Université de Montréal

"Redox regulation of ET-1-induced activation of ERK1/2, PKB and Pyk2 signaling in A-10 vascular smooth muscle cells"

par

Grace Bou Daou

Département de Physiologie

Faculté de médecine

Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de
Maître ès sciences (M.Sc.)
en physiologie

Septembre, 2003

Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé :

"Redox regulation of ET-1-induced activation of Pyk2, ERK1/2 and PKB/Akt signaling in A-10 vascular smooth muscle cells"

Présenté par :

Grace Bou Daou

a été évalué par un jury composé des personnes suivantes:

Président-rapporteur : **Josette Noël**

Directeur de recherche : **Ashok K. Srivastava**

Membre du jury : **Ryszard Grygorczyk**

Mémoire accepté le: _____

SUMMARY

Reactive oxygen species (ROS) have been shown to mediate the effect of several growth factors such as angiotensin II (AII), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Endothelin-1 (ET-1) is an important growth factor for vascular smooth muscle cells (VSMC) which is believed to contribute to the pathogenesis of vascular abnormalities such as atherosclerosis, hypertension and cardiac hypertrophy. However, a possible role of ROS generation in mediating the ET-1 response on ERK1/2 and PKB, key components of growth promoting and proliferative signaling pathways, has not been examined in detail. Therefore, the aim of the present study was to investigate the involvement of ROS in ET-1-mediated activation of ERK1/2 and PKB as well as Pyk2 in A-10 VSMCs. These cells are obtained from rat embryonic thoracic aorta. Pyk2 is a non-receptor Ca^{2+}-dependent protein tyrosine kinase and an upstream regulator of MAPK signaling. ET-1 stimulated the phosphorylation of ERK1/2, PKB and Pyk2 in a dose and time-dependent fashion with maximum response being elicited at 10 nM which peaked at 5 min. Treatment of VSMC with ET-1 resulted in an increase in the generation of ROS that could be blocked with diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase. Furthermore, DPI pretreatment of cells prior to stimulation with ET-1, attenuated ET-1 enhanced phosphorylation of ERK1/2, PKB and Pyk2. N-acetylcysteine (NAC), another ROS scavenger, also exhibited a similar response. Moreover, DPI caused a decrease in the protein synthesis stimulated by ET-1. These results demonstrate that ROS is a critical mediator of ET-1-induced signaling events linked to hypertrophic and growth promoting pathways in VSMC.

There is an emerging evidence suggesting that nitric oxide (NO), a vasoactive substance, contributes to the regulation of several hormone-mediated responses such as EGF, PDGF as well as ET-1 and exerts an anti-mitogenic and anti-proliferative effect *in vitro*. However, the mechanism by which NO

antagonizes ET-1 effect remains unknow. Therefore, the aim of this study was to determine if NO generation would modify ET-1-induced signaling pathways involved in cellular growth and proliferation in A-10 VSMC. NO effect has been evaluated by measuring phosphorylation levels of ERK1/2, PKB and Pyk2 by immunoblot. Treatment of A-10 cells with S-nitroso-N-acetylpenicillamine (SNAP), a NO donor, attenuated the ET-1-enhanced phosphorylation of ERK1/2, PKB and Pyk2. Since, NO mediates principally its effect through a cyclic GMP/soluble guanylate cyclase pathway, we investigated the role of 8-Br-cGMP, a non-metabolizable and cell permeable analogue of cGMP, which exhibites a similar effect to SNAP on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. Furthermore, ODQ, an inhibitor of guanylate cyclase activity, reversed the inhibitory effect of NO on ET-1-induced responses. SNAP also appeared to decrease the protein synthesis induced by ET-1. Taken together, these data demonstrate that NO attenuates selectively ERK1/2, PKB and Pyk2 phosphorylation induced by ET-1 via cGMP, which antagonize the growth promoting and proliferative effects of ET-1.

Key words: ET-1, ROS, NO, MAPKs, ERK1/2, PKB, Pyk2, A-10 cells.

SOMMAIRE

Les espèces réactives oxygénées modulent l'effet de plusieurs facteurs de croissance comme l'angiotensine II (AII), l'EGF ("epidermal growth factor") et le PDGF ("platelet-derived growth factor"). L'endotheline-1 (ET-1) est un important facteur de croissance pour les cellules du muscle lisse vasculaire ("VSMC") et contribue aux anormalités vasculaires, entre autre, l'athérosclérose, l'hypertension ainsi que l'hypertrophie cardiaque. La génération des espèces réactives oxygénées pourraient aussi jouer un rôle dans l'activation des voies de signalisation activées par l'ET-1, en particulier l'activation de ERK1/2 et de PKB. Ces deux composantes clés sont impliquées dans la croissance et la prolifération cellulaire. Ainsi, le but de la présente étude est d'évaluer la participation des espèces réactives oxygénées dans l'activation de ERK1/2, PKB et Pyk2 induite par ET-1 dans les "VSMC" de lignée A-10. Ces cellules sont obtenues à partir de l'aorte thoracique embryonnaire de rat. Pyk2 est une protéine tyrosine kinase dépendante du calcium et constitue ainsi un régulateur ascendant de la voie de signalisation des MAPKs. ET-1 a permis de stimuler la phosphorylation de ERK1/2, PKB et Pyk2 de façon dépendante de la concentration et du temps d'où une réponse maximale a été obtenue à 10 nM et après une stimulation de 5 min. Le traitement des "VSMC" avec ET-1 a augmenté la production des espèces réactives oxygénées, alors qu'une diminution a été observée en présence du diphenyleneiodonium (DPI), un inhibiteur de la NADPH oxydase. En outre, le prétraitement des cellules avec DPI a permis d'atténuer la phosphorylation de ERK1/2, PKB et Pyk2 induite par ET-1. Une réponse similaire a été observée en utilisant un autre antioxidant, le N-acetylcysteine (NAC). De plus, DPI a inhibé la synthèse protéique stimulée par ET-1. Ces résultats démontrent que les espèces réactives oxygénées sont des médiateurs importants dans l'activation des composantes de la voie de signalisation de ET-1, ce qui contