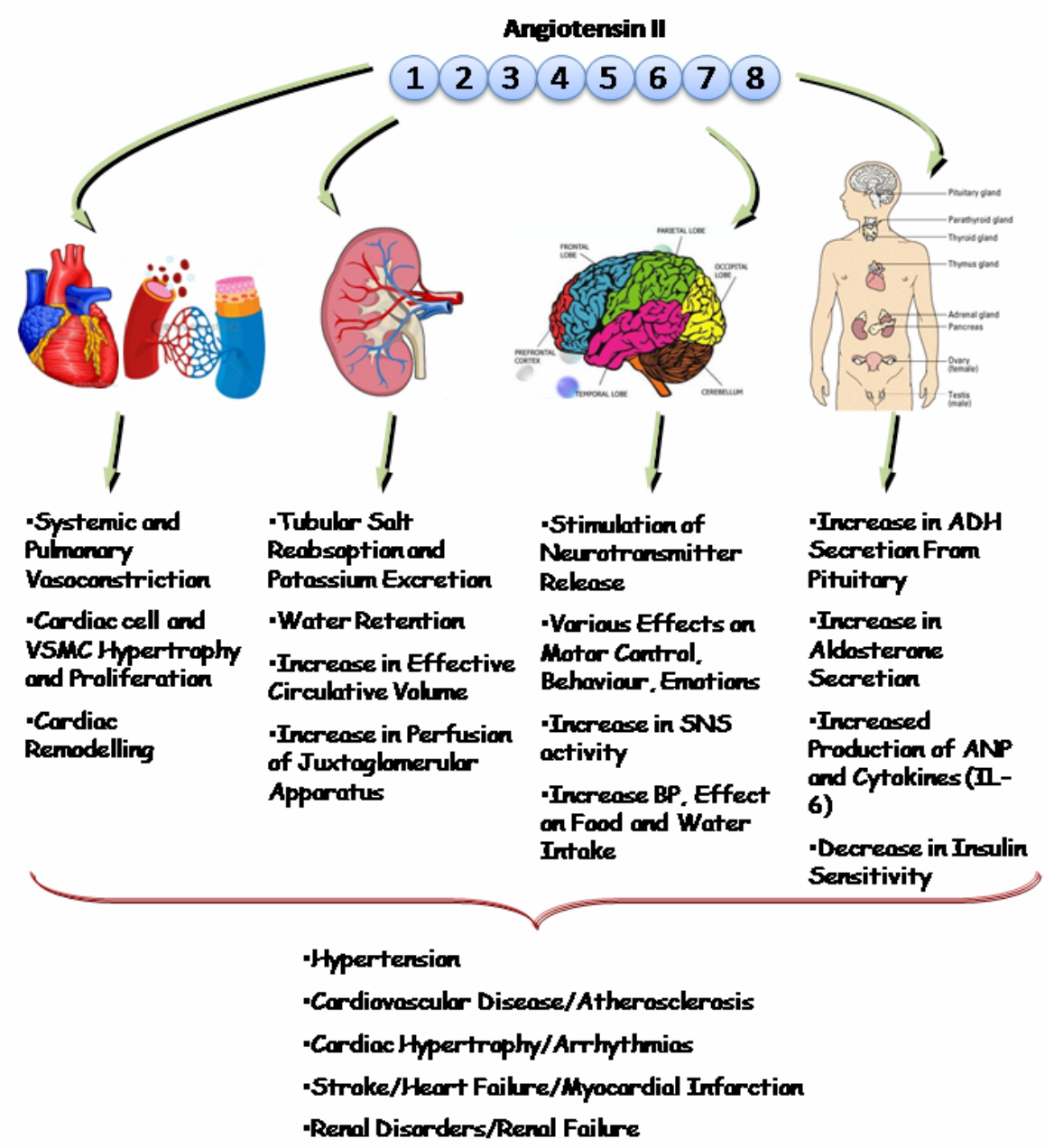

Figure 7: Biological actions of Ang II on various systems leading to multiple pathophysiological states. Ang II plays an important role in maintaining blood pressure and vascular homeostasis, yet a modulation in Ang II production and signaling events can lead to increases in systemic and pulmonary vasoconstriction, cell hypertrophy and proliferation, water and sodium retention, changes in neurotransmitter secretion and SNS activity, and modulation of various hormones. These physiological changes are thought to contribute to the development of vascular pathologies, such as hypertension, atherosclerosis, restenosis, arrhythmias, congestive heart failure, kidney disease, glucose intolerance and diabetes.

1.7 Role of Ang II in Cardiovascular Disease

1.7.1 Role of Ang II in hypertension

While the abnormalities leading to the development of essential hypertension remain obscure, recent studies have suggested that Ang II and the RAS play a critical role in the development of renal dysfunction and essential hypertension [291] (Figure 7). This notion stems from the effectiveness of ACE inhibitors and Ang II receptor blockers in the treatment of essential hypertension, even in subjects with relatively normal Ang II plasma levels [292]. Other studies have shown that transplantation of a kidney from a hypertensive subject to a normotensive recipient causes hypertension in the latter and transplantation of a kidney from a normotensive subject to a hypertensive patient cures the latter's hypertension, in both human and animal experiments (reviewed in [291]), further supporting the involvement of Ang II and the RAS system in the development of essential hypertension. Other studies have also added a more novel mechanism, oxidative stress, which can be activated by both ET-1 and Ang II, and leads to multiple pressor effects potentiating Ang II's vasocontricting effects, in the development of essential hypertension [293] (Figure 7).

Several experimental models of hypertension involving modulation of the RAS system also exist and have been and continue to be useful in elucidating the role of Ang II in the development of hypertension. One such model is the Goldblatt two kidney one clip model, where one renal artery is constricted and the other untouched, resulting in a sustained increase in blood pressure due to increased plasma renin and subsequent increase in Ang II production, as salt and water retention remain normal due to one intact kidney [294]. Approximately six weeks into the kidney constriction, the increase in Ang II levels will lead to excessive aldosterone release from the adrenal cortex, causing salt and water retention, at which point the hypertension of this model will become volume dependent [294] (Figure 7). Subcutaneous infusion of low doses of Ang II for a prolonged period of time (usually four to eight weeks) is also effective in causing hypertension in animal models [295].

Recent studies have also placed the importance of Ang II and the RAS in genetic models of hypertension, such as the SHR, where chronic RAS inhibition improved medularry blood flow, increased renal perfusion and response to Ang II, as well as blocking the development of hypertension in SHR [296]

1.7.2 Role of Ang II in atherosclerosis and heart failure

Atherosclerosis is a pathology affecting whole arteries and can lead to damage to multiple organs, as well as myocardial and cerebral infarction and peripheral artery disease. The development of atherosclerotic plaque involves the migration and proliferation of VSMC whose phenotype changes as the disease progresses [53]. Atherosclerosis is caused by an accumulation of focal or diffuse lipid-packed and fibro-proliferative plaque in the vessel intima, leading to narrowing of the vessel lumen, which is different from the concentric medial thickening observed in hypertension. Many researchers regard atherosclerosis as being an inflammatory disease, with Ang II leading to the progression and destabilization of atherosclerotic plaque through its pro-inflammatory actions [297] (Figure 7).

Both systemic and local RAS and Ang II-AT_1R signaling contribute to the development of atherosclerosis, through the ability of Ang II to increase expression of certain adhesion molecules (which promote monocyte invasion into the vasculature), chemokines and cytokines. These molecules cause endothelial cell dysfunction, proliferation of VSMC and oxidation and uptake of LDL, through the Ang II-induced ROS generation by VSMCs [298]. In more advanced stages of atherosclerosis, MMP and PAI-1 expression are stimulated by Ang II, which causes a destabilization of atherosclerotic plaque [299, 300]. Experimentally, ACE inhibitors have been used to strengthen the link between Ang II and atherosclerosis in a large number of animal studies. The atherosclerosis-prone apoE-deficient ($apoE^{-/-}$) mouse is one of the more frequently used atherosclerosis animal models. In a recent study, treatment with fosinopril, an ACE inhibitor, of the $apoE^{-/-}$ mouse for a period of 12 weeks reduced atherosclerotic lesions by up to 70% [301]. Other studies using olmesartan, an AT_1R blocker, have observed a potent

suppression in atherosclerotic lesion formation in apoE$^{-/-}$ mice [302]. These studies strengthened the notion that Ang II-AT_1R signaling inhibition reduces the size of atherosclerotic lesions, but also stabilizes atherosclerotic plaque [302]. In contrast, several studies have shown that stimulation of AT_2R leads to an inhibition of atherosclerosis through inhibition of oxidative stress. These studies also indicated that AT_1R blockers have the capacity to stimulate AT_2R, as shown in a study using valsartan, whose anti-atherosclerotic effects were attenuated in AT_2R-deficient mice [303].

In regards to heart failure, local Ang II concentrations have been found to be dramatically increased in the failing heart, with cardiac Ang II production increasing as heart failure progresses [304] (Figure 7). However, the full role of Ang II and the RAS in heart failure are not completely understood, as studies have shown contradictory results. For example, several transgenic mouse models overexpressing one or several components of the cardiac RAS have been used in experimentation, with some showing increases in ventricular hypertrophy and fibrosis and others maintaining normal cardiac size and function (reviewed in [305]). Others have further demonstrated that hemodynamic changes play a greater role in ventricular hypertrophy than do local Ang II levels [306]. More recent studies using transgenic mice expressing cardiac myocyte specific Ang II-producing fusion protein have shown that Ang II does not alter ventricular size or cardiac function if hemodynamic loading remains intact, yet in hypertensive animals, cardiac Ang II potently enhanced inflammatory responses and oxidative stress, which lead to an increase in cardiac cell death [306, 307]. These results clearly indicate that Ang II plays a role in heart failure, yet the precise mechanisms by which it does so remain obscure. What seems to be a general consensus in many studies, however, is the role of oxidative stress generated by both ET-1 and Ang II on the vasculature and the heart, in addition to their hypertrophic and proliferative effects on those systems, leading to varied cardiovascular pathologies. To fully understand these effects, we must identify the signaling cascades activated by ET-1 and Ang II, which can lead to both physiological and pathophysiological events.

1.8 ET-1 and Ang II-induced signalling events in VSMC

As mentioned earlier, ET-1 and Ang II signal through ET_A/ET_B receptors and AT_1R/ AT_2R, respectively. Both of these receptors belong to the GPCR family of seven transmembrane-domain G-protein bound receptors. G proteins of the GPCRs are heterotrimetric proteins composed of three distinct subunits; α, β and γ subunits [308] (Figure 8). The GDP bound form of α binds tightly to βγ and is inactive, whereas the GTP bound form of α dissociates from βγ and serves as a regulator of effector proteins. Both α-GTP and βγ subunits can interact with effectors. This activation cycle is terminated by intrinsic GTPase activity of α-subunit. The GDP-bound form of the α-subunit has a high affinity for βγ subunit, with which it re-associates to form the heterotrimer in the basal resting state [308]. $G_{\alpha q}$ is associated to the activation of phospholipase C (PLC), leading to the formation of inositol 1,4,5-trisphosphate (IP3) and diacyglycerol (DAG), modulating downstream calcium signaling to activate calcium/calmodulin and protein kinase C (PKC), leading to the activation of the PI3-K/PKB and MAPK pathways [309, 310] (Figure 8).

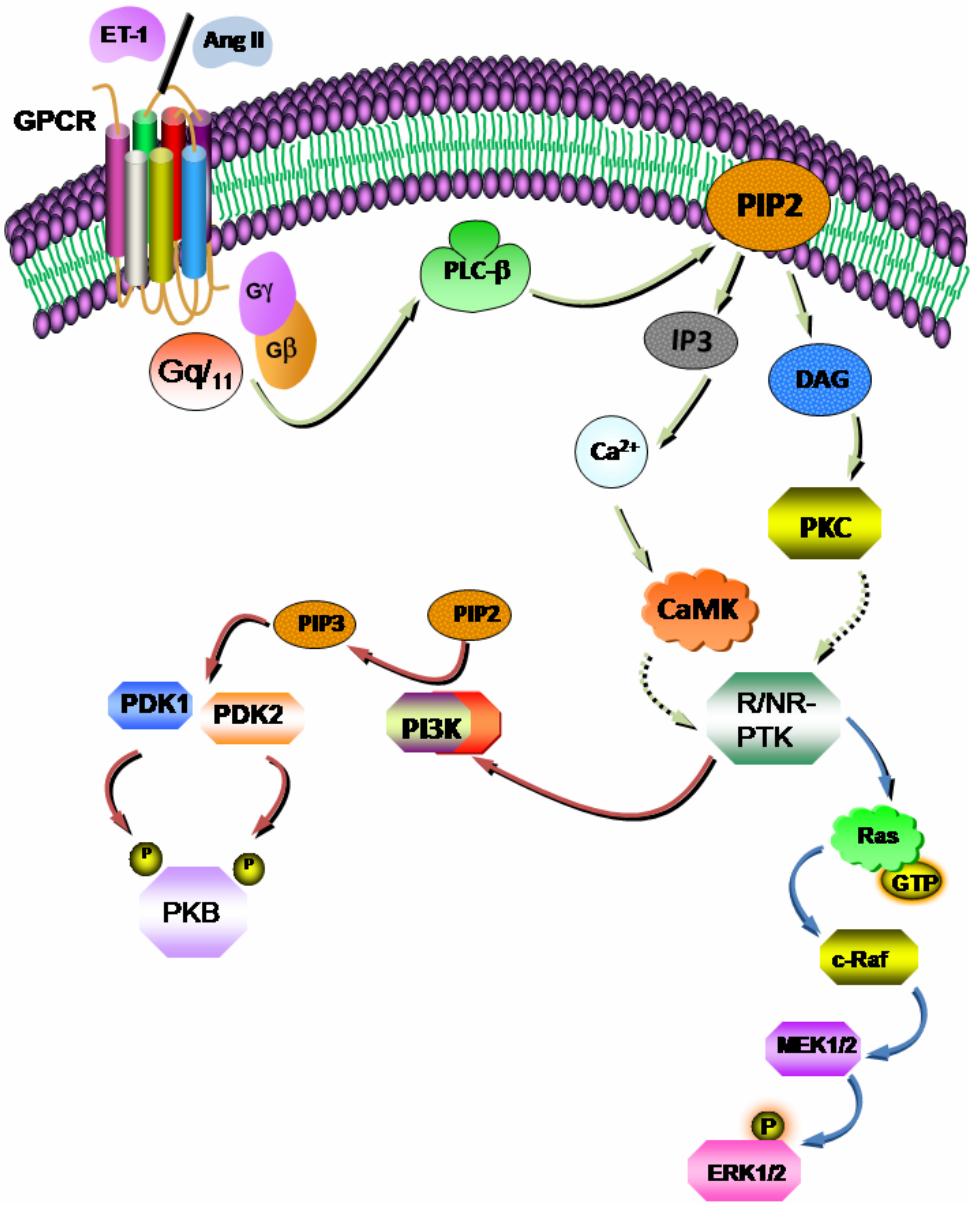

Figure 8: Vasoactive peptide-induced activation of the phosphoinositide cascade through GPCR activation in VSMC. ET-1 and Ang II signal through ET_A/ET_B receptors and AT_1R/AT_2R, respectively. Both of these receptors belong to the GPCR family of seven transmembrane-domain G-protein bound receptors. The GDP bound form of Gα binds tightly to Gβγ and is inactive, whereas the GTP bound form of Gα dissociates from Gβγ and serves as a regulator of effector proteins. Both Gα-GTP and Gβγ subunits can interact with effectors. This activation cycle is terminated by intrinsic GTPase activity of α-subunit. The GDP-bound form of the α-subunit has a high affinity for βγ subunit, with which it re-associates to form the heterotrimer in the basal resting state. $G_{\alpha q}$ is associated to the activation of phospholipase C (PLC), leading to the formation of inositol 1,4,5-trisphosphate (IP3) and diacyglycerol (DAG), modulating downstream calcium signaling to activate calcium/calmodulin and protein kinase C (PKC), leading to the activation of the PI3-K/PKB and MAPK pathways.

1.8.1 ET-1 and Ang II-induced activation of the phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) pathway

PI3-Ks are a family of lipid kinases that phosphorylate the D3 hydroxyl group (3'-OH) of the inositol ring in phosphatidyl inositol (PI) [311]. Products of the PI3-K reaction include phosphatidylinositol-3-monophosphate (PIP), phosphatidylinositol-3,4-bisphosphate (PIP2) and phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) [312]. This lipid kinase has been divided into classes I, II and III. Class I PI3-Ks are heterodimeric proteins, each of which consists of a 110 kDa catalytic subunit and an associated regulatory subunit. This class is further divided into classes IA and IB, of which class IA has three isoforms (α, β and δ) of the catalytic p110 subunit and several forms of regulatory subunits (p85α, p55α, p50α, p85β and p55γ). Class IB, on the other hand, has only one member of the catalytic subunit called p110γ and one form of the regulatory subunit p101. Class IA is activated by receptor PTK (RPTK), while class IB is activated by GPCR [313]. In vitro, PI, PIP, and PIP2 are phosphorylated by class I PI3-K enzymes to form PIP, PIP2 and PIP3. In vivo, phosphorylation of PIP2 is favored, which is responsible for the formation of PIP3 [313, 314].

Class II PI3-Ks, consisting of two major mammalian subclasses, α and β, contain a carboxy-terminal C2 domain, a protein module originally observed in PKC molecules, with phospholipid binding sites. There are no known regulatory subunits in this class, which may not even be necessary.

Class III PI3-Ks are thought to represent the primordial PI3-K that gave existence to the other classes, due to the fact that it is the only class of PI3-K enzymes present in yeast. PI is the only substrate recognized by this class and is phosphorylated to generate PI3P [315, 316]. Class III PI3-Ks induce local increases in PIP3, which are thought to be required for agonist-independent membrane trafficking processes [316].

Generally, the p85 subunit of PI3-K binds to and is activated by IRS-1, in turn activating the p110 catalytic subunit of PI3-K, which catalyses the phosphorylation PI lipids, forming PIP3 and PIP2 [317] (Figure 9). It is important to note that PI3-K activation in

response to other growth factors, e.g. EGF, PDFG, etc, has been shown to signal through an IRS-independent pathway, leading to PKB activation [318, 319]. Following PI3-K activation, formation of PIP3 and PIP2 generates recognition sites for Pleckstrin Homology (PH) domain containing proteins, principally 3'-phosphoinositide-dependent kinase 1 (PDK1), which is translocated to the plasma membrane along with PKB [317, 320], and other related serine/threonine protein kinases, which are responsible for phosphorylating and activating several downstream signaling protein kinases, such as PKB, protein kinase C-zeta (PKC-ζ), and p70 ribosomal S6 kinase ($p70^{s6k}$) [321, 322] (Figure 9). PKB is a 57 kDa protein, given its name due to its high homology with protein kinase A (PKA) and PKC. PKB, also known as Akt, exists as three isoforms, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 [323]. All PKB isoforms have an amino-terminal PH domain, a central catalytic Ser/Thr kinase domain and a carboxy-terminal regulatory domain that contains the hydrophobic motif (HM) [324, 325]. PDK1, which is thought to be constitutively active, phosphorylates Thr 308 in PKB [326], stabilizing the activation loop in an active form. This phosphorylation is a prerequisite for kinase activation, but phosphorylation of Ser 473 is necessary for full PKB activation. The nature of putative Ser 473-PKB kinase called PDK2 is still controversial [327], although several candidates have been suggested [328]. Aside from its well characterized role as regulator of glucose transport, glycogen synthesis, gluconeogenesis and lipogenesis, PKB also plays an important role in protein synthesis, cell growth and cell survival [329-333] (Figure 9).

Several studies have demonstrated a role of the PI3-K/PKB pathway in vasoactive peptide-induced signaling events in multiple cell types, including VSMC, cardiomyocytes and human umbilical vein endothelial cells, leading to increased cell survival, hypertrophy and proliferation. These physiological responses contribute to the development of cardiovascular diseases, such as atherosclerosis and stenosis after angioplasty [64, 203, 334, 335].

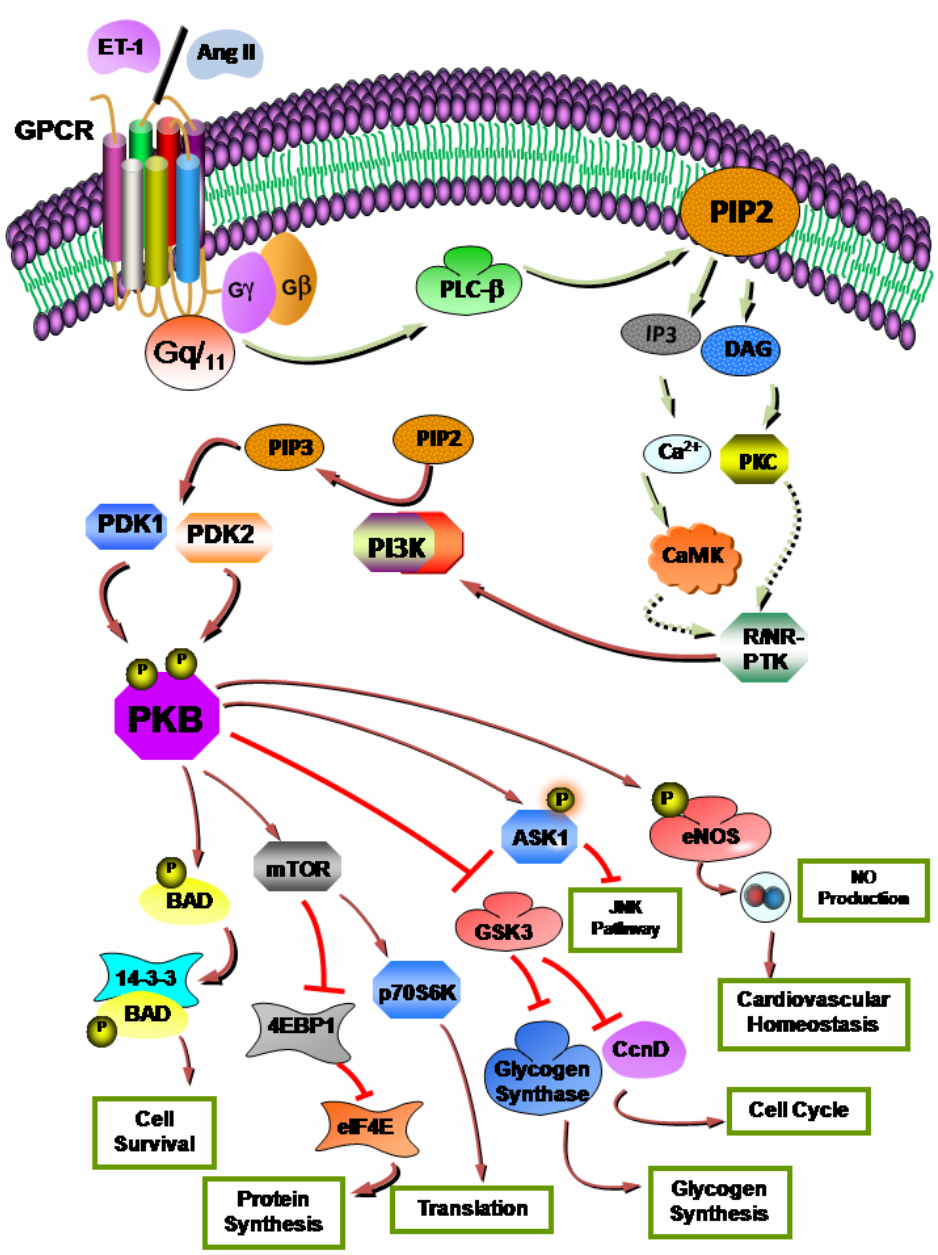

Figure 9: Vasoactive peptide-induced activation of the PI3-K/PKB cascade in VSMC. ET-1 and Ang II bind to their respective GPCRs, causing enhanced activity of PLCβ through Gαq/11 activation, leading to the conversion of phosphatidylinositol 4, 5 biphosphate (PIP2) to inositol 1, 4, 5 trisphosphate (IP3) and diacylglycerol (DAG). This increase in IP3 causes an elevation of intracellular calcium (Ca^{2+}) and DAG-dependent PKC activation. PKC/ Ca^{2+} or other signaling intermediates activate R- and/or NR-PTKs, like EGFR, IGF-1R, PYK2 and c-Src, by inducing their phosphorylation on target tyrosine residues. Activation of these PTKs leads to the phosphorylation of docking proteins, such as IRS-1. Phosphorylated IRS-1 and/or R-PTK bind to the p85 subunit of PI3-K and lead to its activation. Activated PI3-K catalyzes the phosphorylation of PIP2 to phosphatidylinositol 3, 4, 5 triphosphate (PIP_3). PIP3 recruits PKB and PDK-1/2, Pleckstrin homology (PH) domain-containing proteins, to the plasma membrane, where PDK-1 and -2 phosphorylate PKB on threonine and serine residues, which in turn phosphorylates several downstream effectors, which contribute to protein synthesis, cell growth, survival, and gene transcription.

1.8.2 ET-1 and Ang II-induced activation of the Mitogen-Activated Protein Kinase (MAPK) pathway

MAPKs constitute a family of serine/threonine protein kinases which are widely conserved among eukaryotes, and are involved in many cellular responses, such as cell proliferation, cell differentiation, cell movement and cell death [336, 337]. In mammalian cells, 5 MAPK families have been identified, including ERK1/2, c-Jun N-terminal kinase 1, 2 and 3 (JNK1/2/3), also called stress-activated protein kinase (SAPK), p38α/β/γ/δ MAPK, ERK5 and ERK7 [336, 337].

The groups of vertebrate MAPK studied most extensively to date are ERK1/2, JNKs, and p38 kinases [336, 337]. ERK1/2 are stimulated by mitogens, such as polypeptide growth factors (IGF-1, platelet-derived growth factor (PDGF), colony stimulating factor-1 (CSF-1), etc.) as well as insulin and phorbol 12-myristate 13-acetate (PMA). In contrast, SAPKs and p38 MAPK are potently induced by a wide variety of stressors, including ultraviolet irradiation, gamma irradiation, anisomycin, heat shock and chemotherapeutic drugs, yet recent studies have also implicated SAPK/JNK and p38 MAPK in vasoactive peptide-induced proliferative responses [338-344]. The JNK and p38 MAPK pathways are also activated by ischemia or reperfusion after ischemia, and by inflammatory cytokines [345].

The ERK 1/2, JNK and p38 MAPK pathways follow a similar sequence of activation, in which a stimulus activates a MAPKKK, which will then activate a MAPKK, which is an upstream activator of MAPK, the final effector of the cellular response. MAPKKKs are Ser/Thr kinases, and are activated through phosphorylation and/or as a result of their interaction with a small GTP-binding protein of the Ras/Rho family in response to extracellular stimuli (Figure 10). Activation of MAPKKKs phosphorylate and activate MAPKKs, which then phosphorylates Thr and Tyr residues in the activation loop of the kinase, stimulating MAPK activity (Figure 10). Once activated, MAPKs phosphorylate target substrates on Ser or Thr residues followed by a proline. ERK1/2 is the primary MAPK activated (Figure 10). This pathway consists of the MAPKKKs (A-Raf, B-Raf and Raf-1), the MAPKKs (mitogen and extracellular signal regulated kinase 1 and 2

(MEK1/2)), and the MAPKs (ERK1 and ERK2) (Figure 10). Signals from activated receptor tyrosine kinase (RTK) or GPCR to Raf/MEK/ERK are transmitted through different isoforms of the small GTP-binding protein such as Ras. As mentioned earlier, IGF-1R transduces its effect through IRS proteins, where phosphorylated IRS serves as a docking site for Grb-2/Son of Sevenless (SOS), whereas Grb-2/SOS binds directly on the activated EGFR to turn on MAPK signaling. This triggers a conformational change in Ras, a GTP exchanger, resulting in its activation (Figure 10). Once activated, Ras binds to a wide range of downstream effector proteins, including isoforms of the Ser/Thr kinase Raf. Raf then binds to, and phosphorylates, the dual-specificity protein kinase MEK-1 and -2, the latter of which phosphorylates ERK1/2 within a conserved Thr-Glu-Tyr (TEY) motif in their activation loop. ERK1/2 can then be translocated to the nucleus where it can phosphorylate and activate a number of transcription factors involved in gene activation [337, 346] (Figure 10). It can also activate a number of cytosolic proteins, such as $p90^{rsk}$ through its proline directed Ser/Thr kinase activity. Other members of the MAPK family, such as JNK and p38 MAPK, are activated in a similar fashion and mediate cellular functions by phosphorylating downstream targets.

Overwhelming evidence exists to support a vasoactive peptide-induced MAPK activation in multiple systems, including oligodendrocyte progenitor cells, pancreatic stellate cell, aortic and mesenteric artery-derived VSMC, increasing cell growth and hypertrophy, leading to complications such as atrial fibrillation due to left ventricle hypertrophy, vascular remodelling, cardiac hypertrophy and coronary artery disease [203, 347-351].

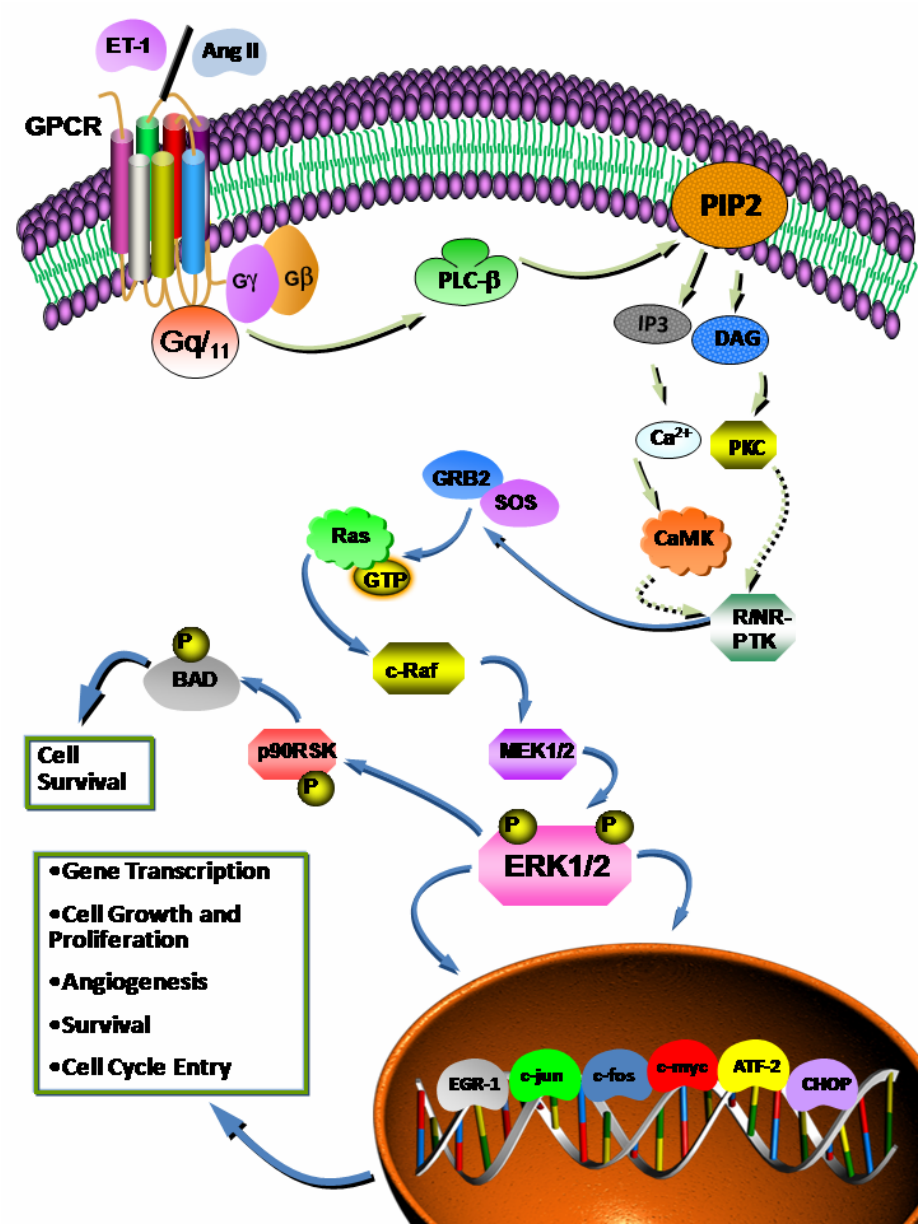

Figure 10: Vasoactive peptide-induced activation of the MAPK pathway in VSMC. ET-1 and Ang II bind to their respective GPCRs, causing enhanced activity of PLCβ through Gαq/11 activation, leading to the conversion of phosphatidylinositol 4, 5 biphosphate (PIP2) to inositol 1, 4, 5 trisphosphate (IP3) and diacylglycerol (DAG). This increase in IP3 causes an elevation of intracellular calcium (Ca^{2+}) and DAG-dependent PKC activation. PKC/ Ca^{2+} or other signaling intermediates activate R- and/or NR-PTKs, like EGFR, IGF-1R, PYK2 and c-Src, by inducing their phosphorylation on target tyrosine residues. Activation of these PTKs leads to the phosphorylation of docking proteins, such as IRS-1, which serve as docking sites for Grb-2/SOS. The latter can bind directly to activated R-PTK, triggering a conformational change in Ras, a GTP exchanger, resulting in its activation. Once activated, Ras binds to Raf, a Ser/Thr kinase, which binds to and phosphorylates the dual-specificity protein kinases MEK-1/2, which phosphorylate ERK1/2. ERK1/2 can then translocate to the nucleus, and regulate cell growth, proliferation and hypertrophy through phosphorylation of transcription factors. Other members of the MAPK family, such as JNK and p38 MAPK, are activated in a similar fashion and mediate cellular functions by phosphorylating downstream targets.

1.8.3 ET-1 and Ang II-induced transactivation of receptor and non-receptor protein tyrosine kinases (R-/NR-PTK)

Activation of growth factor receptor and/or non-receptor protein tyrosine kinases (R-PTK or NR-PTK) has been implicated in transducing the downstream effects of GPCR, leading to the stimulation of the PI3-K/PKB and MAPK cascades, which are involved in mediating the migratory, proliferative, and hypertrophic responses of ET-1 and Ang II [50]. GPCR ligands activate R- and/or NR-PTKs through a process termed "transactivation". Daub et al. first identified this process in 1996, by showing that ET-1 enhanced the tyrosine phosphorylation of epidermal growth factor receptor (EGFR) [352].

1.8.3.1 Epidermal Growth Factor Receptor (EGFR)

The EGFR is one of four members belonging to the Erythroblastic Leukemia Viral Oncogene (erbB) family of R-PTK. As such, EGFR is also known as ErbB1 (ErbB2, ErbB3, and ErbB4 are the other 3 members of this family). This family of transmembrane receptors undergoes homodimerization or heterodimerization to induce autophosphorylation and receptor tyrosine kinase activation in response to ligand binding [353, 354]. The EGFR is universally expressed in a variety of cell types, and is most abundant in epithelial cells and many cancer cells [353, 355, 356]. This receptor contains an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase autophosphorylation and regulatory domain (reviewed in [357]). Recent work has shown that in basal conditions, the kinase domain of the EGFR exists in an autoinhibitory conformational state, and ligand binding-induced dimerization and changes in conformation allow its autophosphorylation, resulting in its activation [358]. This event activates the intrinsic tyrosine kinase activity of the intracellular domain, leading to receptor autophosphorylation on several key tyrosine residues located in the COOH-terminal tail of the receptor [359]. These phosphorylated tyrosine residues then act as docking sites for SH2 and phosphotyrosine-binding domain containing cytoplasmic proteins, such as Grb-2/SOS and the p85 subunit of PI3-K [360], involved in the activation of multiple signaling pathways,

including MAPK and PKB. Both ligand-dependent and -independent mechanisms for its activation have been suggested [361]. To date, seven different ligands, in addition to EGF, are known to bind to the EGFR and cause its activation [362, 363].

Multiple studies have shown that Ang II, and more recently, ET-1, can cause the activation, and subsequent phosphorylation of EGFR through receptor transactivation, a phenomenon stemming from the binding of Ang II to its AT1R [364], and ET-1 to the ET_A receptor [365]. Furthermore, a role of ET_B receptor in ET-1-induced EGFR transactivation has also been demonstrated in VSMC, through the use of the N-terminally truncated or full-length ET_B receptor [365]. ET-1 and Ang II-induced EGFR activation has been demonstrated in multiple cell types, including VSMC, cardiomyocytes, intestinal epithelial cells, preglomerular VSM, C9 cells and pancreatic stellate cells [351, 366-373]. Ang II and ET-1-induced ERK1/2 and PKB phosphorylation were also found to be EGFR-dependent based on studies using pharmacological inhibitors of EGFR, like AG1478 and PD153035. For example, Ang II-induced p38 MAPK and ERK1/2 phosphorylation was attenuated by AG1478 [339]. Furthermore, Ang II-induced PKB phosphorylation was associated with EGFR transactivation in rat aorta VSMC and in intestinal epithelial IEC-18 cells [369, 374]. ET-1-induced ERK1/2 phosphorylation through EGFR activation was also demonstrated in rat cardiomyocytes as well as in rat renal tubular cells [372, 375]. More importantly, the transactivation of EGFR by ET-1 and Ang II has been suggested to play an important role in vasoactive peptide-induced physiological responses linked to MAPK and PKB signaling, such as growth, hypertrophy and proliferation, in multiple cell types, including VSMC [364, 367, 372, 376]. For example, ET-1-induced EGFR transactivation has been implicated in protein and DNA synthesis and c-Fos gene transcription in VSMC [373, 377]. In addition, a role of EGFR in vascular contraction in mouse aortic ring segments and rabbit basilary artery rings has been reported [378, 379].

It is well known that VSMC from spontaneously hypertensive rats (SHR) exhibit exaggerated cell proliferation compared to VSMC from normotensive Wistar Kyoto (WKY) rats [380]. This was thought to occur due to elevated levels of Giα proteins in VSMC from SHR. However, recent reports suggest that endogenously produced ET-1 and Ang II

contribute to the enhanced proliferation of VSMC from SHR, through EGFR transactivation [381], linking growth factor receptor transactivation to the deleterious hypertrophic effects of elevated ET-1 and Ang II levels found in hypertensive states. In these studies, EGFR, AT1R, ET_A and ET_B receptor inhibition decreased exaggerated ERK1/2 phosphorylation found in VSMC from SHR to levels found in VSMC from WKY, suggesting that endogenous ET-1 and Ang II-induced MAPK signaling contributes to the enhanced cell growth of VSMC in SHR through EGFR transactivation [381].

1.8.3.2 Insulin-like Growth Factor Type 1 Receptor (IGF-1R)

IGF-1R, another transmembrane R-PTK, is a tetrameric protein consisting of 2 α- and 2 β-subunits, and has a high degree of homology with the insulin receptor [382]. IGF-1R is activated by IGF-1 and associated growth factors, such as IGF-2, albeit with lower affinity. Importantly, IGF-1R differs from other R-PTKs in that it exists on the cell surface as a covalent dimeric structure, which requires domain rearrangement for activation [383], whereas other R-PTKs, like the EGFR described earlier, dimerize or oligomerize upon ligand binding to trigger receptor activation [384]. The α-subunit of IGF-1R contains the IGF-1-binding site. The β-subunit comprises an intracellular PTK domain that is critical for transducing most of the downstream signaling [382]. The α-chain and the 195 residues of the β-chain make up the extracellular part of the IGF-1R [383]. The mature α2β2 receptor also has a single transmembrane sequence (906-929 residues) and a 408-residue cytoplasmic domain, which possesses tyrosine kinase activity. The N-terminal half of the IGF-1R also contains 2 homology domains, L1 and L2, separated by the Cys-rich region (Cys148 to Cys298). It has been shown that residues 131-315 (Cys-rich and L1 and L2 flanking regions) are required for binding IGF-1 [385]. This cytoplasmic domain is flanked by 2 regulatory regions: a juxtamembrane region, which plays a major role in docking of IR substrates (IRSs), Shc and receptor internalization [386], and a 108-residue long carboxy-terminal tail consisting of 2 phosphotyrosine-binding sites [386, 387]. IGF-1-binding to extracellular α-subunits triggers a conformational change in the β-subunit, resulting in its trans-autophosphorylation in multiple tyrosine residues and evoking the PTK catalytic

activity of the receptor [388]. Activated IGF-1R phosphorylates several downstream substrates, such as Shc and IRSs 1-4, in multiple tyrosine residues [389, 390]. Phosphorylated IRSs serve as docking proteins for many SH2 domain-containing molecules, including growth factor receptor-binding protein 2 (Grb2), the p85 subunit of PI3-K, NcK and SH-phosphatase 2 Grb2 binding to the activated receptor, which recruits SOS, leading to the subsequent activation of the MAPK and/or PI3-K/PKB pathways [391, 392].

While EGFR transactivation by vasoactive peptides has been well described, the requirement of IGF-1R in vasoactive peptide-induced signaling is only more recently coming to light in studies showing a requirement of IGF-1R activation in AngII-induced downstream signaling [393]. These studies bridge the gap with studies done over a decade ago, demonstrating the stimulatory effect of AngII on the tyrosine phosphorylation of IGF-1R β-subunit as well as on IRS-1 rat aortic VSMC [394]. Ang II treatment enhanced tyrosine phosphorylation of the β-subunit of IGF-1R in smooth muscle cells (SMC) isolated from porcine arteries [395]. IGF-1 neutralizing antibody was also used in these experiments, confirming that Ang II-induced IGF-1R phosphorylation was in fact ligand-independent [395]. While this study also showed that AngII-induced phosphorylation of p85 and $p70^{s6k}$ was significantly attenuated by pre-treatment of SMC with AG1024, a selective pharmacological inhibitor of IGF-1R-PTK activity, this drug failed to attenuate AngII-induced ERK1/2 activation [395]. In contrast, AG1024 attenuated AngII-induced phosphorylation of ERK1/2/5 and p38MAPK in mesenteric VSMC [396, 397]. Further consolidating the potential cross-talk of between the Ang II and IGF-1/IGF-1R systems are studies showing that Ang II increases IGF-1mRNA and protein in heart tissue and VSMC [398, 399], and that IGF-1 can increase AT1R expression in VSMC [400]. Moreover, Nguyen et al. have also demonstrated that IGF-1R antisense-induced reduction in IGF-1R was associated with an inhibition of Ang II-induced vascular responses, as well as AT1R expression and functionality, in spontaneously hypertensive rats (SHR) and Wistar Kyoto rats (WKY) [401, 402]. These, and other studies showing that dominant negative or antisense oligonucleotide of IGF-1R are able to attenuate neointima formation in an injured carotid

artery rat model [403], support a potential pathogenic role of upregulated IGF-1R signalling in vascular disease.

1.8.3.3 c-Src

c-Src, also known as Src or $p60^{c\text{-}Src}$, is a NR-PTK and is the cellular homologue of the v-Src avian oncogene of the Rous sarcoma virus, a chicken tumour virus, discovered by Peyton Rous [404]. It is a member of the Src family of NR-TK, which contains 10 other members, including Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes [405]. Src family of NR-TK play a vital role in cell differentiation, proliferation, and survival signaling mechanisms, as well as in cell adhesion, morphology and motility (reviewed in [406]). Src, Yes and Fyn are ubiquitously expressed in all cells, yet expression levels vary among certain cell types, such as platelets, neurons, and osteoclasts, which have been found to express 5–200-fold higher levels of Src protein than other cells [407]. Structurally, each member of the Src family contains a short N-terminal membrane anchor, or SH4 domain, which contains a 14 carbon myristoyl group. This domain is followed by a poorly-conserved 'unique' region of 40 to 70 residues, an SH3 domain of 50 residues, which can bind to specific proline-rich sequences, an SH2 domain of 100 residues, which can bind to Tyr phosphorylation sites, an SH2-linker domain rich in proline residues, an SH1 250 residue tyrosine kinase catalytic domain, and a short C-terminal regulatory region containing conserved tyrosine residues [407]. Each domain of the Src structure plays an important role in Src function. For example, studies have demonstrated that myristoylation allows the membrane localization of Src and is essential for proper functioning of Src in cells [407], yet others have demonstrated that myristoylated Src is found free in the cytosol, and as such, does not guarantee Src binding to the cell membrane [406]. Myristoylation takes place on the myristoyl-rich SH4 (N-terminal) domain, which then binds to the cell membrane. The SH1 domain is the most conserved domain in all the tyrosine kinases, and contains an ATP-binding domain. Tyr 416/418 (416 in chicken and 418 in human) and Tyr 527/530 (527 in chicken and 530 in human) are the two major tyrosine phosphorylation sites which regulate Src activity. Phosphorylation of Tyr 416/418, through an auto-

phosphorylation mechanism, is required for full c-Src activity, while phosphorylation of Tyr 527/530, a site located in the C-terminal regulatory region, is a negative regulator, whose phosphorylation inhibits c-Src activity [408]. In a physiologically normal state, c-Src is found in an inactive state, or "closed" conformation, where the phosphorylated Tyr 527/530 site is linked to the SH2 domain, and the SH2-linker domain is bound to the SH3 domain, keeping the Tyr 416/418 dephosphorylated. Dephosphorylation of Tyr 527/530 causes a conformational change and disrupts the bond of this site with the SH2 domain, thus "opening" the conformation of the c-Src molecule, allowing for access of ATP to the catalytic site and release of ADP, leading to its auto-phosphorylation on Tyr 416/418, and causing c-Src activation [409]. In growth factor receptor signaling, the SH2 domain of c-Src binds to Tyr phosphorylated subunits of the growth factor receptor in question, leading to a conformational change and allowing for Tyr 416/418 phosphorylation and subsequent c-Src activation.

In the vasculature, especially VSMC, GPCR-linked contractile responses, induced by Ang II and phenylephrine, have been associated with c-Src activation [410, 411]. Furthermore, the use of PP-1 and PP-2, pyrazolopyrimidine-based selective inhibitors of Src family tyrosine kinases [412], has indicated a role of Src in mediating H_2O_2, ET-1 and Ang II-induced transactivation of the EGFR and PDGFR in several cell types [64, 413-416]. ET-1 and Ang II-induced MAPK activation has also been reported to be primarily dependent on the activation of c-Src in different cell types, including VSMC [417]. In Src-deficient VMSC derived from c-Src knockout mice, activation of ERK1/2 by Ang II was significantly decreased, as compared to VSMC from wild type mice [418, 419]. c-Src rescue in these cells through retroviral vector transfection caused a significant increase in Ang II-induced ERK1/2 phosphorylation, demonstrating that c-Src activation is necessary for Ang II-induced signal transduction in VSMC [419].

Both c-Src and IGF-1R have been implicated in the activation of NAD(P)H oxidase system [396], and since both ET-1 and Ang II trigger their effect through the generation of ROS [203], activation of IGF-1R and c-Src may be an early event in tranducing vasoactive peptide-induced ROS generation, leading to the activation of growth promoting and

hypertrophic signaling pathways. In fact, c-Src has also been shown to be activated in response to ROS, including H_2O_2, in different cell types [414, 416, 420-422]. Furthermore, through the use of PP-1 and PP-2, a role of c-Src in mediating H_2O_2–induced ERK1/2 phosphorylation in VSMCs derived from mesenteric arteries [423], in CHO-IR cells [422] and in renal cells [414, 424] has been shown. A requirement of c-Src has also been shown in H_2O_2-induced PKB phosphorylation CHO-IR cells [422], renal cells [414], rat-2 fibroblasts [425] and, more recently, in A10 VSMCs [421]. Thus, c-Src appears to play a key role in signaling the effects of H_2O_2, as well as vasoactive peptides.

1.8.3.4 Proline-rich Tyrosine Kinase (PYK2)

PYK2 is a cytosolic Ca^{2+}-dependent, proline-rich tyrosine kinase [426], also known as calcium-dependent tyrosine kinase, cell adhesion kinase β, or related adhesion focal tyrosine kinase (RAFTK), as it has a similar overall structural organization to the focal adhesion kinase (FAK) non-receptor tyrosine kinases [427]. Like FAK, PYK2 has a C-terminal focal-adhesion targeting domain, a catalytic tyrosine kinase domain which is centrally located, an unstructured proline-rich region, and a N-terminal FERM domain, which can bind and auto-inhibit the kinase activity of the tyrosine kinase domain [428]. However, recent reports demonstrate that the PYK2 FERM domain and the FAK FERM domain regulate the activity of PYK2 and FAK, respectively, but do so in different ways [429], indicating a role of the PYK2 FERM domain in the regulation of PYK2 activity [429]. PYK2 is activated by autophosphorylation in Tyr 402, located in its catalytic domain.

PYK2 has been proposed to facilitate the linkage between integrin receptors, heterodimeric transmembrane receptors that connect the extracellular matrix to the cytoskeleton, and the activation of signaling pathways, such as the PI3-K/PKB and MAPK pathways [426, 430]. Additionally, G-protein-associated ERK signaling was shown to be enhanced by PYK2 overexpression [426], which is further supported by studies showing that PYK2 inactivation, or mutation of its auto-phosphorylation sites, attenuates G-protein-induced ERK phospohorylation [431]. Moreover, the increases in intracellular Ca^{2+} and PKC activation via Gαq activation by Ang II have been well characterized, and appear to be the

main mechanism by which PYK2 is phosphorylated on Tyr 402, and thus fully activated by, Ang II [432]. The activated PYK2 forms a complex with shc and Grb/SOS, resulting in the activation of the Ras/MAPK and PI3-K pathways [433]. ET-1 was also shown to induce PYK2 phosphorylation in several cell types, including rabbit and rat VSMC [203, 349, 434]. This phosphorylation was blocked by BQ123, an ET_A receptor inhibitor, but not by BQ788, an ET_B receptor blocker, demonstrating that ET-1-induced PYK2 phosphorylation is dependent of ET_A receptor activation [434]. Furthermore, this study also showed that the Ca^{2+} channel blocker SK&F 96365 inhibited ET-1-induced PYK2 phosphorylation, linking ET-1-induced PYK2 phosphorylation to an increase in Ca^{2+} levels caused by ET_A receptor G-protein activation [434], similar to Ang II-induced PYK2 phosphorylation [435]. Furthermore, vasoactive peptide-induced MAPK and PKB activation have been linked to c-Src and PYK2 complex formation with either EGFR and/or IGF-1R, leading to their subsequent activation and downstream signaling [436, 437]. However, a recent report has suggested that Ang II activates MAPK signaling through a c-Src-dependent mechanism, while ET-1 activates MAPK signaling through a c-Src-independent mechanism [418]. Thus, PYK2 may activate EGFR signaling in a c-Src-independent fashion, yet the mechanism through which ET-1 signals MAPK activation still remains controversial. Nevertheless, it is quite evident that PYK2 plays an important role in mediating vasoactive peptide-induced signaling pathways involved in cell migration, proliferation, and hypertrophy.

1.8.3.5 Potential mechanisms contributing to ET-1 and Ang II-induced R-/NR-PTK transactivation

While the precise events that trigger the transactivation of R-/NR-PTK in response to vasoactive peptides are not clear, several mechanisms have been suggested. Studies have shown that inhibitors of matrix metalloproteinases (MMP), such as GM6001 and doxycyclin, attenuated ET-1 and Ang II-induced EGFR transactivation [438, 439]. MMPs act mainly by disrupting the link between growth factors and their transmembrane precursors, as is the case for heparin-bound-EGF (HB-EGF), allowing EGF to bind to EGFR, and

leading to tyrosine kinase activation and stimulation of hypertrophic and growth promoting signaling cascades [440].

Another proposed mechanism of R-PTK transactivation by vasoactive peptides is through their capacity to induce the generation of reactive oxygen species (ROS). ET-1 has been shown to activate reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase, resulting in ROS generation in endothelial cells [441], and increased H_2O_2 levels via ET_A receptor binding in pulmonary smooth muscle cells [442]. This increase in ROS generation has been linked with the ET-1-induced activation of ERK1/2, JNK, p38 MAPK, PKB and PYK2 [203, 443]. For its part, Ang II has also been shown to induce ROS generation in multiple cell types, including cardiomyocytes, endothelial cells and VSMC [444-446], which appears to play a direct role in Ang II-induced vascular hypertrophy through the activation of hypertrophic signaling pathways [447, 448]. ET-1-induced ROS generation in VSMC was suppressed by N-Acetylcysteine (NAc), a ROS scavenger, and diphenyleneiodonium (DPI), an inhibitor of NAD(P)H oxidase [203]. DPI and NAc pre-treatment of VSMC also inhibited ET-1-induced ERK1/2, PKB, and PYK2 phosphorylation, demonstrating that ROS are critical mediators of ET-1-induced signaling events linked to growth-promoting proliferative and hypertrophic pathways in VSMCs. Observations that both ROS e.g. H_2O_2, and vasoactive peptides, induce the tyrosine phosphorylation of IGF-1R and EGFR, and pharmacological blockade or genetic ablation of the R- and/or NR-PTK activity resulted in the attenuation of ET-1 and Ang II-induced ERK1/2 and PKB phosphorylation have suggested that ROS may serve as intermediates to enhance the tyrosine phosphorylation of R- and NR-PTKs [64, 415, 421, 423, 449]. It should be noted that ROS molecules have been shown to inhibit the activity of protein tyrosine phosphatases (PTPases), such as PTP-1B [450], and SH-2 domain-containing tyrosine phosphatase-2 (SHP-2) [451]. PTPase inhibition can cause a shift in the phosphorylation-dephosphorylation cycle, leading to a net increase of tyrosine phosphorylation of R- and/or NR-PTK[421, 422], which may contribute to the activation of the ERK1/2 and PKB signaling cascades. It has also been reported that PTEN, which catalyzes PIP_3 dephosphorylation, becomes inactivated by oxidation of Cys 124 in its catalytic domain subsequent to treatment with H_2O_2 or ROS-

generating peptides [452-455], leading to an increase of PIP_3 levels and a subsequent increase in PKB activation.

1.9 Objectives of the present study

Among various growth factor PTKs, the role of transactivation of EGFR in promoting the downstream responses to both ET-1 and Ang II in VSMC has been studied in some detail. Ang II has also been shown to induce tyrosine phosphorylation of EGFR in VSMC and a role of EGFR transactivation in Ang II-induced activation of ERK1/2 and PKB signaling, hypertrophy and proliferation of VSMC has been reported. Similarly, a critical role of EGFR transactivation in mediating ET-1-induced signaling events such as ERK1/2, $p70^{S6K}$ activation as well as protein and DNA synthesis, gene expression has been reported in several cell types. However, in contrast to the well-studied role of EGFR transactivation in mediating the responses of both of these vasoactive peptides, the involvement of other growth factor receptors in this process has not been investigated in detail. Furthermore, the role of c-Src, a non-receptor tyrosine kinase, in ET-1-induced MAPK signaling remains controversial in VSMC. While both ET-1 and Ang II seem to activate PKB through the same c-Src-dependent mechanism, a recent report has suggested that Ang II activates MAPK signaling through a c-Src-dependent mechanism, whereas ET-1 activates MAPK signaling through a c-Src-independent mechanism [418]. In addition, studies have suggested that the early growth transcription factor-1 (Egr-1) plays an important role in multiple cardiovascular pathological processes, including the pathogenesis of atherosclerotic lesions and neointimal thickening after vascular injury. While a growing number of studies have examined Egr-1 expression in response to Ang II in several cell types, including VSMC, little is known on ET-1-induced Egr-1 responses in VSMC.

Our laboratory has previously demonstrated that transactivation of insulin-like growth factor type 1 receptor (IGF-1R) plays a key role in triggering H_2O_2-induced phosphorylation of PKB in VSMC and that a cross-talk exists between the c-Src family of PTK and IGF-1R in mediating this effect. Since ET-1 and Ang II have been shown to increase the production of ROS, such as $O_2^{\cdot -}$ and H_2O_2 in VSMC, the objective of the present study was to elucidate the following aims:

1. Whether IGF-1R transactivation contributes to ET-1 and Ang II-induced activation of PKB and on hypertrophic and proliferative responses in VSMC.

2. Study the role of a cross talk between c-Src and IGF-1R PTK in ET-1 and Ang II-induced activation of PKB and subsequent hypertrophic and proliferative events induced by these vasoactive peptides.

3. Examine if the non-receptor protein tyrosine kinase c-Src differentially regulates ET-1 and Ang II-induced ERK 1/2, JNK and p38MAPK activation.

4. Study a possible role of c-Src NR-PTK in ET-1 and Ang II-induced modulation of early growth transcription factor-1 (Egr-1) in VSMC.

Chapter 2

Role of insulin-like growth factor 1 receptor and c-Src in endothelin-1 and angiotensin II-induced PKB phosphorylation, and hypertrophic and proliferative responses, in vascular smooth muscle cells.

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Role of the insulin-like growth factor-1 receptor and c-Src in endothelin-1 and angiotensin II-induced PKB phosphorylation as well as hypertrophic and proliferative responses in vascular smooth muscle cells

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Running title: IGF-1R transactivation in ET-1 and Ang II-induced PKB phosphorylation.

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2.1 Abstract

Endothelin-1 (ET-1) and angiotensin II (Ang II) are believed to contribute to the pathogenesis of vascular abnormalities such as hypertension, atherosclerosis, hypertrophy and restenosis. During the last several years, the concept of transactivation of growth factor receptors, such as epidermal growth factor receptor (EGFR) in triggering vasoactive peptide-induced signaling events, has gained much recognition. We demonstrated that insulin-like growth factor-1 receptor (IGF-1R) plays a role in tranducing the effect of H_2O_2, leading to protein kinase B (PKB) phosphorylation. Since vasoactive peptides elicit their responses through generation of reactive oxygen species, including H_2O_2, we investigated whether IGF-1R transactivation plays a similar role in ET-1 and Ang II-induced PKB phosphorylation and hypertrophic responses in VSMC. AG-1024, a specific inhibitor of IGF-1R protein tyrosine kinase (PTK), attenuated both ET-1 and Ang II-induced PKB phosphorylation in a dose-dependent manner. ET-1 and Ang II treatment also induced the phosphorylation of tyrosine residues in the autophosphorylation sites of IGF-1R, which was blocked by AG-1024. In addition, both ET-1 and Ang II evoked tyrosine phosphorylation of c-Src, a non-receptor PTK, and pharmacological inhibition of c-Src PTK activity by PP-2, a specific inhibitor of Src-family tyrosine kinase, significantly reduced PKB phosphorylation as well as tyrosine phosphorylation of IGF-1R induced by the two vasoactive peptides. Furthermore, protein and DNA synthesis enhanced by ET-1 and Ang II were also attenuated by AG-1024 and PP-2. In conclusion, these data suggest that IGF-1R and c-Src PTK play a critical role in mediating PKB phosphorylation as well as hypertrophic and proliferative responses induced by ET-1 and Ang II in A-10 VSMC.

Key words: Endothelin-1, Angiotensin II, IGF-1R, c-Src, PKB, Growth, VSMC.

2.2 Introduction

Endothelin-1 (ET-1) and angiotensin II (Ang II) are considered important vasoactive peptides in the vascular system. ET-1 is a potent vasoconstrictor peptide (Webb 1997) predominantly secreted by endothelial cells, whereas Ang II is the main component of the renin-angiotensin system (Touyz and Schiffrin 2000). Both of these vasoactive peptides exert their biological effects through interaction with specific heterotrimeric GTP binding protein coupled receptor (GPCR) in vascular smooth muscle cell (VSMC) (Bouallegue et al. 2007a; Touyz and Schiffrin 2000). ET-1 exerts its biological actions through the activation of two receptor subtypes, ET_A and ET_B (Arai et al. 1990; Sakurai et al. 1990), whereas Ang II acts via AT_1 and AT_2 receptors (de Gasparo et al. 2000). In recent years, it has become evident that both peptides play a critical role in vascular complications such as atherosclerosis (Mathew et al. 1996), hypertension (Haynes and Webb 1998) and restenosis after angioplasty (Burke et al. 1997). These actions are believed to be mediated through the activation of multiple signaling pathways which include: the mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases 1/2 (ERK1/2); c-Jun-NH2-terminal kinase (JNK) and p38mapk (Bogoyevitch et al. 1994; Yamboliev et al. 1998; Yoshizumi et al. 1998; Sorokin et al. 2001; Bouallegue et al. 2007a); as well as phosphatidylinositol 3-kinase (PI-3K) and its downstream effectors such as protein kinase B (PKB) (Foschi et al. 1997; Bouallegue et al. 2007a). An intermediary role of the transactivation of growth factor receptor protein tyrosine kinases (PTK) in transducing ET-1 and Ang II-induced signaling responses has been suggested (Yamauchi et al. 2002; Kodama et al. 2002; Kodama et al. 2003; Sorokin et al. 2001; Flamant et al. 2003). Among various growth factor PTK, the role of transactivation of epidermal growth factor receptor (EGFR) in promoting the downstream responses to both ET-1 and Ang II in VSMC has been studied in some detail (Chansel et al. 2006; Li and Malik 2005). Ang II has also been shown to induce tyrosine phosphorylation of EGFR in VSMC (Eguchi et al. 1999b; Eguchi et al. 1999a) and a role of EGFR transactivation in Ang II-induced activation of ERK1/2 and PKB signaling, hypertrophy and proliferation of VSMC has been reported (Eguchi et al. 1999a; Ohtsu et al. 2006; Bokemeyer

et al. 2000; Eguchi et al. 2001; Li and Malik 2005). Similarly, a critical role of EGFR transactivation in mediating ET-1-induced signaling events such as ERK1/2, $p70^{S6K}$ activation as well as protein and DNA synthesis, gene expression (Iwasaki et al. 1998) and contraction (Chansel et al. 2006; Kawanabe et al. 2004) has been reported in several cell types. However, in contrast to the well-studied role of EGFR transactivation in mediating the responses of both of these vasoactive peptides, the involvement of other growth factor receptors in this process has not been investigated in detail. We have demonstrated that transactivation of insulin-like growth factor type 1 receptor (IGF-1R) plays a key role in triggering H_2O_2-induced phosphorylation of PKB in VSMC and that cross-talk exists between the c-Src family of PTK and IGF-1R in mediating this effect (Azar et al. 2007). Since ET-1 and Ang II have been shown to increase the production of reactive oxygen species (ROS) such as O_2^- and H_2O_2 in VSMC (Daou and Srivastava 2004; Griendling et al. 1994; Cheng et al. 1999), we investigated whether IGF-1R transactivation also contributes to ET-1 and Ang II-induced activation of PKB and on hypertrophic and proliferative responses in VSMC and whether c-Src plays a role in this process.

2.3 Materials and Methods

2.3.1 Materials

ET-1 and Ang II were purchased from American Peptide Inc (USA). AG-1024, PP-2 and PP-3 were from obtained from Calbiochem. The phospho-specific-Ser^{473}-PKB, total PKB and horseradish peroxidase-conjugated anti-rabbit antibodies were procured from New England Biolabs (Beverly, MA). Anti-phospho-specific-IGF-1R (phospho-$Tyr^{1131/1135/1136}$) and anti-phospho-specific-c-Src (phospho-Tyr^{418}) antibodies were obtained from Biosource. Anti-IGF-1R and anti-cSrc antibodies were obtained from Santa Cruz Biotech (Santa Cruz, CA). The enhanced chemiluminescence (ECL) detection system kit was purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada). Leucine L-(4,5-^{3}H) and (^{3}H) Thymidine were obtained from MP Biomedicals (OH, USA)

2.3.2 Methods

Cell culture

A-10 VSMC were maintained in culture with DMEM containing 10% fetal bovine serum at 37^0 C in a humidified atmosphere of 5% CO_2, as previously described (Bouallegue et al. 2007b). Cells were grown to 80-90% confluence in 60-mm plates and incubated in serum-free DMEM 5h prior to the treatments.

Cell lysis and Immunoblotting

Cells incubated in the absence or presence of various agents were washed twice with ice-cold PBS and lysed in 200 µl of buffer (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 2 mM benzamidine, 2 mM ethylenebis(oxyethylenenitrolo)-tetraacetic acid, 2 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5 µg/ml leupeptin) on ice. Cell lysates were centrifuged at 12,000g for 10 min at 4^0 C. Protein concentrations were measured by Bradford assay. Equal amounts of protein were subjected to 7.5% SDS-polyacrylamide gel (SDS-PAGE), transferred to PVDF membranes (Millipore, MA, USA) and incubated with respective primary antibodies, polyclonal phospho-specific-Ser^{473}-PKB (1:1,000) or phospho-cSrc (1:2,000) or phosphor-IGF-1R (1:1,000) antibodies. The antigen-antibody complex was detected by horseradish peroxidase-conjugated second antibody (1:2000) and protein bands were visualized by ECL. The intensity of specific bands was quantified by NIH Image software as described previously (Pandey et al. 1999).

Measurement of [^{3}H]Leucine and [^{3}H]Thymidine incorporation.

Subconfluent A-10 VSMC were plated in 24-well plates and treated for 20 h with ET-1 or Ang II (100nM). To assess the role of IGF-1R or c-Src, cells were pretreated for 30 min with 5 µM of AG-1024 or PP-2, respectively, prior to stimulation with either ET-1 or Ang II. Protein and DNA synthesis were assessed by addition of 2 µCi/mL of [^{3}H]leucine or

[^{3}H]Thymidine respectively as described previously (Bouallegue et al. 2007b). Following the completion of the experimental protocol, A-10 cells were washed four times with ice-cold NaCl (150 mM) and incubated with 1 ml of cold 5% trichloroacetic acid for 30 min. Subsequently, cells were washed twice with ice-cold water and incubated with 500 µl of 0.4 N NaOH. Aliquots were counted in a scintillation counter to determine the incorporation of radioactivity.

Statistics

The data are means ± SE of three individual experiments. Statistical significance was determined with paired or unpaired Student's *t* test.

2.4 Results

2.4.1 Attenuation of ET-1 and Ang II-induced PKB phosphorylation by AG-1024 in A-10 VSMCs.

Both ET-1 and Ang II have been shown to transactivate EGFR in many cell types (Kodama et al. 2002; Iwasaki et al. 1998) and an important role of this transactivation in mediating ET-1-induced ERK1/2 activation has been reported (Iwasaki et al. 1998). However, involvement of IGF-1R in ET-1 and Ang II-induced phosphorylation of PKB has not been investigated in VSMC. Therefore, by using AG-1024 (*3-bromo-5-t-butyl-4-*hydroxy-benzylidenemalonitrile), a specific pharmacological inhibitor of IGF-1R-PTK activity (Parrizas et al. 1997), we examined the involvement of IGF-1R in this process. As shown in Fig.1, pretreatment of A-10 VSMCs with AG-1024 for 30 min dose-dependently attenuated both ET-1 and Ang II-induced phosphorylation of PKB and 10μM AG-1024 was found to completely abrogate PKB phosphorylation induced by both these vasoactive peptides.

2.4.2 ET-1 and Ang II induce tyrosine phosphorylation of IGF-1R

Since tyrosine phosphorylation of IGF-1R β subunit is essential for its activation, we examined whether stimulation of cells with these two vasopeptides would result in an enhanced tyrosine phosphorylation of IGF-1R β subunit. This was achieved by using a phospho-specific antibody that recognizes IGF-1R phosphorylated on tyrosine residues 1131, 1135 and 1136, critical sites of autophosphorylation necessary for the activation of IGF-1R (Hernandez-Sanchez et al. 1995). As shown in Fig.2, both ET-1 and Ang II were able to evoke an increase in tyrosine phosphorylation of IGF-1R, which was blocked by pretreatment of cells with AG-1024.

2.4.3 Attenuation of ET-1 and Ang II-induced PKB phosphorylation by PP-2 in A-10 VSMCs.

The Src family of non-receptor PTKs has been implicated in triggering some signaling responses of Ang II (Eguchi et al. 1999b) and ET-1(Shah et al. 2006), however, involvement of these PTKs in mediating ET-1 and Ang II-induced PKB phosphorylation in VSMC has not been examined. Therefore, by using PP-2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazole(3,4-d) pyrimidine), a specific blocker of Src-family PTK (Hanke et al. 1996), we investigated the role of Src PTK in this process. As shown in Fig.3, pretreatment of VSMC with PP-2 prior to stimulation with ET-1 or Ang II inhibited PKB phosphorylation induced by both ET-1 and Ang II in a dose-dependent manner. At 5 μM, PP-2 almost completely inhibited PKB phosphorylation. However, PP-3 (4-amino-7-phenylpyrazole(3,4-d) pyrimidine), an inactive analog of PP-2, had no effect.

2.4.4 ET-1 and Ang II induce tyrosine phosphorylation of c-Src

Since c-Src activation requires enhancement in the phosphorylation of a Tyr ^{418} conserved tyrosine residue (referring to human Src sequence) located in its activation loop (Thomas and Brugge 1997), we determined whether ET-41 and Ang II were capable of inducing phosphorylation of this tyrosine residue in A-10 VSMC. As shown in Fig.4, both ET-1 and Ang II treatment resulted in increased phosphorylation of Tyr^{418} on c-Src. Moreover, pretreatment with PP-2 prior to stimulation of cells with these vasoactive peptides blocked this response, yet PP-3 had no effect (Fig.4).

2.4.5 Attenuation of ET-1 and Ang II-induced tyrosine phosphorylation of IGF-1R β subunit by PP-2.

c-Src has been shown to increase the phosphorylation of autophosphorylation sites of the IGF-1R β subunit both *in vitro* and *in vivo* (Peterson et al. 1996). Therefore, we determined the contribution of c-Src in ET-1 and Ang II-induced tyrosine phosphorylation of IGF-1R β subunit in A-10 VSMC. As shown in Fig.5, pretreatment of VSMC with PP-2

significantly attenuated vasoactive peptide-induced phosphorylation of IGF-1R β subunit. PP-3, on the other hand, was unable to inhibit IGF-1R phosphorylation induced by vasoactive peptides (Fig.5)

2.4.6 Lack of Involvement of IGF-1R in ET-1/Ang II-induced c-Src phosphorylation.

We have shown that H_2O_2-induced c-Src phosphorylation is mediated through IGF-1R PTK (Azar et al. 2006). As a result, we investigated whether a similar mechanism was also involved in vasoactive peptide-induced increase in c-Src phosphorylation. As shown in Fig.6, pretreatment of cells with AG-1024 failed to inhibit Tyr^{418} phosphorylation of c-Src by either ET-1 or Ang II treatment.

2.4.7 Attenuation of ET-1 and Ang II-induced protein and DNA synthesis by AG-1024 and PP-2.

Both ET-1 and Ang II have been shown to induce increases in protein and DNA synthesis in mediating cell hypertrophy and proliferation (Bouallegue et al. 2007b; Di et al. 2005; Haider et al. 2002; Yang et al. 2005; Hashim et al. 2006). Therefore, we examined whether IGF-1R and/or c-Src-PTK contributed in mediating the effect of ET-1 or Ang II on protein and DNA synthesis in A-10 VSMC. As shown in Fig.7, both of these vasoactive peptides increased the rate of protein (Fig.7.A) and DNA (Fig.7.B) synthesis, as determined by incorporation of [^{3}H] Leucine into total cellular proteins and [^{3}H] Thymidine into DNA of VSMC, respectively. However, the blockade of IGF-1R by AG-1024 or c-Src PTK activity by PP-2 significantly decreased both protein and DNA synthesis induced by these two vasoactive peptides. PP-3, an inactive analog of PP-2 had no effect (Fig.7).

2.5 Discussion

There is an increasing body of evidence that suggests an important role of growth factor receptor transactivation in mediating vasoactive peptide-induced signaling pathways in VSMC. Among various growth factor receptors, the transactivation of EGFR has been studied in great detail in response to Ang II. However, transactivation of IGF-1R in triggering the responses of both ET-1 and Ang II remains poorly characterized. We provide evidence to suggest that ET-1 and Ang II, important GPCR ligands with a critical role in the pathogenesis of hypertensive vascular disease, induce the phosphorylation of PKB in an IGF-1R-dependent fashion. We also show that both of these vasoactive peptides enhance the phosphorylation of key tyrosine residues in the autophosphorylation site of IGF-1R β subunit. Although Ang II has been shown to increase tyrosine phosphorylation of IGF-1R in VSMC (Lauzier et al. 2007; Zahradka et al. 2004; Du et al. 1996), and a role of IGF-1R transactivation in Ang II-induced phosphorylation of ERK1/2 and $p70^{S6K}$ has been suggested, no studies investigating the role of IGF-1R transactivation in either ET-1 or Ang II induced PKB phosphorylation in VSMC have been conducted. Thus, to our knowledge, the results reported here are the first to identify an involvement of IGF-1R in tranducing the downstream effects of ET-1 and Ang II in activating PKB in VSMC.

Another important finding of these studies is that the Src family of non-receptor PTKs are key mediators of ET-1/Ang II-induced PKB phosphorylation. We demonstrated that both of these vasoactive peptides can induce phosphorylation of Tyr ^{418} located in the activation loop of c-Src and that pharmacological blockade of c-Src-PTK activity not only inhibited ET-1 and Ang II evoked phosphorylation of PKB, but also blocked IGF-1R tyrosine phosphorylation. These data suggest that c-Src is upstream of IGF-1R in the signaling cascade leading to PKB phosphorylation by both ET-1 and Ang II in VSMC. In fact, previous studies have shown that c-Src can catalyze the phosphorylation of IGF-1R β subunit on the same tyrosine residues that are autophosphorylated subsequent to the binding of IGF-1R to its receptor (Peterson et al. 1996). Thus, c-Src-induced phosphorylation of these sites in IGF-1R in VSMC can trigger downstream effects leading to PKB phosphorylation. Earlier

work reported the phosphorylation of c-Src by ET-1 (Shah et al. 2006; Mishra et al. 2005) and Ang II (Eguchi et al. 1999b; Kyaw et al. 2004), and a requirement of c-Src in ET-1 and Ang II-induced PKB and $p70^{S6K}$ activation has been demonstrated (Bokemeyer et al. 2000; Zahradka et al. 2004). However, current studies indicate that c-Src activation is essential in triggering IGF-1R phosphorylation by ET-1 and, provide the molecular basis by which the ligand-independent transactivation of IGF-1R occurs in VSMC in response to vasoactive peptides.

Both c-Src and IGF-1R have been implicated in the activation of NAD(P)H oxidase system (Touyz et al. 2003b; Touyz et al. 2003a), and since both ET-1and Ang II trigger their effect through ROS generation (Daou and Srivastava 2004; Touyz et al. 2004), it may be suggested that phosphorylation and activation of IGF-1R and c-Src is one of the early events in tranducing the signals of ET-1 and Ang II for ROS generation and subsequent activation of signaling pathways, linked to cellular hypertrophy and proliferation. This notion is supported by our results showing that blockade of IGF-1R PTK by AG-1024 or c-Src by PP-2 not only attenuated PKB phosphorylation but also attenuated protein as well as DNA synthesis induced by ET-1 and Ang II in A-10 VSMC.

Involvement of PKB and its downstream effectors in regulating the hypertrophic and proliferative responses in VSMC has been suggested (Bouallegue et al. 2007a; Ivey et al. 2008). Pharmacological blockade of PI3K activity with wortmannin was reported to inhibit ET-1 and Ang II-induced protein as well as DNA synthesis in VSMC (Li et al. 2006; Hashim et al. 2006). PKB activation results in the phosphorylation of several of its downstream substrates including mammalian target of rapamycin (mTOR) which has been implicated in the translational initiation of protein synthesis (Gingras et al. 2001). mTOR complex 1 (mTORC1), through phosphorylation of ribosomal S6 kinases (S6K) and eukaryotic initiation factor 4E binding protein (eIF4EBPI) enhances translational efficiency of cap-dependant mRNA translation leading to enhanced protein synthesis and cell growth (Gingras et al. 2004). PKB via its effects on cell cycle regulatory proteins such as p21cip1 and p27 kip1 is believed to control cell cycle progression and cell proliferation (Shin et al. 2002).

In conclusion, the results of this investigation demonstrate that ET-1 and Ang II induce the phosphorylation of PKB through a c-Src and IGF-1R PTK-dependent pathway, with c-Src being upstream to IGF-1R in this signaling cascade. Moreover, IGF-1R and c-Src appear to be key mediators of hypertrophic and proliferative responses of both ET-1 and Ang II in A-10 VSMC.

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2.7 Figure legends

Figure 1. Pharmacological blockade of IGF-1R abolishes ET-1 and Ang II-induced PKB phosphorylation in A-10 VSMCs.

Serum-starved quiescent A-10 VSMC were pretreated in the absence (0) or presence of the indicated concentration of AG-1024 for 30 min followed by stimulation with 100nM of ET-1 (section A) or Ang II (section B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Ser^{473}-PKB antibodies (top panels in each section). Blots were also analyzed for total PKB (middle panels of each section). Bottom panels (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the top panel. Values are the means ± SE of at least three independent experiments and are expressed as percentages of phosphorylation, where phosphorylation observed with ET-1 alone (for section A) or Ang II alone (for section B) is defined as 100%. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone for section A or versus Ang II alone for section B. * indicates that $P<0.05$; ** indicates that $P<0.005$ and † indicates that $P<0.0005$.

Figure 2. Pharmacological blockade of IGF-1R attenuates ET-1 and Ang II-induced IGF-R phosphorylation in A-10 VSMCs.

Serum-starved quiescent A-10 VSMC were pretreated in the absence (0) or presence of 10μM AG-1024 for 30 min followed by stimulation with 100nM of ET-1 (section A) or Ang II (section B) for 5 min. Cell lysates were immunoblotted with phospho-specific-$Tyr^{1131,1135,1136}$ IGF-1R antibodies (top panel in each section). Blots were also analyzed for total IGF-1R (middle panels of each section). Bottom panels (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the top panel. Values are the means ± SE of at least three independent experiments and are expressed as percentages of phosphorylation, where phosphorylation observed with ET-1 alone (for section A) or Ang II alone (for section B) is defined as 100%. $P<0.05$ was

considered as statistically significance versus ET-1 stimulation alone for section A or versus Ang II alone for section B. † indicates that $P<0.0005$.

Figure 3. Pharmacological blockade of c-Src abolishes ET-1 and Ang II-induced PKB phosphorylation in A-10 VSMCs.

Serum-starved quiescent A-10 VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or presence of the indicated concentration of PP-2 for 30 min followed by stimulation with 100nM of ET-1 (section A) or Ang II (section B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Ser^{473}-PKB antibodies (top panels in each section). Blots were also analyzed for total PKB (middle panels of each section). Bottom panels (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the top panel. Values are the means ± SE of at least three independent experiments and are expressed as percentages of phosphorylation, where phosphorylation observed with ET-1 alone (for section A) or Ang II alone (for section B) is defined as 100%. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone for section A or versus Ang II alone for section B. * indicates that $P<0.05$; ** indicates that $P<0.005$ and † indicates that $P<0.0005$.

Figure 4. Pharmacological blockade of c-Src attenuates ET-1 and Ang II-induced c-Src phosphorylation in A-10 VSMCs.

Serum-starved quiescent A-10 VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or presence of 10μM PP-2 for 30 min followed by stimulation with 100nM for 5 min of ET-1 (section A) or Ang II (section B). Cell lysates were immunoblotted with phospho-specific-Tyr^{418}-c-Src antibodies (top panels in each section). Blots were also analyzed for total cSrc (middle panels of each section). Bottom panels (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the top panel. Values are the means ± SE of at least three independent experiments and are expressed as percentages of phosphorylation, where phosphorylation observed with ET-1 alone (for section A) or Ang II alone (for section B) is defined as

100%. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone for section A or versus Ang II alone for section B. † indicates that $P<0.0005$.

Figure 5. Pharmacological blockade of c-Src attenuates ET-1 and Ang II-induced IGF-1R phosphorylation in A-10 VSMCs.

Serum-starved quiescent A-10 VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or presence of 10μM PP-2 for 30 min followed by stimulation with 100nM for 5 min of ET-1 (section A) or Ang II (section B). Cell lysates were immunoblotted with phospho-specific-$Tyr^{1131/1135/1136}$-IGF-1R antibodies (top panels in each section). Blots were also analyzed for total IGF-1R (middle panels of each section). Bottom panels (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the top panel. Values are the means ± SE of at least three independent experiments and are expressed as percentages of phosphorylation, where phosphorylation observed with ET-1 alone (for section A) or Ang II alone (for section B) is defined as 100%. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone for section A or versus Ang II alone for section B.

Figure 6. Pharmacological blockade of IGF-1R had no effect on ET-1 and Ang II-induced c-Src phosphorylation in A-10 VSMCs.

Serum-starved quiescent A-10 VSMC were pretreated in the absence (0) or presence of 10μM AG-1024 for 30 min followed by stimulation with 100nM for 5 min of ET-1 (section A) or Ang II (section B). Cell lysates were immunoblotted with phosphor-specific Try^{418}-c-Src antibodies (top panel in each section). Blots were also analyzed for total c-Src (middle panels of each section). Bottom panels (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the top panel. Values are the means ± SE of at least three independent experiments and are expressed as percentages of phosphorylation, where phosphorylation observed with ET-1 alone (for section A) or Ang II alone (for section B) is defined as 100%. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone for section A or versus Ang II alone for section B.

Figure.7. Pharmacological blockade of IGF-1R or c-Src attenuates ET-1 and Ang II-induced DNA and protein synthesis in A-10 VSMC.

Serum-starved quiescent A-10 VSMC were stimulated with ET-1 or Ang II (100 nM). Cells were pretreated with or without either 5 μM of AG-1024, PP-2 or PP-3 for 30 min before ET-1 or Ang II stimulation. Cells were then labeled with [^{3}H]Leucine (section A) or [^{3}H]Thymidine (section B) for 20 h as described in Materials and Methods. Values are the means ± SE of three independent experiments and are expressed as percentage of change in [^{3}H]leucine incorporated into total cellular proteins (section A) and in [^{3}H]Thymidine incorporated into DNA (section B) over the basal values. $P<0.05$ was considered as statistically significance. * versus control, † versus ET-1 stimulation alone and ‡ versus Ang II stimulation alone.

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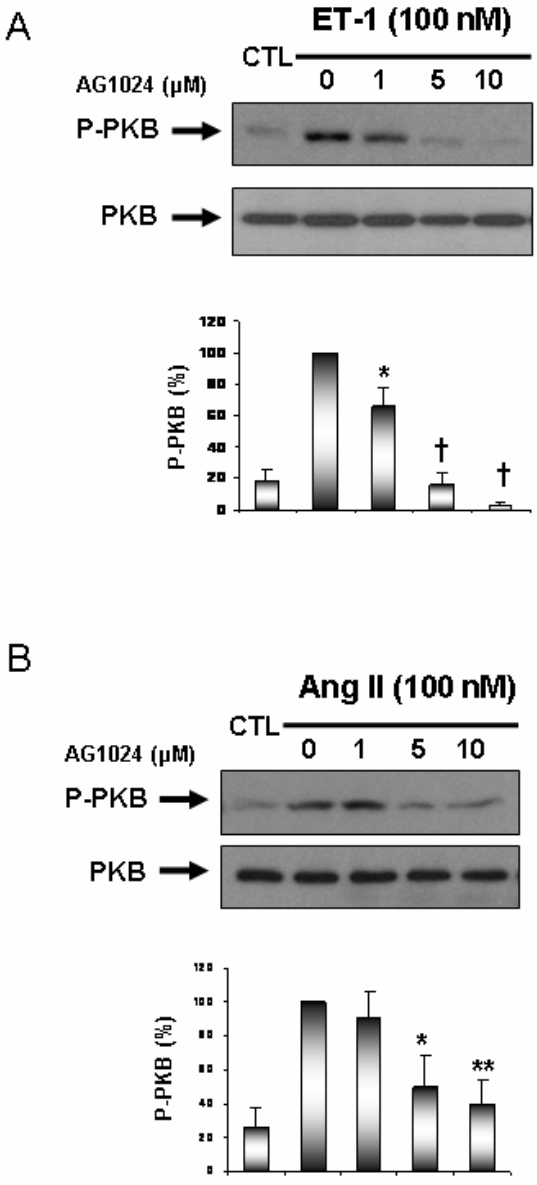
Figure 1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AG1024 (μM)
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PKB
P-PKB (%)
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100
80
60
40
20
0
*
†
†
B
Ang II (100 nM)
CTL
AG1024 (μM)
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P-PKB
PKB
P-PKB (%)
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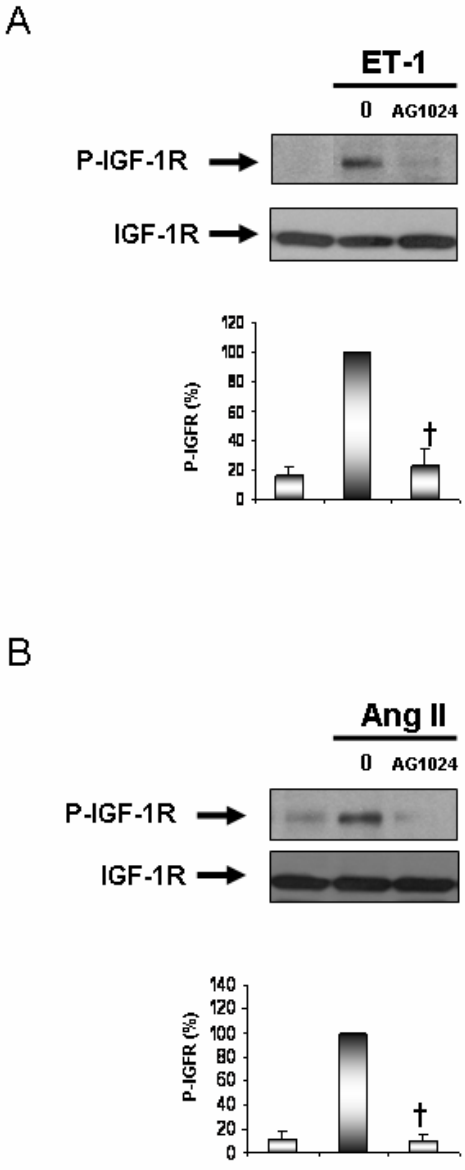
100
80
60
40
20
0
*
**

Figure 2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P-IGFR (%)
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B
Ang II
0
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P-IGF-1R
IGF-1R
P-IGFR (%)
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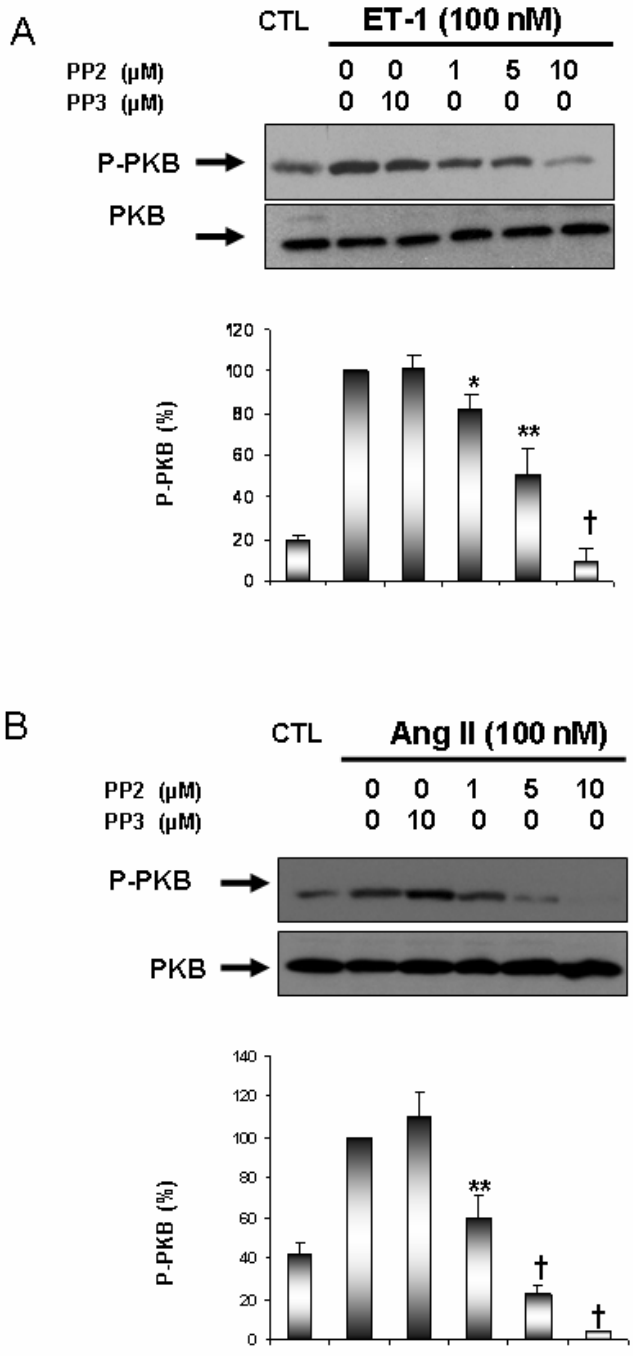
120
100
80
60
40
20
0
†

Figure 3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PP3 (μM)
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0 10 0 0 0
P-PKB
PKB
P-PKB (%)
120
100
80
60
40
20
0
*
**
†
B
CTL
Ang II (100 nM)
PP2 (μM)
PP3 (μM)
0 0 1 5 10
0 10 0 0 0
P-PKB
PKB
P-PKB (%)
140
120
100
80
60
40
20
0
**
†
†

Figure 4

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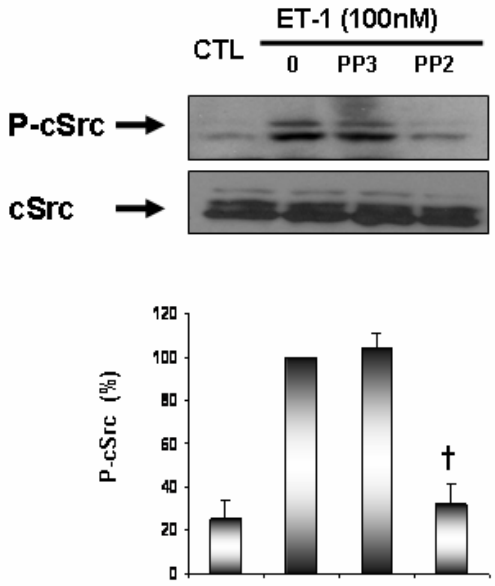
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0
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PP2
P-cSrc
cSrc
P-cSrc (%)
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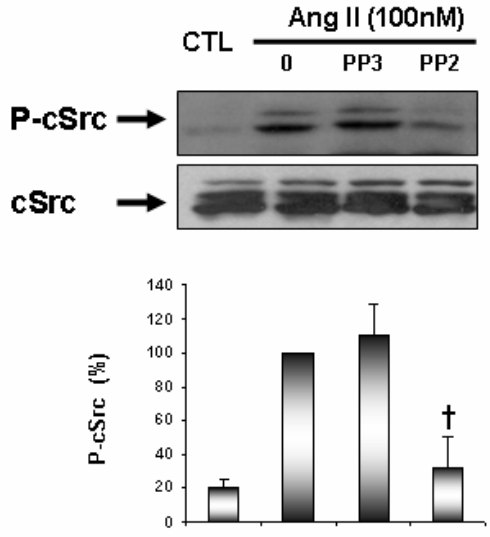
80
60
40
20
0
†

B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0
PP3
PP2
P-cSrc
cSrc
P-cSrc (%)
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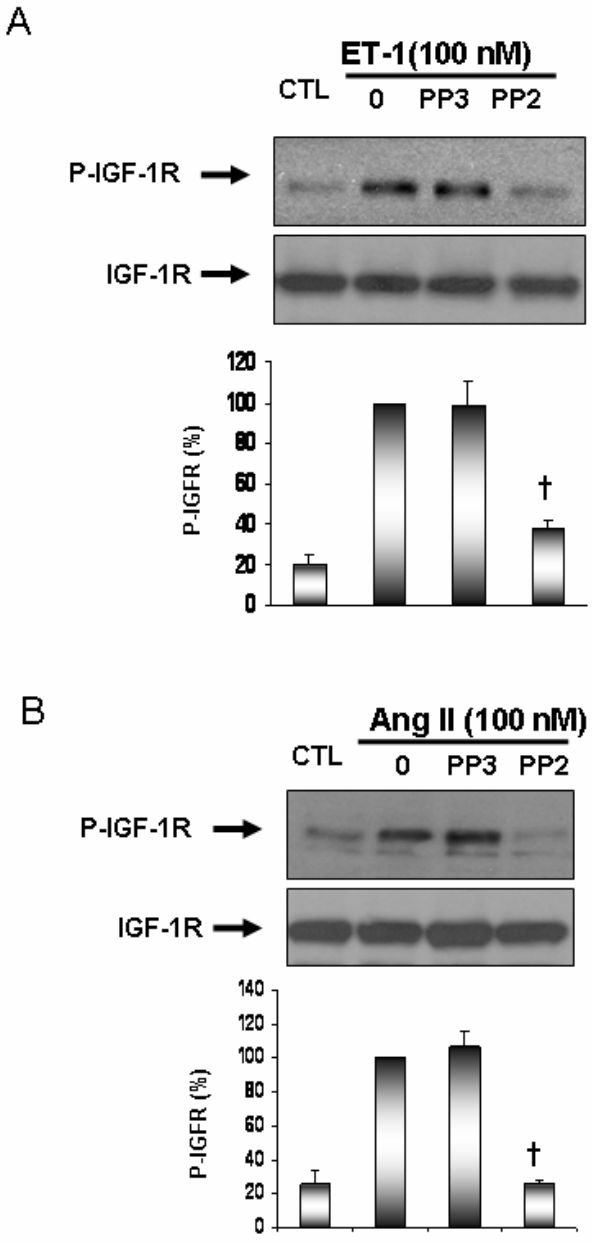
120
100
80
60
40
20
0
†

Figure 5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PP3
PP2
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IGF-1R
P-IGFR (%)
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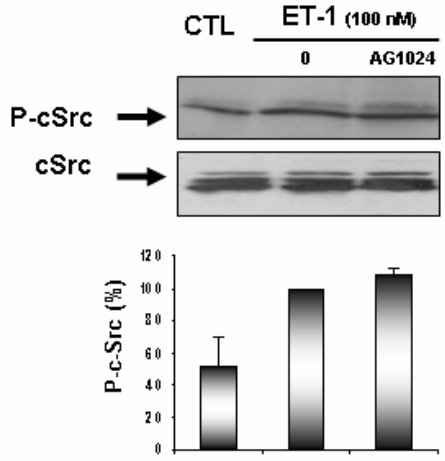
100
80
60
40
20
0
†
B
Ang II (100 nM)
CTL
0
PP3
PP2
P-IGF-1R
IGF-1R
P-IGFR (%)
140
120
100
80
60
40
20
0
†

Figure 6

A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AG1024
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cSrc
P-c-Src (%)
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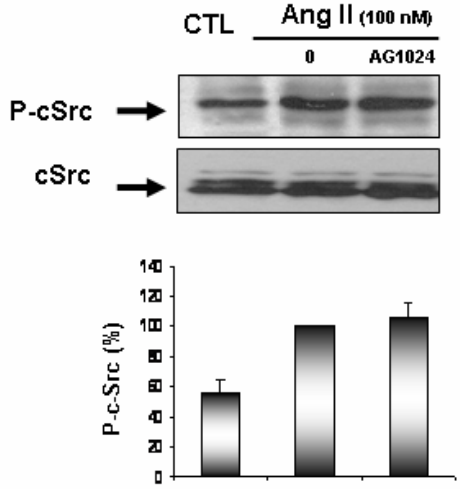
100
80
60
40
20
0

B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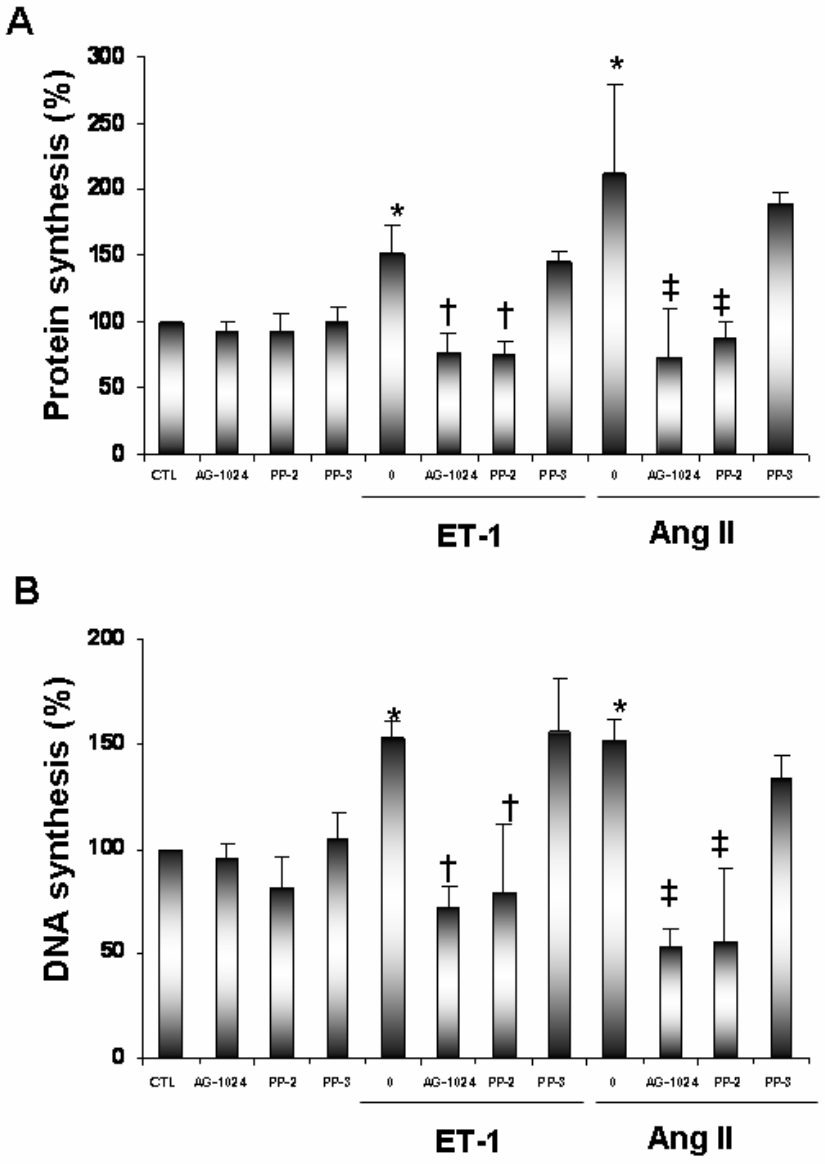
P-cSrc
cSrc
P-c-Src (%)
140
120
100
80
60
40
20
0

Figure 7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200
150
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†
‡
‡
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PP-3
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AG-1024
PP-2
PP-3
0
AG-1024
PP-2
PP-3
ET-1
Ang II
B
DNA synthesis (%)
200
150
100
50
0
*
*
†
†
‡
‡
CTL
AG-1024
PP-2
PP-3
0
AG-1024
PP-2
PP-3
0
AG-1024
PP-2
PP-3
ET-1
Ang II

Chapter 3

Requirement of c-Src, a Non-Receptor Tyrosine Kinase in both Endothelin-1 and Angiotensin II-induced ERK 1/2, JNK and p38 MAPK, as well as Egr-1 activation in Vascular Smooth Muscle cells.

To be submitted to Biochemical Pharmacology

Requirement of c-Src, a Non-Receptor Tyrosine Kinase in both Endothelin-1 and Angiotensin II-induced ERK 1/2, JNK and p38 MAPK, as well as Egr-1 activation in Vascular Smooth Muscle cells.

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Running title: MAPK activation and Egr-1 expression through a c-Src dependent mechanism.

Address for Correspondence:

3.1 Abstract

A heightened activity of Endothelin-1 (ET-1) and angiotensin II (Ang II), key vasoactive peptides implicated in the maintenance of blood pressure and vascular homeostasis, is thought to contribute to the development of vascular pathologies, such as hypertension, atherosclerosis, hypertrophy and restenosis. This occurs through the hyperactivation of growth promoting signal transduction pathways, including PI3K/PKB and the MAPK pathways, and regulation of transcription factors, such as early growth response factor-1 (Egr-1), which was recently shown to be expressed in atherosclerotic plaque. We have previously shown that c-Src, a non-receptor protein tyrosine kinase (NR-PTK), is an upstream regulator of ET-1 and Ang II-induced activation of PKB in vascular smooth muscle cells (VSMC). However, the role of c-Src in ET-1-induced MAPK signaling remains controversial in VSMC. Therefore, in the present studies, we have investigated the involvement of c-Src in ET-1 and Ang II-induced ERK 1/2, JNK and p38 MAPK activation, as well as Egr-1 regulation. ET-1 and Ang II-induced the phosphorylation of ERK 1/2, JNK and p38mapk, and enhanced the expression of Egr-1 in rat aortic A10 VSMC, as well as VSMC derived from thoracic aorta of adult Sprague Dawley rats. This increased phosphorylation was decreased by PP-2, a specific pharmacological inhibitor of Src. Further proof for the role of c-Src in this process was obtained by using mouse embryonic fibroblasts (MEF) deficient in c-Src (SYF). ET-1-induced Egr-1 expression, as well as MAPK activation, was found to be downregulated in SYF, as compared to MEF expressing normal Src levels. In summary, these data indicate that c-Src PTK plays a critical role in mediating ET-1 and Ang II-induced MAPK phosphorylation and Egr-1 expression through a c-Src dependent mechanism in VSMC.

Keywords: Endothelin-1, Angiotensin II, VSMC, ERK, JNK, p38 MAPK, c-Src, Egr-1.

3.2 Introduction

Alterations in vascular smooth muscle cell (VSMC) growth, migration, proliferation and plasticity is believed to contribute to abnormal vascular functions associated with or leading to CVDs, such as hypertension, atherosclerosis, and stenosis after angioplasty [1-3]. Under normal physiological conditions, vasoactive peptides, such as angiotensin II (Ang II) and endothelin-1 (ET-1), normalize blood pressure through the regulation of salt and/or water homeostasis, sympathetic nervous system modulation, as well as VSMC contraction and relaxation [4-7]. Increased levels of both ET-1 and Ang II, present in certain pathophysiological states, such as essential hypertension, obesity, or advanced stages of diabetes, have been suggested to contribute to the pathogenesis of CVDs, by activating signaling events intimately linked to migration and proliferation of VSMC [4, 8-10]. Ang II acts primarily through the activation of its two main receptors, Ang II type 1 receptor (AT_1R) and Ang II type 2 receptor (AT_2R), both of which are 7 transmembrane domain guanine nucleotide-binding protein (G Protein)-coupled receptors (GPCR) [11]. These receptors are primarily found in blood vessels, but also in heart, lung liver and brain tissues [4]. ET-1 exerts its biological actions through the activation of its two receptor subtypes, ET_A and ET_B [12, 13], both of which, like the Ang II receptors, belong to the GPCR family. GPCR stimulation leads to the activation of several downstream growth and proliferative signaling cascades, which include members of the mitogen-activated protein kinase (MAPK) family, as well as the phosphatidyl-inositol 3-kinase (PI3-K)/protein kinase B (PKB) pathway.

MAPKs constitute a family of serine/threonine protein kinases which are widely conserved among eukaryotes, and are involved in many cellular responses, such as cell proliferation, cell differentiation, cell movement and cell death [14, 15]. The groups of vertebrate MAPK studied most extensively to date are ERK1/2, JNKs, and p38 MAPK [14, 15]. ERK1/2 are stimulated by mitogens, such as polypeptide growth factors (IGF-1, platelet-derived growth factor (PDGF), colony stimulating factor-1 (CSF-1), etc.) as well as insulin and phorbol 12-myristate 13-acetate (PMA). In contrast, SAPKs and p38 MAPK are potently induced by a wide variety of stressors, including ultraviolet irradiation, gamma

irradiation, anisomycin, heat shock and chemotherapeutic drugs. Recent studies have also implicated SAPK/JNK and p38 MAPK in vasoactive peptide-induced proliferative responses [16-22]. These pathways are also activated by ischemia or reperfusion after ischemia, and by inflammatory cytokines [23]. Overwhelming evidence exists to support a vasoactive peptide-induced MAPK activation in multiple systems, including oligodendrocyte progenitor cells, pancreatic stellate cell, aortic and mesenteric artery-derived VSMC, increasing cell growth and hypertrophy, leading to complications such as atrial fibrillation due to left ventricle hypertrophy, vascular remodelling, cardiac hypertrophy and coronary artery disease [24-29].

c-Src, also known as Src or $p60^{c\text{-}Src}$, is a non-receptor protein tyrosine kinase (NR-PTK). In the vasculature, especially VSMC, GPCR-linked contractile responses, induced by Ang II and phenylephrine, have been associated with c-Src actvation [30, 31]. Furthermore, the use of PP-1 and PP-2, pyrazolopyrimidine-based selective inhibitors of Src family tyrosine kinases [32], has indicated a role of Src in mediating H_2O_2, ET-1 and Ang II-induced transactivation of the EGFR, IGF-1R and PDGFR in several cell types [33-37]. We have shown a role of c-Src in mediating ET-1 and Ang II-induced PKB phosphorylation, cell hypertrophy and proliferation [37]. Another study showed that, in VMSC derived from c-Src knockout mice, activation of ERK1/2 by Ang II was significantly decreased, as compared to VSMC from wild type mice [38, 39]. c-Src rescue in these cells through retroviral vector transfection caused a significant increase in Ang II-induced ERK1/2 phosphorylation, demonstrating that c-Src activation is necessary for Ang II-induced signal transduction in VSMC [39]. While both ET-1 and Ang II seem to activate PKB through the same c-Src-dependent mechanism, a recent report has suggested that whereas Ang II activates MAPK signaling through a c-Src-dependent mechanism, ET-1 activates MAPK signaling through a c-Src-independent mechanism [38]. Despite the fact that the role of c-Src as a mediator of Ang II signaling is clear [37, 40, 41], its role in ET-1-induced signaling events remains controversial.

In the present studies, we examined the role of c-Src as an upstream regulator of both ET-1 and Ang II-induced ERK 1/2, JNK and p38 MAPK phosphorylation and

modulation of early response growth factor-1 (Egr-1), a transcription factor downstream of ERK1/2 that plays a regulatory role in several cardiovascular pathological processes, by down-regulating c-Src through pharmacological inhibition in aortic VSMC, as well as using a c-Src knockdown cell model.

3.3 Materials and Methods

3.3.1 Materials

Chemicals. Cell culture reagents were purchased from Gibco (Burlington, ON). ET-1 was purchased from American Peptide (Sunnyvale, CA) and Ang II from Sigma Chemical (Oakville, ON). PP-2 and PP-3 were purchased from Calbiochem (Carlsbad, CA). The enhanced chemiluminescence (ECL) detection system kit was purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC).

Antibodies. Phospho-SAPK/JNK (Thr183/Tyr185) (#4668), phospho-p38 MAPK (Thr180/Tyr182) (#4631), phospho-c-Src (Tyr416) (#2101), total SAPK/JNK (#9252), total GAPDH (#5174) and Anti-rabbit IgG, horseradish peroxidase-linked secondary antibody (#7074) were procured from Cell Signaling Technologies (Danvers, MA). Phospho-ERK1/2 (Thr202/Tyr204) (sc-16982-R), total ERK (sc-154), total p38 MAPK (sc-7972), total c-Src (sc-8056), total Egr-1 (sc-110) and anti-mouse IgG, horseradish peroxidase-linked secondary antibody (sc-2005) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-phospho-specific-c-Src (phospho-Tyr418) antibody was obtained from Invitrogene (Camarillo, CA).

3.3.2Methods

Cell culture. Rat aorta A-10 VSMC (CRL-1476, ATCC) were maintained in 75-cm^2 flasks culture with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics at 37ºC in a humidified atmosphere of 5% CO_2, as previously described [37]. Cells were passed upon reaching confluence with 0.5% trypsin-containing

0.2% EDTA and plated in 60mm dished. Cells were grown to 90% confluence and incubated in serum-free DMEM 18h prior to the treatments.

VSMC derived from thoracic aorta of eight week old Sprague Dawley rats were isolated as previously described [42]. Briefly, male Sprague-Dawley rats were sacrificed and the descending thoracic aorta was excised. Adhering fat and connective tissue were removed by blunt dissection. Vessels were then opened longitudinally and preincubated in DMEM containing 1 mg/ml type II collagenase, 0.5 mg/ml type I elastase, penicillin (100 U/ml), and streptomycin (100 g/ml) for 15-20 min at 37ºC. The adventitia was carefully removed under a dissecting microscope and the luminal surface scraped with forceps to remove endothelial cells. After dissection, aortas were placed in fresh enzyme solution, minced and incubated (37°C) for an additional 60-90 min with tituration at 30-min intervals. Solution was then centrifuged, and the pellet was gently resuspended in fresh DMEM containing 10%FBS with antibiotics, and maintained in 75-cm^2 flasks. Cells were then maintained in the same fashion as A10 VSMC. Cells from passages 3-8 were used for experimentation.

Mouse embryonic fibroblasts deficient for c-Src, Yes and Fyn (SYF) (CRL-2459, ATCC) and expressing endogenous wild type c-Src but not Yes and Fyn (Src +/+)(CRL-2497, ATCC) were maintained in 75-cm^2 flasks culture with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics at 37ºC in a humidified atmosphere of 5% CO_2. Cells were passed upon reaching confluence with 0.5% trypsin-containing 0.2% EDTA and plated in 60mm dished. Cells were grown to 90% confluence and incubated in serum-free DMEM 18h prior to the treatments.

Cell lysis and Immunoblotting. Cells incubated in the absence or presence of various agents were washed three times with ice-cold PBS and lysed in 200 µl of lysis buffer (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na-pyrophosphate, 2 mM benzamidine, 2 mM ethylenebis (oxyethylenenitrolo)-tetra acetic acid, 2 mM ethylenediamine tetra acetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 1% Protease Inhibitor Cocktail (PIC)) on ice. Cell lysates were centrifuged at 12,000g for 10 min at 4ºC. Protein concentrations were measured by Bradford assay. Equal amounts of protein were subjected

to 7.5% SDS-polyacrylamide gel (SDS-PAGE), transferred to PVDF membranes (Millipore, Billerica, MA) and incubated with respective primary antibodies. The antigen-antibody complex was detected by horseradish peroxidase-conjugated second antibody and protein bands were visualized by ECL. The intensity of specific bands was quantified by Quantity One Image Software (Bio-Rad, Hercules, CA).

Egr-1 nuclear extraction protocol. To decrease dilution and interference by cytosolic proteins and to amplify the signal of Egr-1 in cell lysates, cells incubated in the absence or presence of pharmacological agents were lysed and nuclear protein was isolated for subsequent immunoblotting. Briefly, cells were washed twice with ice-cold PBS and collected in 500µl of buffer solution containing 10mM Hepes, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM protease cocktail inhibitor and 1mM NaOV. Lysates were put on ice for 15 minutes before the addition of 10% NP40 detergent. Lysates were then vortexed for 10 seconds at highest setting before being centrifuged at 13000RPM for 4 minutes at 4^{o}C. The supernatant (corresponding to the cytoplasmic fraction) was saved and transferred in a clean tube and stored at -80^{o}C for future use. The pellet was resuspended in 60µl, by pipeting up and down several times, in buffer containing 10mM Hepes, 400mM NaCl, 0.1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM protease cocktail inhibitor and 1mM NaOV. Lysates were sonicated by performing 6 cycles at 10 seconds per cycle with 30 second intervals and then centrifuged at 13000 RPM for 5 minutes at 4^{o}C. Pellet was discarded and the supernatant, corresponding to the nuclear fraction, was collected. Protein concentrations were measured using Bradford assay.

Statistics. The results presented are means ± SE of three independent experiments, performed in duplicate, with standard errors. Statistical analyses were performed by analysis of variance (one-way ANOVA), followed by Dunnett's multiple comparison post test, where applicable, using Prism 5 (GraphPAD software Inc.). p values < 0.05 were considered significant.

3.4 Results

3.4.1 Inhibition of c-Src PTK attenuates ET-1 and Ang II-induced phosphorylation of ERK 1/2 in A10 VSMC.

Several studies have previously produced evidence supporting vasoactive peptide-induced ERK 1/2 activation in multiple systems, including oligodendrocyte progenitor cells, pancreatic stellate cell, aortic and mesenteric artery-derived VSMC [24-29]. In this study, we confirmed that both ET-1 and Ang II, at a 100nM concentration, induced the phosphorylation of ERK 1/2, JNK and p38MAPK in a time dependent fashion in both A10 VSMC and isolated rat aortic VSMC and determined the time points at which peak phosphorylation occurred (data not shown). Our lab has also recently demonstrated a role of c-Src in mediating ET-1 and Ang II-induced PKB phosphorylation through Tyr 418 phosphorylation of c-Src in VSMC, which was blocked by PP-2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazole(3,4-d) pyrimidine), a specific blocker of Src family of PTK [37]. Here, by using PP-2, we have investigated a role of c-Src in ERK 1/2 phosphorylation in A10 VSMC. As shown in Fig. 1, both ET-1 (panel A) and Ang II (panel B) potently enhanced the phosphorylation of ERK 1/2. However, pre-treatment of A10 VSMC with PP-2 for 30 minutes dose-dependently attenuated both ET-1 and Ang II-induced phosphorylation of ERK 1/2. However, PP-3 (4-amino-7-phenylpyrazole(3,4-d) pyrimidine), an inactive analog of PP-2, had no effect. No alterations on the total amounts of ERK 1/2 were observed under these experimental conditions. Similar experiments were performed in VSMC isolated from rat aorta and identical results were obtained (supplementary figure S1).

3.4.2 ET-1 and Ang II-induced phosphorylation of JNK/SAPK and p38 MAPK is attenuated by c-Src PTK inhibition in A10 VSMC.

c-Jun N-terminal kinase (JNK) and p38 MAPK are both expressed in multiple cell types, including endothelial and VSMC. While ERK 1/2 has been extensively studied in

terms of vasoactive peptide induced activation, the activation of JNK and p38mapk, being associated more often with inflammatory cytokines and cellular stress, is not. Nonetheless, a few studies have shown an activation of JNK and p38 MAPK by Ang II and norepinephrine in cardiac myocytes, VSMC, and human mesangial cell. Furthermore, the role of c-Src in the activation of JNK and p38 MAPK in VSMC has been examined in very few studies, which provide conflicting results as to the location of p38mapk in the cascade of signaling events [38, 43]. Therefore, by using PP-2, we have investigated a role of c-Src in JNK and p38 MAPK phosphorylation in A10 VSMC. Both ET-1 (panel A) and Ang II (panel B) potently enhanced the phosphorylation of JNK (Fig.2) and p38mapk (Fig.3). Treatment of A10 VSMC with PP-2 for 30 minutes prior to either ET-1 or Ang II stimulation dose-dependently inhibited JNK (Fig.2) and p38 MAPK (Fig.3) phosphorylation by both peptides. PP-3, on the other hand, was unable to inhibit JNK or p38 MAPK phosphorylation induced by vasoactive peptides. No alterations on the total amounts of JNK or p38 MAPK were observed under these experimental conditions. Similar experiments were performed in VSMC isolated from rat aorta and identical results were obtained (Supplementary figures S2 and S3).

3.4.3 Inhibition of c-Src PTK attenuates ET-1-induced early growth response factor-1 (Egr-1) transcription factor expression in A10 VSMC.

Recent studies have suggested that the Egr-1 transcription factor plays an important role in multiple cardiovascular pathological processes, including the pathogenesis of atherosclerotic lesions and neointimal thickening after vascular injury [44, 45]. Low levels of Egr-1 are generally found in normal vessels, yet its expression is rapidly increased in both endothelial and VSMC in response to injury, a state in which Egr-1 exerts potent chemotactic and mitogenic effects, possibly contributing to the vascular remodeling commonly observed in various pathological forms of vascular disease [46]. A growing number of studies have examined Egr-1 expression in response to Ang II in several cell types, including VSMC [47, 48], yet very little work has been done on ET-1-induced Egr-1 response. Therefore, we wished to determine if ET-1 would upregulate Egr-1 expression in A10 VSMC. As shown in Fig.4, stimulation of serum-starved VSMC with 100nM ET-1 time-dependently increased

expression of Egr-1 protein (panel A). To examine a role of c-Src in ET-1-induced Egr-1 expression, cells were treated with either 10 μM PP-2 or PP-3 prior to ET-1 treatment for one hour. PP-2 treatment decreased ET-1-induced Egr-1 expression in A10 VSMC (Fig.4 panel B) confirming the essential role of c-Src in Egr-1 expression. PP-3, an inactive analog of PP-2, was without any effect. Similar experiments were performed in VSMC isolated from rat aorta and identical results were obtained (Supplementary figure S4).

3.4.4 Effect of c-Src knockdown in ET-1 and Ang-II-induced ERK 1/2, JNK and p38 MAPK phosphorylation and Egr-1 expression

To further confirm a role of c-Src PTK in vasoactive peptide-induced MAPK activation, we utilized mouse embryonic fibroblasts harvested from mouse embryos which have a functional null mutation in both alleles of the Src family PTK coding for c-Src (SYF) [49]. Mouse embryonic fibroblasts expressing endogenous wild type c-Src (Src +/+) were used as control cells in these experiments. Furthermore, to insure the decrease of c-Src in SYF cells as compared to Src +/+ cells, blots were probed with total c-Src antibody, and as seen in panel E of Fig.5, there is a clear decrease in c-Src content in the SYF cells as compared to the Src+/+ cells.

Since previous studies have investigated a role of c-Src in Ang II-induced responses, we have focused on studying the uncertain role of c-Src in ET-1-induced responses. As shown in Fig.5, ET-1 treatment caused a time-dependent increase of ERK 1/2 (panel A), JNK (panel B) and p38 MAPK (panel C) phosphorylation in c-Src +/+ cells, however, this response was blunted in SYF cells. No alterations on the total amounts of ERK 1/2, JNK or p38 MAPK were observed under these experimental conditions. Furthermore, to examine a role of c-Src in ET-1-induced Egr-1 expression, both SYF and Src+/+ cells were treated for 1, 2 or 3 hours with 100 nM ET-1. Cells were lysed and nuclear protein was isolated and immunoblotted for Egr-1. As shown in panel D of Fig.5, ET-1-induced Egr-1 expression is decreased in SYF cells as compared to Src +/+ MEF.

3.5 Discussion

c-Src is a member of the Src family of NR-TK, which contains at least 10 other members, including Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes [50]. The Src family of NR-TK play a vital role in cell differentiation, proliferation, and survival signaling mechanisms, as well as in cell adhesion, morphology and motility (reviewed in [51]). The studies performed here demonstrate a role of c-Src in both ET-1 and Ang II-induced ERK 1/2, JNK and p38 MAPK phosphorylation, through pharmacological inhibition of c-Src in immortalized VSMC, primary culture of aortic VSMC derived from aorta of eight week old Sprague Dawley rats, as well as mouse embryonic fibroblast from c-Src knockout mice.

The present data further suggest that both ET-1 and Ang II signal through a similar c-Src dependent pathway, leading to MAPK activation, opposing the notion of the existence of 2 distinct signaling pathways taken by ET-1 and Ang II, which lead to GPCR-mediated MAPK activation in VSMCs [38]. While Yogi et al. clearly show a role of c-Src in Ang II-induced MAPK activation, they identified ET-1-induced ERK 1/2 and JNK phosphorylation as a c-Src-independent event. Curiously, ET-1-induced p38 MAPK phosphorylation was inhibited in c-Src -/- mesenteric VSMC [38]. Our findings clearly demonstrate that ET-1-induced p38 MAPK phosphorylation is inhibited by PP-2 just as potently as ET-1-induced ERK 1/2 and JNK phosphorylation in aortic VSMC and mouse embryonic fibroblasts. A recent study showing that incubation of aortic rings with ET-1 leads to an increase in ERK 1/2 phosphorylation, which was suppressed by co-incubation with PP-2, further supports a role of c-Src as an upstream regulator of MAPK activation [52]. Thus, the work presented here, as well as previous work from our laboratory [37] and that of others [52], supports the notion that c-Src is an important regulator of both ET-1 and Ang II-induced hypertrophic and proliferative signaling pathways in VSMCs. This is in contrast to work showing that Src tyrosine kinases do not contribute to vascular trophic signaling of ET-1 [53].

Previous studies from our laboratory have detected c-Src Tyr 418 phosphorylation induced by ET-1, Ang II and H_2O_2 in as little as five minutes in VSMC [37, 54] which is in contrast to studies in whole aorta, where c-Src phosphorylation was detected only after

several hours of treatment with ET-1 [52]. In these studies, it was demonstrated that both of these vasoactive peptides can induce the Tyr 418 phosphorylation of c-Src activity, in VSMC and that pharmacological inhibition of c-Src by PP-2 not only inhibited ET-1 and Ang II-induced c-Src phosphorylation, but also inhibited IGF-1R tyrosine phosphorylation, as well as PKB phosphorylation and activation [37]. These studies imply that c-Src activation is essential in triggering IGF-1R phosphorylation by both Ang II and ET-1, and therefore provide a molecular basis by which the ligand-independent transactivation of IGF-1R occurs in VSMC, in response to vasoactive peptides [37]. Thus, our present and past findings provide evidence to support that ET-1 and Ang II lead to phosphorylation and subsequent activation of c-Src through Tyr 418 phosphorylation, and not solely through *de novo* synthesis of c-Src, as suggested by other studies [52].

The results presented here also reveal a role of c-Src in ET-1-induced Egr-1 expression in VSMC. Egr-1, which plays an important role in vascular biology, belongs to the family of zinc finger transcription factors which also includes Egr-2, Egr-3, and Egr-4 [55]. It is an 80-82 kDa nuclear phosphoprotein expressed in multiple cell types, including both endothelial and VSMCs, following stimulation by either cytokines or inflammatory stimuli (i.e. hypoxia, shear stress, etc...) or growth factors (EGF, PDGF). These studies lead to vascular remodelling and the development of vascular diseases like atherosclerosis and restenosis [56-58]. However, Egr-1 is very weakly expressed in normal, healthy vessel wall tissue. Several studies, including one in VSMCs, have investigated the capacity of Ang II to stimulate Egr-1 protein and mRNA expression [59], yet few have investigated the role of ET-1-induced Egr-1 expression. Furthermore, these studies were not performed in VSMC, but either in cardiomyocytes or mouse muscle cells, and were not able to correlate ET-1-induced Egr-1 mRNA with an increase in functional Egr-1 protein in these cells [60, 61]. Our results clearly demonstrate that ET-1 treatment of VSMC and mouse embryonic fibroblasts induces the expression of Egr-1 protein in as little as 1 hour. We further show that pharmacological blockade or genetic knockdown of c-Src PTK inhibits ET-1-induced Egr-1 expression in both cell types. Therefore, to our knowledge, the results presented in this study are the first to explore the role of c-Src in ET-1-induced Egr-1 expression in VSMC. ERK1/2 has also

proven to play an important role in Egr-1 activation in both endothelial and VSMC [59, 62] through work demonstrating that inhibition of Egr-1 gene expression inhibits VSMC migration and proliferation [63]. Thus, it may be suggested that the downregulation of ET-1-induced VSMC hypertrophy and proliferation by c-Src inhibition, demonstrated in our previous studies [37], may be due to its capacity to regulate MAPK signaling and Egr-1 protein expression. This notion is supported by work showing that the blockade of ET-1-induced Egr-1 mRNA through antisense oligonucleotides (ASO) inhibited ET-1-induced hypertrophy in isolated rat cardiomyocytes [64].

In summary, the results of this investigation demonstrate that ET-1 and Ang II induce the phosphorylation of ERK 1/2, JNK and p38 MAPK through a c-Src PTK-dependent pathway. Moreover, c-Src appears to be a key mediator of ET-1-induced Egr-1 expression, further clarifying the signaling mechanisms implicated in mediating the hypertrophic and proliferative responses of both ET-1 and Ang II in aortic VSMC.

3.6 Acknowledgments

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3.7 Figure legends

Figure 1. Pharmacological blockade of c-Src abolishes ET-1 and Ang II-induced ERK1/2 phosphorylation in A10 VSMCs. Serum-starved quiescent A10 VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or the indicated concentration of PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel A) or Ang II (100nM) (panel B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Thr202/Tyr204-ERK1/2 antibodies. Blots were also analyzed for total ERK. Bar diagrams in each panel represent average data quantified by densitometric scanning of immunoblots shown in the same panel. Values are the means ± SE of three independent experiments and are expressed as a fold increase in phosphorylation, where basal phosphorylation observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone in panel A or versus Ang II alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

Figure 2. Pharmacological blockade of c-Src attenuates ET-1 and Ang II-induced JNK phosphorylation in A10 VSMCs. Serum-starved quiescent A10 VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or the indicated concentration of PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel A) or Ang II (100nM) (panel B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Thr183/Tyr185-JNK antibodies. Blots were also analyzed for total JNK. Bar diagrams in each panel represent average data quantified by densitometric scanning of immunoblots shown in the same panel. Values are the means ± SE of three independent experiments and are expressed as a fold increase in phosphorylation, where basal phosphorylation observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone in panel A or versus Ang II alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

Figure 3. Pharmacological blockade of c-Src attenuates ET-1 and Ang II-induced p38 MAPK phosphorylation in A10 VSMCs. Serum-starved quiescent A10 VSMC were

pretreated in the absence (0) or presence of 10μM PP-3 or the indicated concentration of PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel A) or Ang II (100nM) (panel B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Thr180/Tyr182-p38 MAPK antibodies. Blots were also analyzed for total p38 MAPK. Bar diagrams in each panel represent average data quantified by densitometric scanning of immunoblots shown in the same panel. Values are the means ± SE of three independent experiments and are expressed as a fold increase in phosphorylation, where basal phosphorylation observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone in panel A or versus Ang II alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

Figure 4. Pharmacological blockade of c-Src attenuates ET-1-induced Egr-1 protein expression in A10 VSMCs. Serum-starved quiescent A10 VSMC were treated without (0) or with 100nM ET-1 for the indicated time periods (section A) or were pretreated in the absence (0) or presence of 10μM PP-3 or 10μM PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel B). Nuclear protein was isolated from cell lysates and immunoblotted by Egr-1 antibody as shown in the top panels of each section. Blots were analyzed for total nuclear protein by GAPDH (middle panels of each section). Bottom panels (bar diagrams) represent average data quantified by densitometric scanning of immunoblots showing in the middle panel. Values are the means ± SE of three independent experiments and are expressed as fold increase of protein expression where basal expression observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus no stimulation (0), or versus ET-1 stimulation alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

Figure 5: ET 1-induced ERK 1/2, JNK and p38 MAPK phosphorylation, as well as Egr-1 expression is downregulated in SYF MEF but not in SRC +/+ MEF. Confluent, serum-starved SRC +/+ and SYF MEF were treated with 100 nM ET-1 for indicated time points. The cells were lysed and lysates were subjected to immunoblotting using phospho-

specific Thr 202/Tyr 204-ERK and total ERK antibodies (panel A), phospho-specific-Thr183/Tyr185-JNK and total JNK antibodies (panel B) and phospho-specific-Thr180/Tyr182-p38 MAPK and total p38 MAPK antibodies (panel C). To detect Egr-1, nuclear protein was isolated from cell lysates and immunoblotted with Egr-1 antibody and GAPDH (panel D). Immunoblots were also probed with total Src antibody to measure for levels of Src in SYF and Src +/+ MEF (panel E). Values are the means ± SE of three independent experiments and are expressed as fold increase of protein expression where basal expression observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus no stimulation (0). * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

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3.9 Figures

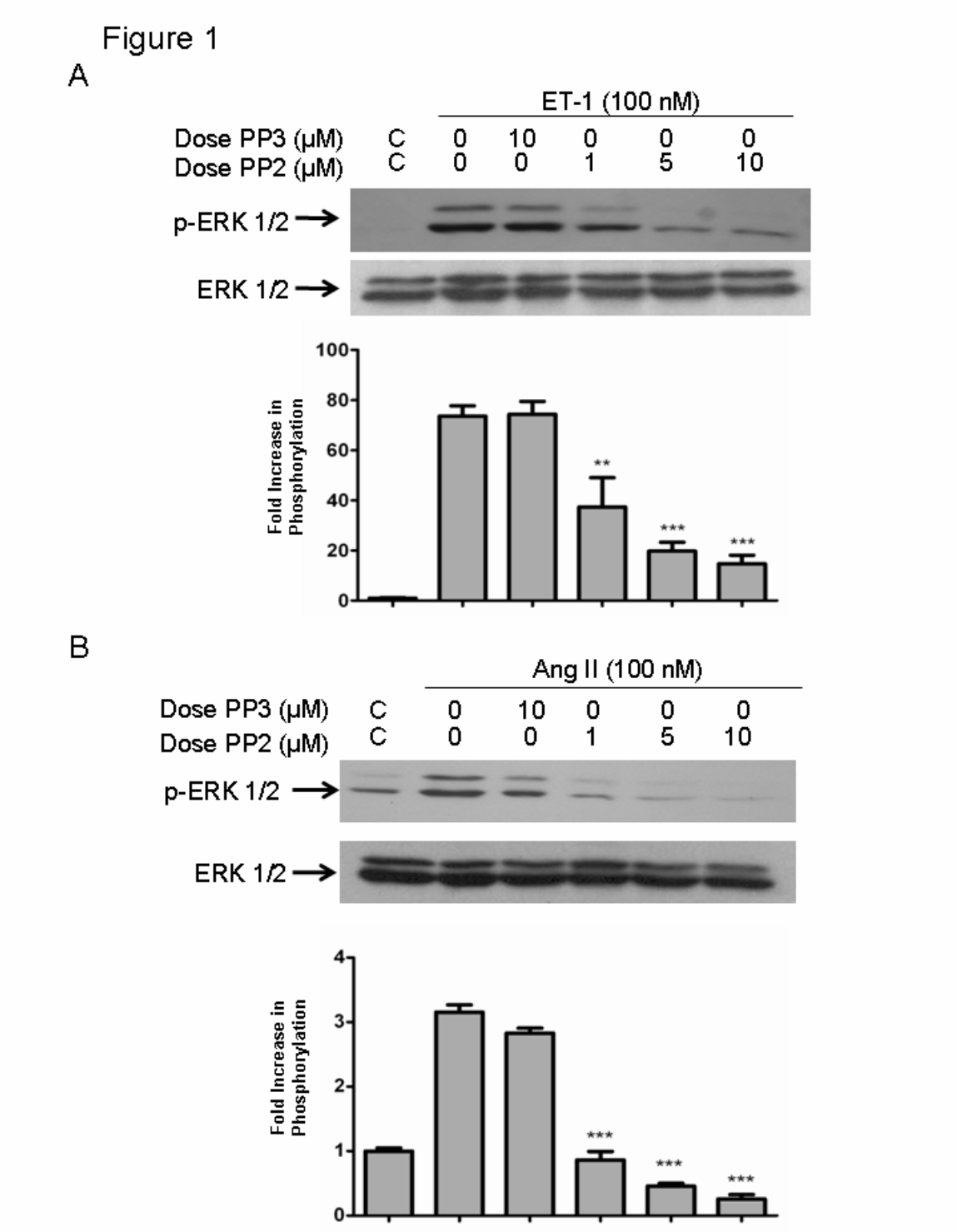
Figure 1
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Dose PP2 (μM) C 0 0 1 5 10
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ERK 1/2
Fold Increase in Phosphorylation
100
80
60
40
20
0
**
***
***
B
Ang II (100 nM)
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Dose PP2 (μM) C 0 0 1 5 10
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ERK 1/2
Fold Increase in Phosphorylation
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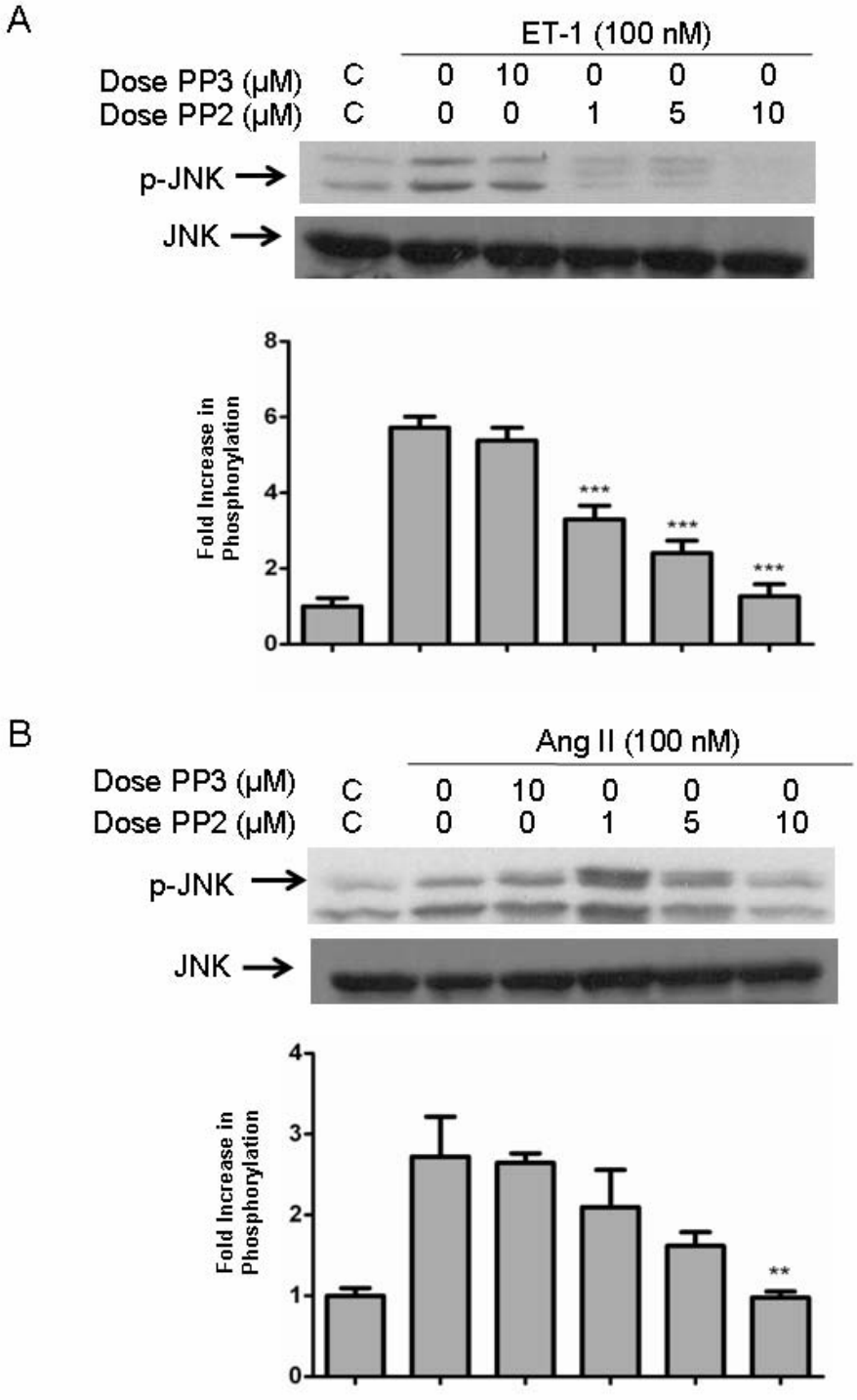
3
2
1
0
***
***
***

Figure 2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Dose PP2 (μM) C 0 0 1 5 10
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JNK
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8
6
4
2
0
***
***
***
B
Ang II (100 nM)
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Dose PP2 (μM) C 0 0 1 5 10
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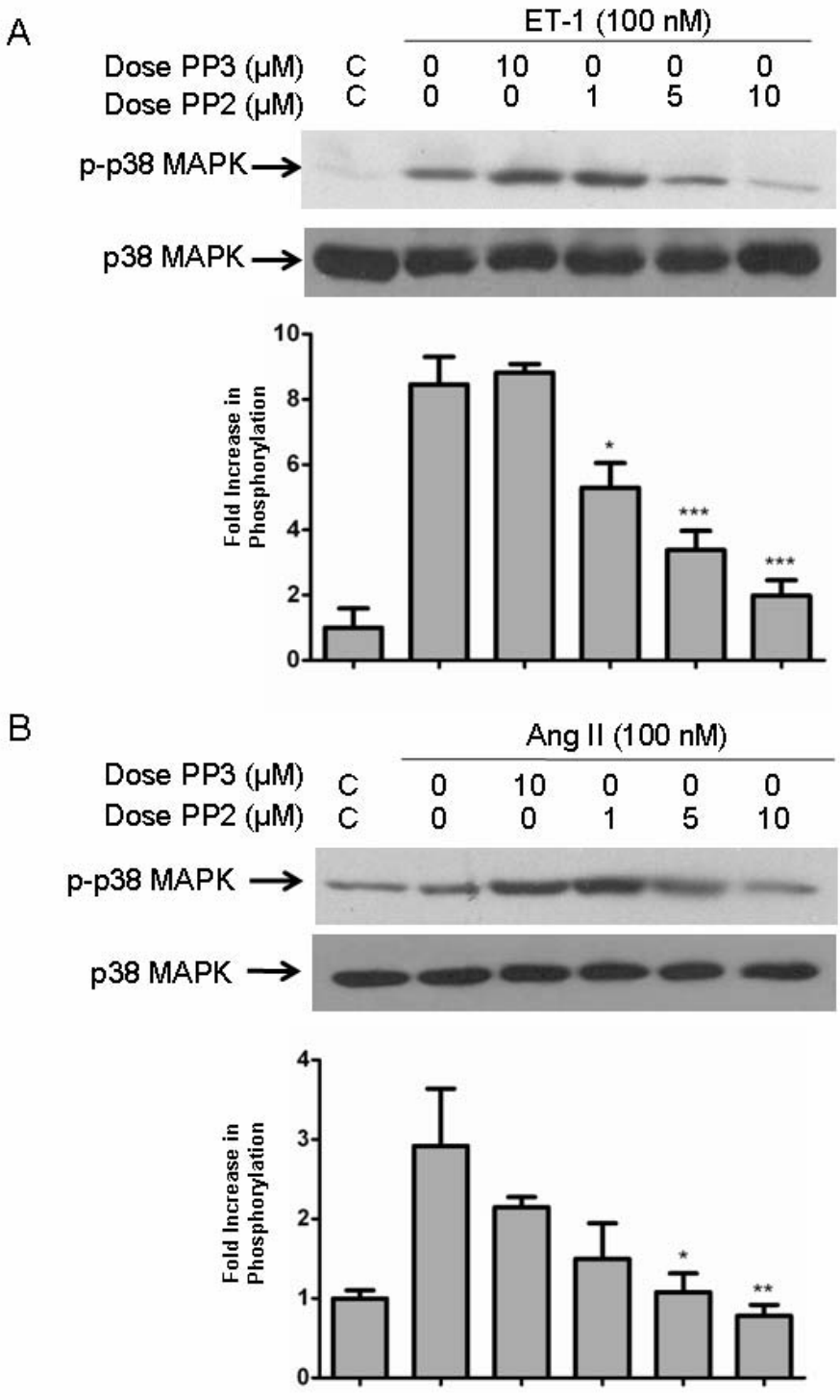
JNK
Fold Increase in Phosphorylation
4
3
2
1
0
**

Figure 3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Dose PP2 (μM) C 0 0 1 5 10
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p38 MAPK
Fold Increase in Phosphorylation
10
8
6
4
2
0
*
***
***
B
Ang II (100 nM)
Dose PP3 (μM) C 0 10 0 0 0
Dose PP2 (μM) C 0 0 1 5 10
p-p38 MAPK
p38 MAPK
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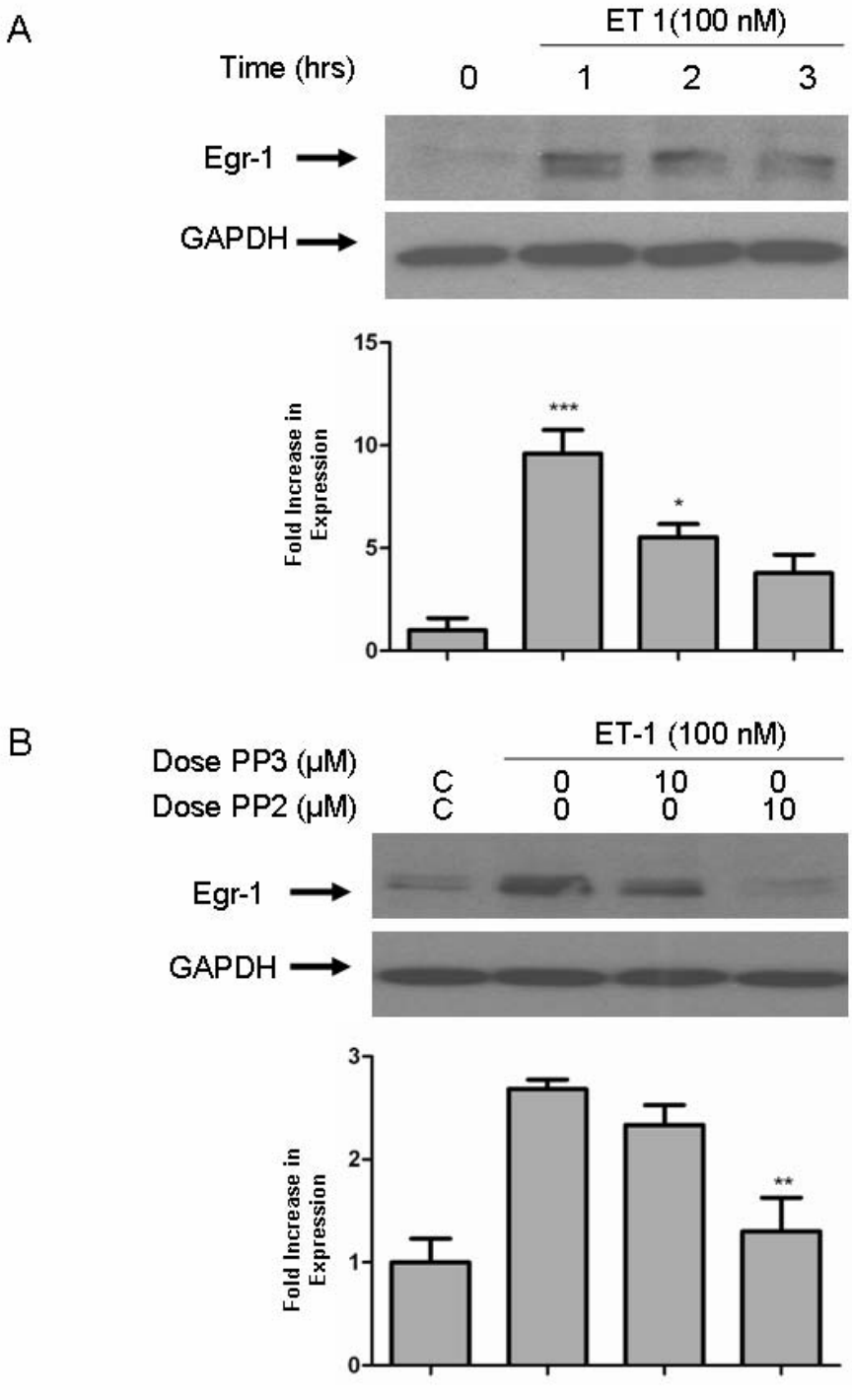
4
3
2
1
0
*
**

Figure 4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Time (hrs)
0
1
2
3
Egr-1
GAPDH
Fold Increase in Expression
15
10
5
0
***
*
B
ET-1 (100 nM)
Dose PP3 (μM)
C
0
10
0
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10
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GAPDH
Fold Increase in Expression
3
2
1
0
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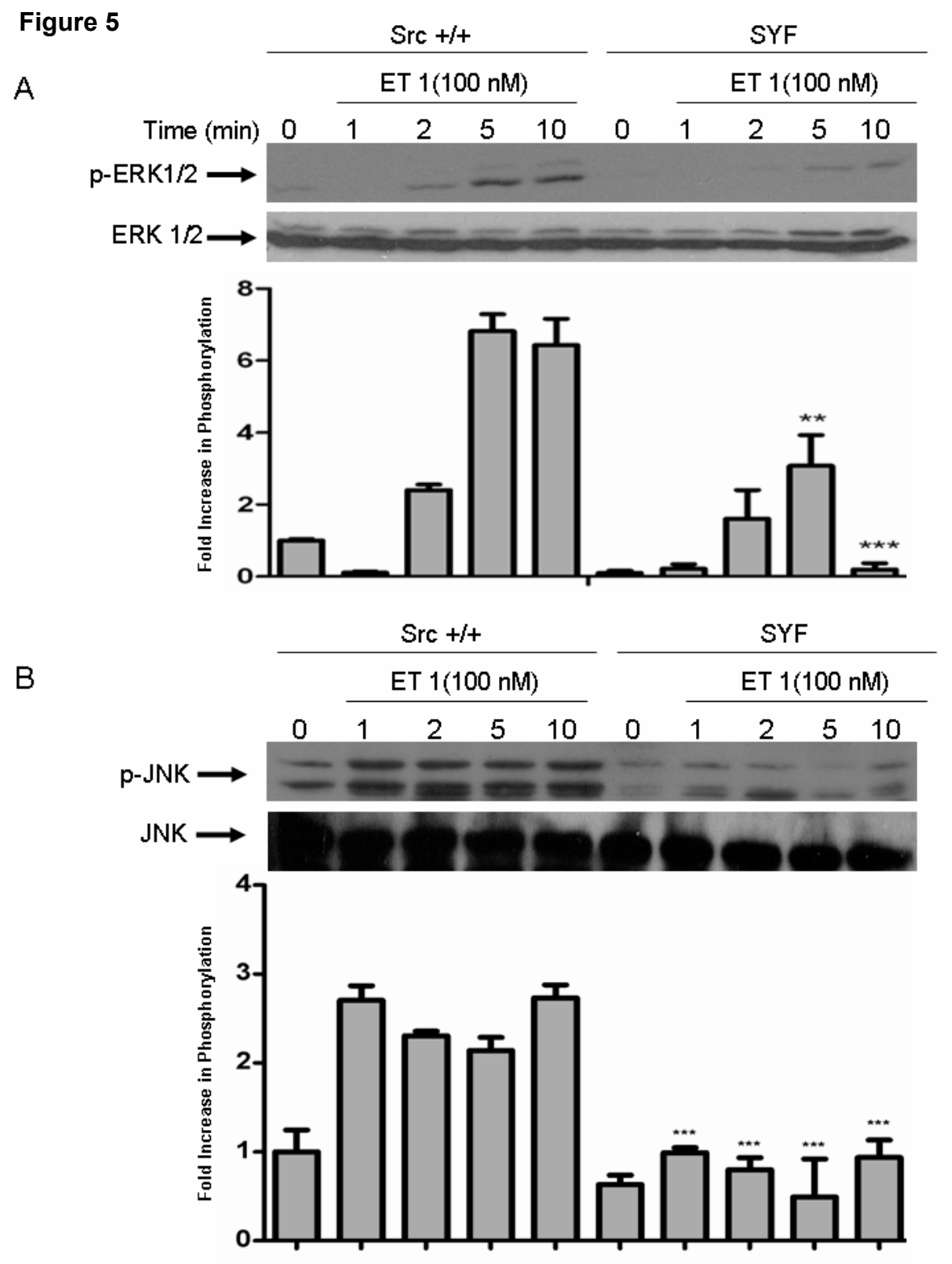
Figure 5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SYF
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ET 1(100 nM)
Time (min) 0 1 2 5 10 0 1 2 5 10
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ERK 1/2
Fold Increase in Phosphorylation
8
6
4
2
0
**
***
B
Src +/+
SYF
ET 1(100 nM)
ET 1(100 nM)
0 1 2 5 10 0 1 2 5 10
p-JNK
JNK
Fold Increase in Phosphorylation
4
3
2
1
0
***
***
***
***

Figure 5

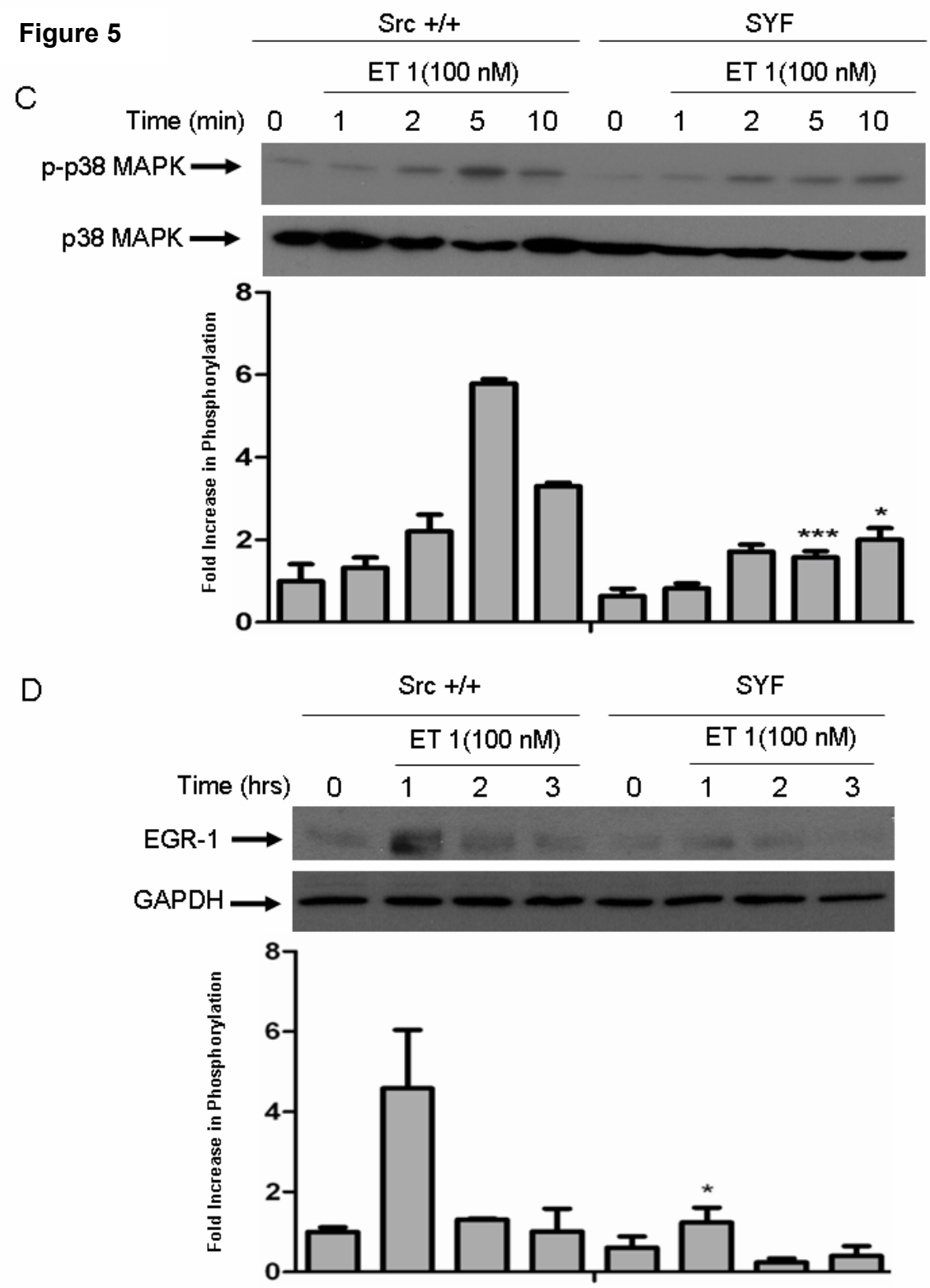
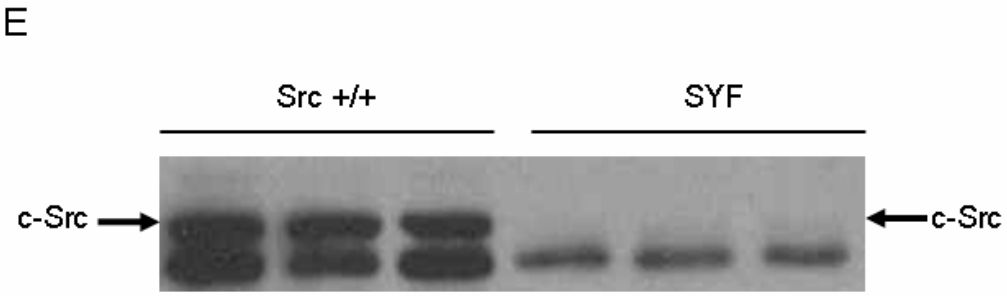
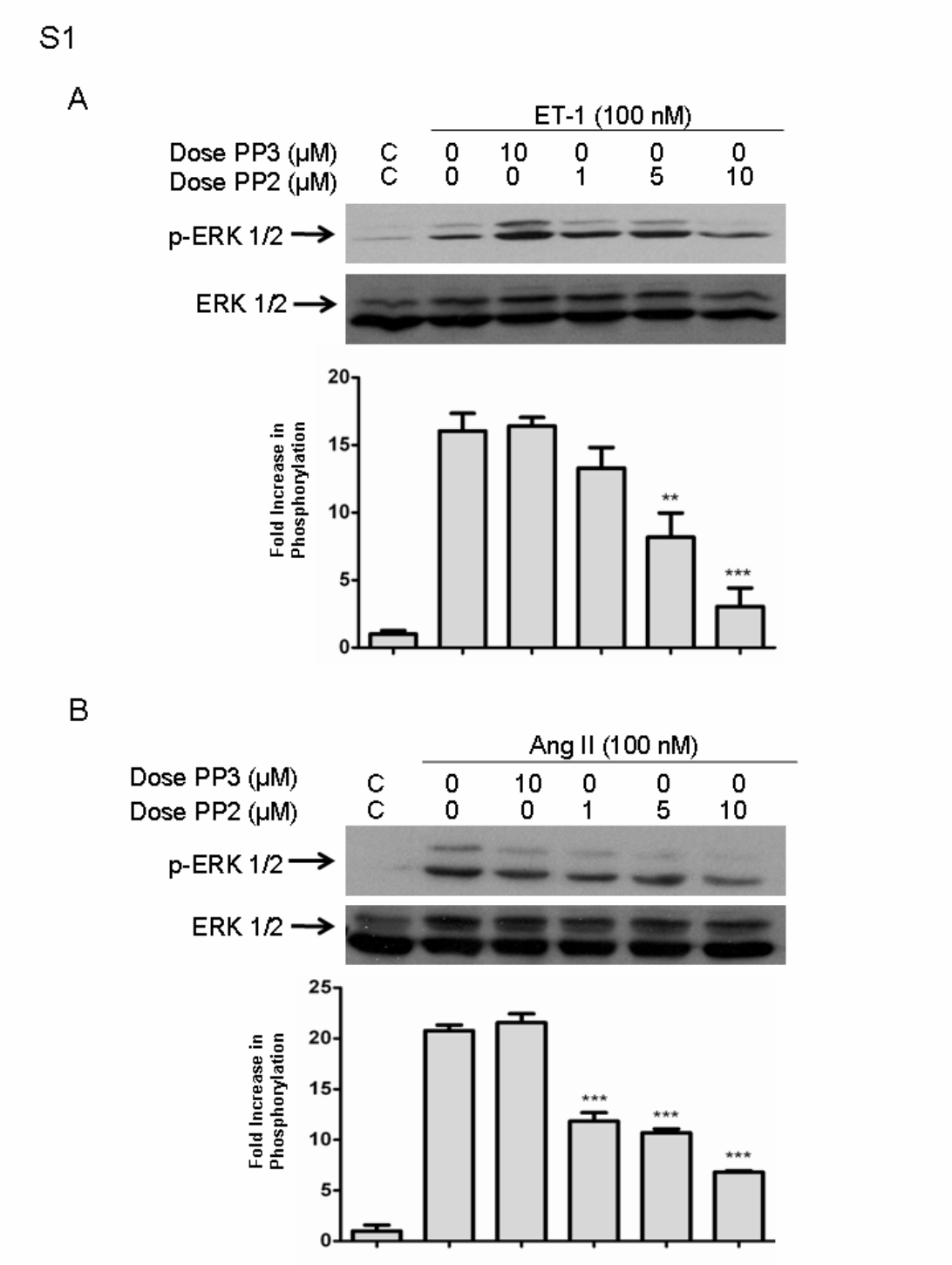

C
Src +/+
SYF
ET 1(100 nM)
ET 1(100 nM)
Time (min) 0 1 2 5 10 0 1 2 5 10
p-p38 MAPK
p38 MAPK
Fold Increase in Phosphorylation
8
6
4
2
0
***
*
D
Src +/+
SYF
ET 1(100 nM)
ET 1(100 nM)
Time (hrs) 0 1 2 3 0 1 2 3
EGR-1
GAPDH
Fold Increase in Phosphorylation
8
6
4
2
0
*

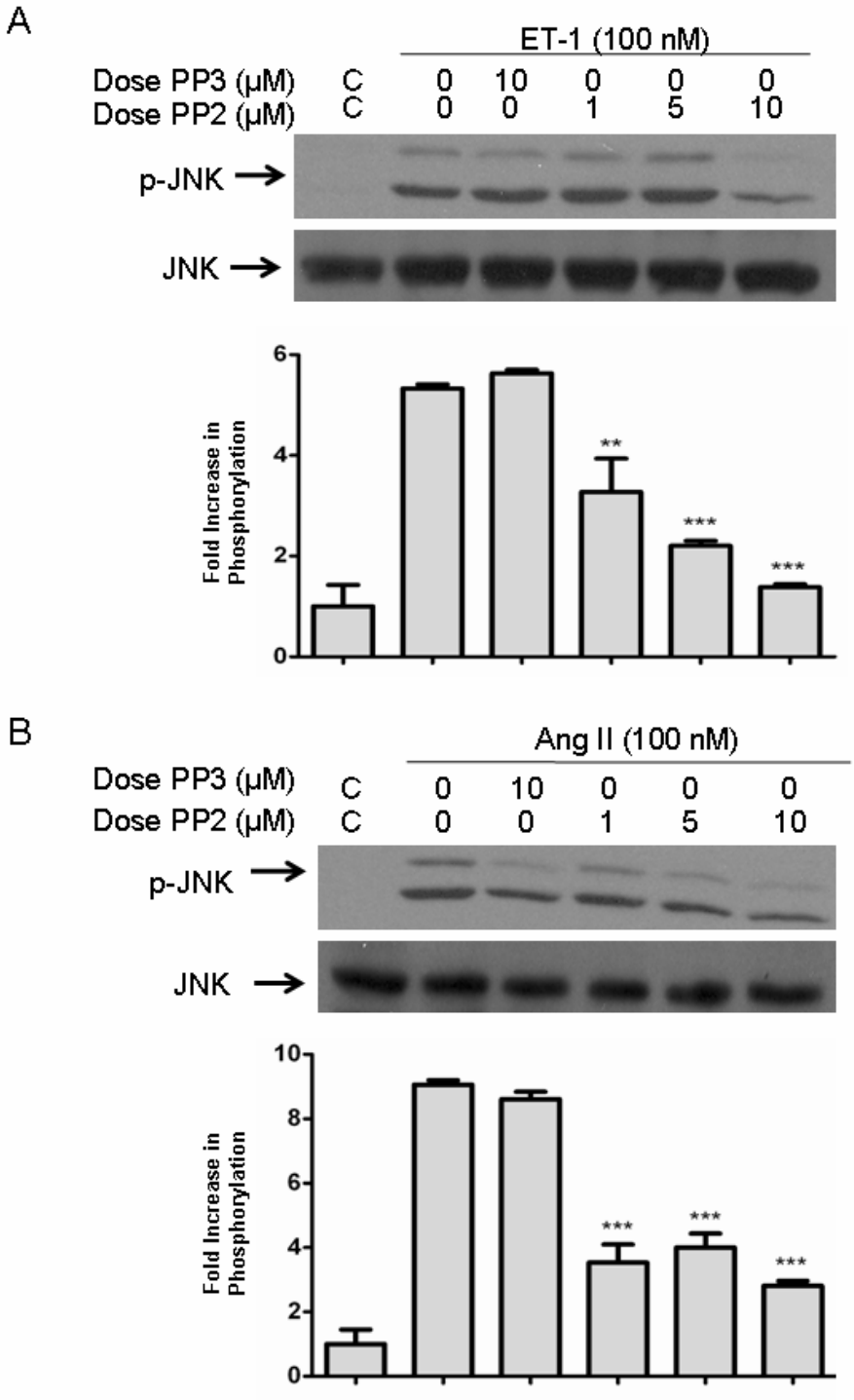
Figure 5



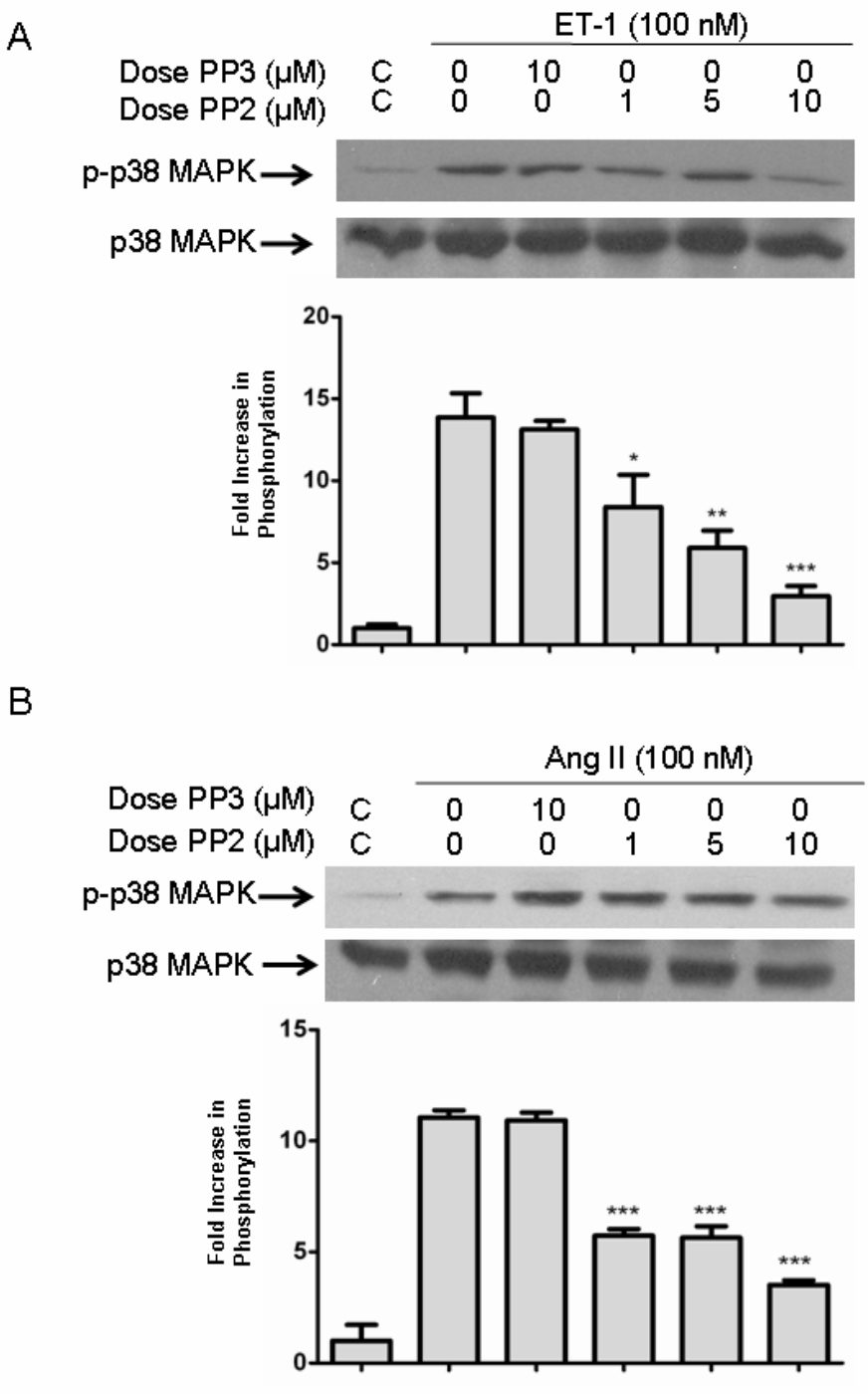
3.10 Supplementary Figures



S2

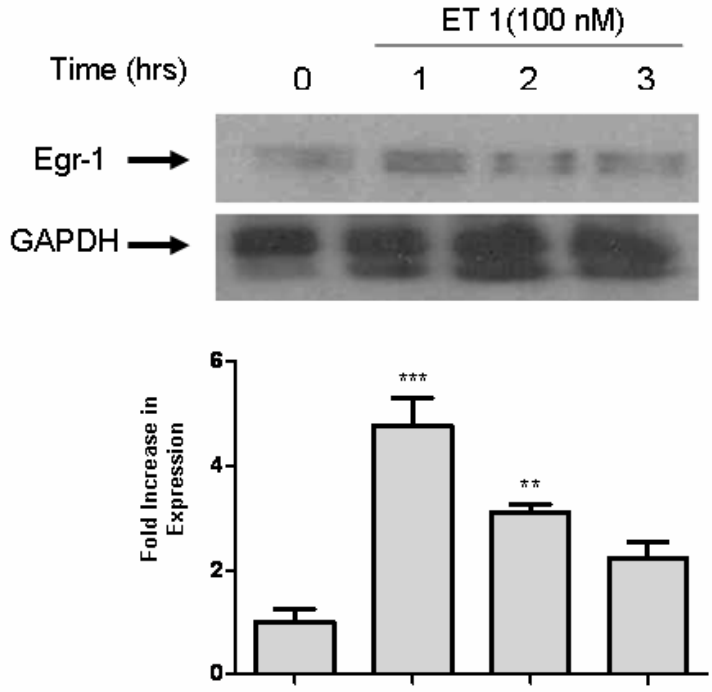

A
ET-1 (100 nM)
Dose PP3 (μM) C 0 10 0 0 0
Dose PP2 (μM) C 0 0 1 5 10
p-JNK
JNK
Fold Increase in Phosphorylation
0
2
4
6
**
***
***
B
Ang II (100 nM)
Dose PP3 (μM) C 0 10 0 0 0
Dose PP2 (μM) C 0 0 1 5 10
p-JNK
JNK
Fold Increase in Phosphorylation
0
2
4
6
8
10
***
***
***

S3

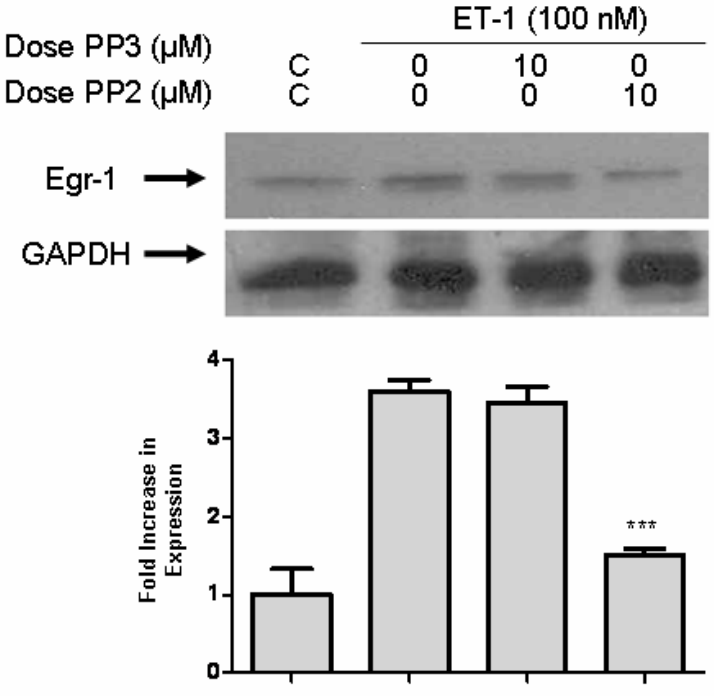

A
ET-1 (100 nM)
Dose PP3 (μM) C 0 10 0 0 0
Dose PP2 (μM) C 0 0 1 5 10
p-p38 MAPK
p38 MAPK
Fold Increase in Phosphorylation
20
15
10
5
0
*
**
***
B
Ang II (100 nM)
Dose PP3 (μM) C 0 10 0 0 0
Dose PP2 (μM) C 0 0 1 5 10
p-p38 MAPK
p38 MAPK
Fold Increase in Phosphorylation
15
10
5
0
***
***
***

S4

A


ET 1(100 nM)
Time (hrs)
0
1
2
3
Egr-1
GAPDH
Fold Increase in Expression
6
4
2
0
***
**

B


ET-1 (100 nM)
Dose PP3 (μM)
C
0
10
0
Dose PP2 (μM)
C
0
0
10
Egr-1
GAPDH
Fold Increase in Expression
4
3
2
1
0
***

3.11 Supplementary Figure Legends

S1. Pharmacological blockade of c-Src abolishes ET-1 and Ang II-induced ERK1/2 phosphorylation in SD VSMCs. Serum-starved quiescent SD VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or the indicated concentration of PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel A) or Ang II (100nM) (panel B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Thr202/Tyr204-ERK1/2 antibodies. Blots were also analyzed for total ERK. Bar diagrams in each panel represent average data quantified by densitometric scanning of immunoblots shown in the same panel. Values are the means ± SE of three independent experiments and are expressed as a fold increase in phosphorylation, where basal phosphorylation observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone in panel A or versus Ang II alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

S2. Pharmacological blockade of c-Src attenuates ET-1 and Ang II-induced JNK phosphorylation in SD VSMCs. Serum-starved quiescent SD VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or the indicated concentration of PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel A) or Ang II (100nM) (panel B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Thr183/Tyr185-JNK antibodies. Blots were also analyzed for total JNK. Bar diagrams in each panel represent average data quantified by densitometric scanning of immunoblots shown in the same panel. Values are the means ± SE of three independent experiments and are expressed as a fold increase in phosphorylation, where basal phosphorylation observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone in panel A or versus Ang II alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

S3. Pharmacological blockade of c-Src attenuates ET-1 and Ang II-induced p38 MAPK phosphorylation in SD VSMCs. Serum-starved quiescent SD VSMC were pretreated in the absence (0) or presence of 10μM PP-3 or the indicated concentration of

PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel A) or Ang II (100nM) (panel B) for 5 min. Cell lysates were immunoblotted with phospho-specific-Thr180/Tyr182-p38 MAPK antibodies. Blots were also analyzed for total p38 MAPK. Bar diagrams in each panel represent average data quantified by densitometric scanning of immunoblots shown in the same panel. Values are the means ± SE of three independent experiments and are expressed as a fold increase in phosphorylation, where basal phosphorylation observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus ET-1 stimulation alone in panel A or versus Ang II alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

S4. Pharmacological blockade of c-Src attenuates ET-1-induced Egr-1 protein expression in SD VSMCs. Serum-starved quiescent SD VSMC were treated without (0) or with 100nM ET-1 for the indicated time periods (section A) or were pretreated in the absence (0) or presence of 10μM PP-3 or 10μM PP-2 for 30 minutes, followed by stimulation with ET-1 (100nM) (panel B). Nuclear protein was isolated from cell lysates and immunoblotted by Egr-1 antibody as shown in the top panels of each section. Blots were analyzed for total nuclear protein by GAPDH (middle panels of each section). Bottom panels (bar diagrams) represent average data quantified by densitometric scanning of immunoblots showing in the middle panel. Values are the means ± SE of three independent experiments and are expressed as fold increase of protein expression where basal expression observed (no stimulation) is defined as 1. $P<0.05$ was considered as statistically significance versus no stimulation (0), or versus ET-1 stimulation alone in panel B. * indicates that $P<0.05$; ** indicates that $P<0.001$ and *** indicates that $P<0.0001$.

Chapter 4

General Discussion

Ang II is a powerful vasoconstricting octapeptide cleaved from angiotensin I (Ang I) by angiotensin converting enzyme (ACE), and is an integral part of the renin-angiotensin-system (RAS) [456]. The principal physiological function of Ang II is the acute regulation of vascular tone to regulate blood pressure. ET-1 is one of the most potent vasoconstrictor peptides in circulation, responsible for regulating vascular tone and blood pressure through various mechanisms, such as salt and water homeostasis, as well as affecting the sympathetic nervous system [54-56]. It is produced mainly by the cardiovascular system, primarily by endothelial cells in the vasculature [457], but is also produced by the heart, kidney, posterior pituitary and central nervous system [458]. Under normal physiological conditions Ang II and ET-1 normalize blood pressure through the regulation of salt and/or water homeostasis, sympathetic nervous system modulation, as well as VSMC contraction and relaxation [53-56]. Increased levels of both ET-1 and Ang II, present in certain pathophysiological states, such as essential hypertension, obesity, or advanced stages of diabetes, have been suggested to contribute to the pathogenesis of CVDs, by activating signaling events intimately linked to migration and proliferation of VSMC [53, 57-59]. Ang II and ET-1 both exert their biological actions through their own respective GPCR receptor subtypes, described earlier.

Kinases, such as those activated by Ang II and ET-1, are amongst the highest represented known functions in the human genome [459]. Genome sequencing has revealed more than 520 protein kinases and approximately 130 protein phosphatases, which counteract kinase action [460]. Protein kinases can generally be separated into 2 groups, consisting of the tyrosine kinases, which once activated, phosphorylate substrates on tyrosine residues (e.g. IGF-1R, PYK2) or serine/threonine kinases, which phosphorylate substrates on serine and/or threonine residues (e.g. ERK1/2, PKB). Tyrosine kinases play a vital role in regulating multiple intracellular signaling pathways, responsible for cellular metabolism, survival, growth and programmed cell death [382, 422, 461-463]. Tyrosine kinase activity is very well regulated in normal physiological states, yet these kinases are often found in altered or mutated states in pathological conditions involving uncontrolled tissue or cell growth, such as cancers and cardiovascular diseases, due to their regulation of

growth promoting pathways [460, 464, 465]. This group of tyrosine kinases can further be separated into two groups consisting of R-PTK and NR-PTK. The usual mechanism of action consists of the R-PTK being activated through ligand binding, causing its dimerisation (if not already present as a dimer), which leads to the activation of its kinase activity, and subsequent autophosphorylation in tyrosine residues. These phosphorylated tyrosine residues serve as docking sites for other anchoring proteins and/or NR-PTK, which help propagate the downstream signaling of the ligand [466], activating signaling cascades such as the PI3K/PKB and MAPK pathways. As mentioned in earlier chapters, ligand-independent activation, or transactivation, of R- and NR-PTK has been implicated in transducing the downstream effects of GPCR, leading to the stimulation of the PI3-K/PKB and MAPK cascades, which are involved in mediating the migratory, proliferative, and hypertrophic responses of ET-1 and Ang II [50, 64, 417, 462, 467].

The results presented in this thesis demonstrate a requirement of IGF-1R in ET-1-induced PKB phosphorylation in VSMC [64]. In these studies, AG-1024, a specific inhibitor of IGF-1R PTK, attenuated ET-1-induced PKB phosphorylation in a dose-dependent manner. ET-1 treatment also induced the phosphorylation of key tyrosine residues in the auto-phosphorylation sites of the IGF-1R, which was blocked by AG-1024. We have also provided evidence to suggest an involvement of c-Src in ET-1-induced tyrosine phosphorylation of IGF-1R. We found that pre-treatment of VSMC with PP-2, a specific inhibitor of the Src family of PTK, significantly reduced ET-1-induced phosphorylation of IGF-1R. Interestingly, this treatment also inhibited ET-1-induced PKB phosphorylation in VSMC. ET-1-induced phosphorylation of IGF-1R has also been demonstrated in prostate cancer cell lines [468]. Similar to our observations in VSMC, c-Src activation was required to induce ET-1-induced PKB phosphorylation in these cell lines [468]. It thus appears that c-Src serves an upstream role in Ang II and ET-1-induced, ligand-independent phosphorylation of IGF-1R. It should be noted that c-Src is able to increase the tyrosine phosphorylation of the autophosphorylation sites on the IGF-1R β subunit [469].

Furthermore, both protein and DNA synthesis enhanced by Ang II and ET-1 were also attenuated by pharmacological blockade of IGF-1R by AG-1024, suggesting an important

role of IGF-1R in mediating PKB phosphorylation, as well as hypertrophic and proliferative responses induced by ET-1 in A10 VSMC [470].

There is also some evidence indicating that transactivation of EGFR mediates the responses of IGF-1R in some cell types, suggesting the existence of a cross-talk between IGF-R and EGFR transactivation [471]. Moreover, the studies showing that dominant negative or antisense oligonucleotide (ASO) of IGF-1R are able to attenuate neointima formation in an injured carotid artery rat model [403] and reduce Ang II type 1 receptor (AT_1R) expression and function in spontaneously hypertensive rats (SHR) [402] supports a potential pathogenic role of upregulated IGF-1R signalling in vascular disease.

In addition to the PKB pathway, ET-1 and Ang II-induced GPCR stimulation leads to the activation of the mitogen-activated protein kinase (MAPK) family, including ERK 1/2, JNK and p38 MAPK. While both ET-1 and Ang II seem to activate PKB through a c-Src-dependent mechanism, with a role of c-Src as a mediator of Ang II signaling [64, 472, 473], its role in ET-1-induced signaling events remains controversial [474], with recent reports suggesting that Ang II activates MAPK signaling through a c-Src-dependent mechanism, whereas ET-1 activates MAPK signaling through a c-Src-independent mechanism [418].

The results presented in this thesis are aimed at defining a role of this NR-PTK in ET-1-induced MAPK signaling. In these studies, PP-2 was found to reduce both ET-1 and Ang II-induced ERK 1/2, JNK and p38 MAPK phosphorylation in A10 VSMC, as well as in primary cultures of Sprague Dawley rat thoracic aorta. These results are supported by studies showing that ET-1-induced ERK 1/2 phosphorylation is suppressed by co-incubation with PP-2, identifying c-Src as an upstream regulator of MAPK activation in aortic rings [475].

To further confirm our hypothesis that c-Src is an important modulator of ET-1-induced MAPK signaling, we utilized mouse embryonic fibroblast harvested from mouse embryos which have a functional null mutation in both alleles of the Src family PTK coding for c-Src, Yes and Fyn (SYF), the three main members of the Src PTK family [476]. ET-1-induced phosphorylation of all three MAPKs studied was attenuated in SYF cells, yet remained normal in mouse embryonic fibroblasts expressing normal c-Src. We have also

demonstrated that inhibition of c-Src, by pharmacological or genetic approaches, blunted the expression of ET-1-induced Egr-1, a transcription factor implicated in multiple cardiovascular pathological processes, including the pathogenesis of atherosclerotic lesions and neointimal thickening after vascular injury. Therefore, the work presented in this thesis, and that of others [475], provides solid evidence and supports the notion that c-Src is an important regulator of vasoactive peptide-induced hypertrophic and proliferative signaling pathways. However, the specific participation of a precise R- or NR-PTK in mediating vasoactive peptide-induced responses may be dependent on the cell type and the expression levels of the R- and NR-PTKs in those cells. As a result, it may be possible that c-Src-dependent and -independent pathways contribute to both ET-1 and Ang II-induced signaling, which may vary from cell type to cell type, and may be a possible reason for the discrepancy between our work and that of others.

While the precise events that trigger the transactivation of R-/NR-PTK in response to vasoactive peptides are not clear, several mechanisms have been suggested. Studies have shown that inhibitors of matrix metalloproteinases (MMP), such as GM6001 and doxycyclin, attenuated ET-1 and Ang II-induced EGFR transactivation [438, 439]. MMPs act mainly by disrupting the link between growth factors and their transmembrane precursors, as is the case for heparin-bound-EGF (HB-EGF), allowing EGF to bind to EGFR, and leading to tyrosine kinase activation and stimulation of hypertrophic and growth promoting signaling cascades [440].

Another proposed mechanism of R-PTK transactivation by vasoactive peptides is through their capacity to induce the generation of reactive oxygen species (ROS). ROS are formed as intermediates in redox reactions, leading from molecular oxygen (O_2) to water (H_2O). These small, quickly-diffusible and highly-reactive molecules are classified into superoxide anion ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) [477]. A major intracellular source of ROS is the mitochondria, which converts 1-2% of consumed O_2 to $^{\cdot}O_2^-$ [478]. A univalent reduction of O_2 leads to $^{\cdot}O_2^-$, which is relatively unstable and short-lived because of its unpaired electron.

NAD(P)H oxidase is among the primary enzymes responsible for the generation of $\cdot O_2^-$ [446], and is composed of many subunits, including p22phox, p47phox, gp91phox, the GTPase Rac and the recently-identified Nox1 and Nox4 [446, 479-481]. NAD(P)H oxidase catalyzes $\cdot O_2^-$ production by the one electron reduction of O_2, where NAD(P)H is the electron donor. In addition to NAD(P)H oxidases, O_2^- can also be generated by xanthine/xanthine oxidase, lipooxygenase, and cyclooxygenase [482, 483] (Figure 11).

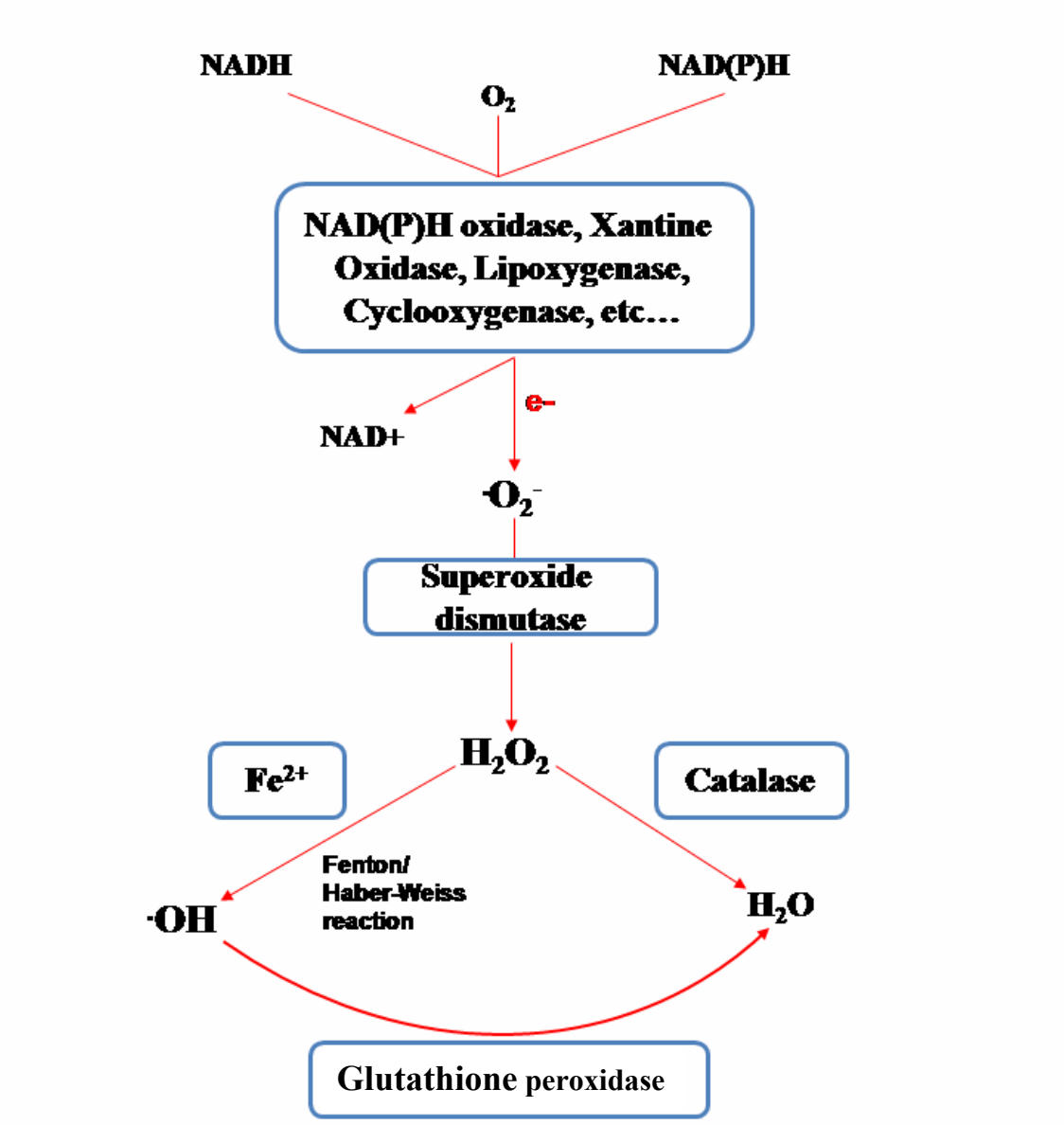


Figure 11: Key steps in the production of reactive oxygen species (ROS) by vasoactive peptides. NAD(P)H oxidase catalyzes superoxide anion ($\cdot O_2^-$) production by the 1 electron reduction of O_2, where NAD(P)H is the electron donor. In addition to NAD(P)H oxidases, O_2^- can also be generated by xanthine/xanthine oxidase, lipooxygenase, and cyclooxygenase. $\cdot O_2^-$ undergoes dismutation either spontaneously or by a reaction catalyzed by superoxide dismutase (SOD) to produce H_2O_2. Normally, it is scavenged by catalase and glutathione peroxidase to produce H_2O. In the presence of metal-containing molecules such as Fe^{2+}, H_2O_2 can also be reduced to generate the extremely-active hydroxyl radical ($\cdot OH$) that causes damage to cell components. (Adapted from Vardatsikos et al., Antioxid Redox Signal., 2009, 11(5):1165-90.)

Under physiological conditions, $^{\cdot}O_2^-$ undergoes dismutation either spontaneously or by a reaction catalyzed by superoxide dismutase (SOD) to produce H_2O_2 (Figure 11). Dismutation of $^{\cdot}O_2^-$ by SOD is favored at low concentrations of $^{\cdot}O_2^-$ and at high concentrations of SOD, which occurs under physiological conditions. H_2O_2 is much more stable than $^{\cdot}O_2^-$, can cross cell membranes and has a longer half-life. Normally, it is scavenged by catalase and glutathione peroxidase to produce H_2O [484] (Figure 11). In the presence of metal-containing molecules such as Fe^{2+}, H_2O_2 can also be reduced to generate the extremely-active hydroxyl radical ($^{\cdot}OH$) that causes damage to cell components [485] (Figure 11). In the glutathione peroxidase reaction, glutathione (GSH) is oxidized to glutathione disulfide (GSSG), which can be converted back to GSH by glutathione reductase in a NAD(P)H-consuming process. Several forms of SOD are known: copper-zinc SOD (Cu/Zn-SOD), mitochondrial or manganese SOD (Mn-SOD), extracellular SOD type C (EC SOD C) and iron-containing SOD (Fe-SOD) [486, 487]. Normally, the rate of ROS production is balanced by the rate of their elimination. However, in pathological conditions, a disequilibrium between ROS generation and elimination results in increased ROS bioavailability, leading to oxidative stress [488]. Under these conditions, $^{\cdot}O_2^-$ can react with nitric oxide (NO) to produce peroxynitrite ONOO- [489], which is reduced to form its conjugate acid, peroxynitrous acid (ONOOH), a reactive and unstable oxidizing species. NO is also implicated in the production of nitrite (NO_2^-), which can be oxidized to produce nitrogen dioxide (NO_2), a nitrogen intermediate, which along with an $^{\cdot}OH$ group forms nitrate (NO_3^-). $ONOO^-$ and its metabolites are capable of tyrosine nitration in multiple proteins, evoking changes in their conformation, structure and catalytic activity [490].

Ang II has also been shown to induce ROS generation in multiple cell types, including cardiomyocytes, endothelial cells and VSMC [444-446], which appears to play a direct role in Ang II-induced vascular hypertrophy through the activation of hypertrophic signaling pathways [447, 448]. ET-1 has been also shown to activate NAD(P)H oxidase, resulting in ROS generation in endothelial cells [441], and increased H_2O_2 levels via ET_A receptor binding in pulmonary smooth muscle cells [442]. This increase in ROS generation

has been linked with the ET-1-induced activation of ERK1/2, JNK, p38 MAPK, PKB and PYK2 [203, 443]. ET-1-induced ROS generation in VSMC was suppressed by N-Acetylcysteine (NAc), a ROS scavenger, and diphenyleneiodonium (DPI), an inhibitor of NAD(P)H oxidase [203]. DPI and NAc pre-treatment of VSMC also inhibited ET-1-induced ERK1/2, PKB, and PYK2 phosphorylation, demonstrating that ROS are critical mediators of ET-1-induced signaling events linked to growth-promoting proliferative and hypertrophic pathways in VSMCs. Observations that both ROS e.g. H_2O_2, and vasoactive peptides, induce the tyrosine phosphorylation of IGF-1R and EGFR, and pharmacological blockade or genetic ablation of the R- and/or NR-PTK activity resulted in the attenuation of ET-1 and Ang II-induced ERK1/2 and PKB phosphorylation have suggested that ROS may serve as intermediates to enhance the tyrosine phosphorylation of R- and NR-PTKs [64, 415, 421, 423, 449]. Further support for a role of ROS in modulating R/NR-PTK function has been provided by reports where Ang II was shown to enhance the expression of IGF-1R through ROS-dependant mechanism in VSMC [491].

ROS may directly target growth factor receptors such as IGF-1R, platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) [483], which can induce intracellular ROS generation [492]. Receptor tyrosine kinases have been implicated in Ang II signaling by Ang II type 1 receptor (AT_1R)-mediated transactivation [493]. This transactivation may involve Ang II-mediated NAD(P)H-dependent ROS formation, followed by MAPK activation. Furthermore, transactivation of IGF-1R and EGFR via Ang II has been shown to activate p38 MAPK, ERK5 and ERK 1/2 in VSMCs [396]. Recent studies have also suggested that growth factors, such as IGF-1, signal through ROS-dependent transactivation of EGFR [471], and a role of NAD(P)H oxidase 4 (NOX4) in IGF-induced production has been demonstrated in VSMC [494]. ROS/RNS are capable of activating NR-PTKs, such as Src and Janus Kinase (JAK), which have been shown to play a role in H_2O_2-induced activation of p21Ras [495], as well as several transcription factors, such as NF-κB and activator protein-1, a transcription complex consisting of dimers of Fos-Jun or Jun-Jun proteins [496], leading to cell growth and differentiation by activation of the

PI3-K-Akt/PKB signaling pathway. Ang II-mediated MAPK and apoptosis signaling-regulated kinase 1 (ASK1) activation may also be involved [341].

In addition to its effects on receptor and non-receptor PTKs, growth factor or vasoactive peptide-induced ROS can potentially oxidize and inactivate multiple protein tyrosine phosphatases (PTPases), such as PTP-1B [450]and SH-2 domain-containing tyrosine phosphatase-2 (SHP-2) [451], in vitro, as well as in vivo [451]. PTPases catalyze the rapid dephosphorylation and inactivation of multiple R-/NR-PTK, including IR and the IGF-1R β-subunit, and their substrates [497, 498], leading to a downregulation of proliferative and hypertrophic signaling. PTPase inhibition can cause a shift in the phosphorylation-dephosphorylation cycle, leading to a net increase of tyrosine phosphorylation of R- and/or NR-PTK[421, 422], which may contribute to the activation of the ERK1/2 and PKB signaling cascades. For example, it has been reported that PTEN, which catalyzes PIP_3 dephosphorylation, becomes inactivated by oxidation of Cys 124 in its catalytic domain subsequent to treatment with H_2O_2 or ROS-generating peptides [452-455], leading to an increase of PIP_3 levels and a subsequent increase in PKB activation. PTPases can respond to oxidative stresses from the environment, as well as to intracellular ROS, generated in response to physiological activation of growth factor receptors [451].

In summary, vasoactive peptides have a profound effect on ROS production and R-/NR-PTK activation through PTPase regulation, yet vasoactive peptides also affect and regulate the synthesis of growth factors themselves, which further compound vasoactive peptide-induced proliferative effects. For example, Ang II has been shown to increase the levels of IGF-1 mRNA and protein in heart and VSMC [398, 399]. Conversely, IGF-1 was also found to up-regulate the expression of AT_1R in VSMCs [400], suggesting the existence of a potential cross-talk between Ang II and IGF-1 system. This notion is further supported by the studies showing that IGF-1R antisense-induced reduction in IGF-1R was associated with an inhibition of Ang II-induced vascular responses in SHR and WKY rats [401, 402]. In addition, native unmodified LDL has been found to increase IGF-1 mRNA, whereas oxidatively-modified LDL (oxy-LDL) decreases IGF-1 mRNA and protein expression in a dose-dependent fashion [499]. IGF-1 also stimulates ROS-mediated transactivation of EGFR

via Src activation, leading to ERK-1/2 phosphorylation, which plays a critical role in VSMC proliferation [471]. Antioxidant treatment can inhibit IGF-1-induced EGFR transactivation by lowering H_2O_2 production [471]. In VSMCs, H_2O_2 can enhance IGF-1R and Src-PTK-dependent PKB activation [500].

Taken together, the results presented in this body of work identify c-Src as a clear mediator of vasoactive peptide-induced IGF-1R, leading to increases in PKB and MAPK signaling, as well as Egr-1 transcription factor regulation (Figure 12). To our knowledge, the results reported here are the first to identify an involvement of IGF-1R in transducing the downstream effects of ET-1 and Ang II in activating PKB in VSMC. They are also the first to explore the role of c-Src in ET-1-induced Egr-1 expression in VSMC, providing a possible link between vasoactive peptide-induced signaling events and the hypertrophic and proliferative responses they induce in pathophysiological states.

Chapter 5

Conclusion

Overall, the studies presented in this thesis demonstrate that receptor and non-receptor protein tyrosine kinase transactivation by ET-1 and Ang II are crucial in propagating downstream signaling responses induced by these vasoactive peptides. Although EGFR transactivation has been studied in detail with regards to vasoactive peptide-induced signaling, the transactivation of other receptor and/or non-receptor tyrosine kinases is only recently coming to light as an important mechanism implicated in ET-1 and Ang II-induced migratory, hypertrophic and proliferative signaling, leading to the deleterious effects of hypertensive states.

We have provided evidence demonstrating that both ET-1 and Ang II can induce the phosphorylation and subsequent activation of IGF-1R and c-Src PTK. We have also demonstrated that IGF-1R and c-Src are required for ET-1 and Ang II-induced PKB phosphorylation. Our studies have placed c-Src upstream of both PKB and IGF-1R in this signaling mechanism. In addition, we have provided proof that IGF-1R and c-Src are key mediators of ET-1 and Ang II-induced proliferation and hypertrophy of VSMC, a key phenomenon in the development of vascular pathologies such as atherosclerosis and restenosis. Furthermore, we have provided data demonstrating that MAPK activation by ET-1 and ANG II is not differentially regulated, and that c-Src is implicated in the activation of ERK 1/2, JNK and p38 MAPK induced by both vasoactive peptides. Our results also highlight for the first time the role of c-Src in the modulation of the Egr-1 transcription factor by ET-1 in VSMC, a mechanism which has been linked to the hypertrophic actions of ET-1 (Figure 12).

Despite the work presented here and those of others, the precise mechanism of vasoactive peptide-induced R-/NR-PTK transactivation still remains unclear; however, evidence points towards the implication of signal intermediates, such as matrix metalloproteinase and the generation of ROS, in inducing the activation and subsequent phosphorylation of R-/NR-PTK, through either ligand-dependent or –independent mechanisms. Examples of this are the inhibition of protein tyrosine phosphatases by ROS, allowing for EGFR or IGF-1R phosphorylation, or the cleavage of heparin from EGF to

allow for EGF-induced EGFR activation. Notwithstanding the lack of a clear explanation as to how R-/NR-PTK transactivation occurs, evidence is accumulating to indicate that activation of R-/NR-PTK play a critical role in triggering the vasoactive peptide-induced signaling that mediates the hypertrophic, proliferative and migratory responses in VSMC. Further research using pharmacological or genetic approaches will likely help to decipher the exact mechanisms by which R-/NR-PTK transactivation occurs, leading to potential developments in therapeutic tools to help restore dysregulated signaling events associated with vascular disorders.

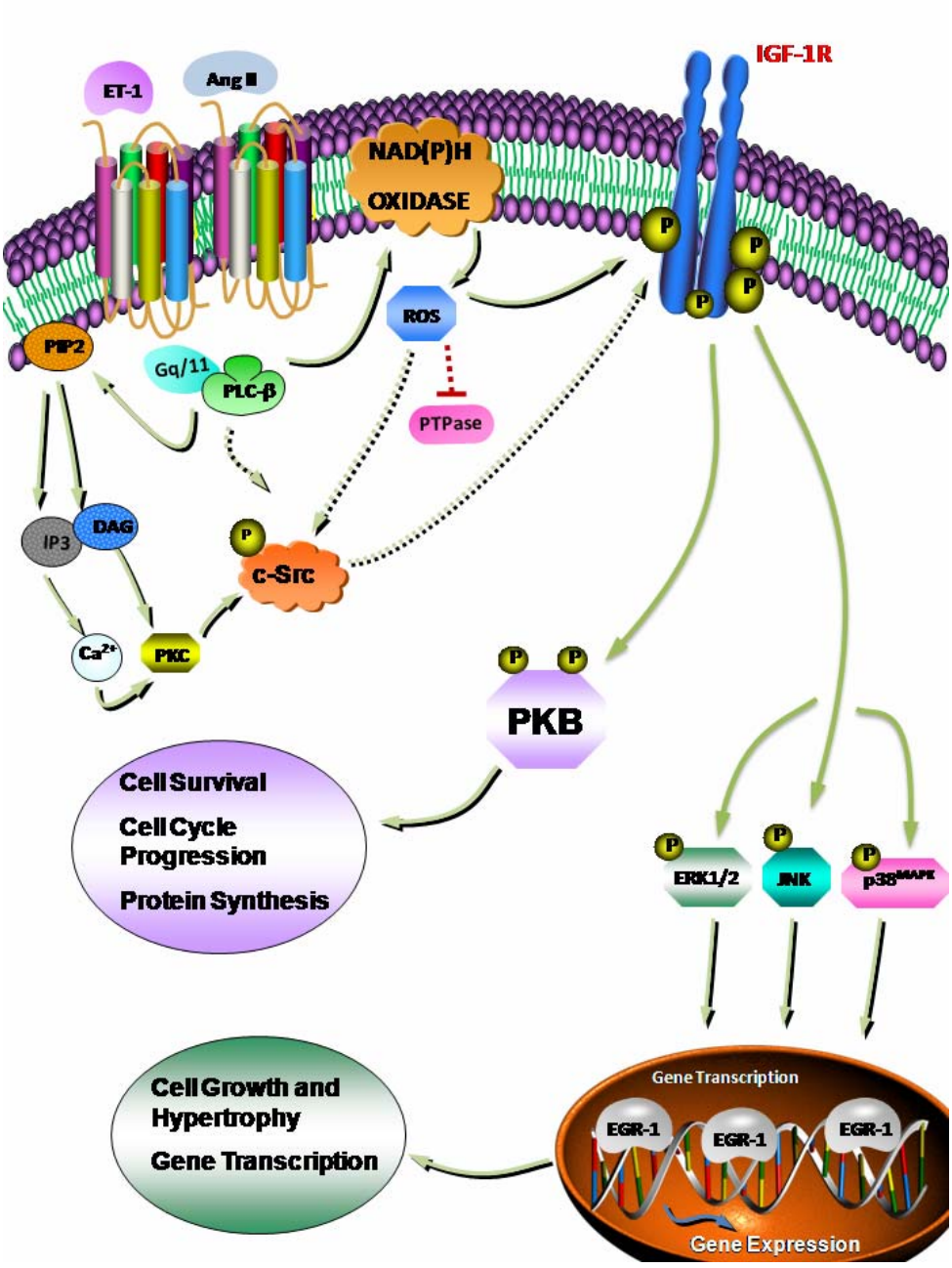


Figure 12: Schematic model summarizing the mechanism by which ET-1 and Ang II induce downstream activation and modulation of PKB, ERK1/2, JNK, p38mapk and Egr-1, leading to subsequent hypertrophic and proliferative events in VSMCs. ET-1 and/or Ang II receptor activation is known to generate reactive oxygen species (ROS) through NAD(P)H oxidase system. ROS are able to inhibit protein tyrosine phosphatases (PTPase) through the oxidation of cystein residues in their catalytic domain. Inhibition of PTPases favors an increase in the tyrosine phosphorylation of NR-PTK, such as c-Src, and R-PTKs, such as IGF-1R, resulting in the ligand-independent activation of IGF-1R, triggering the PI3-K/PKB and MAPK signaling cascades. Activation of PKB, ERK1/2, JNK and p38 MAPK leads to modulation of many downstream effectors, including transcription factors such as Egr-1, contributing to hypertrophy, proliferation and migration of VSMCs.

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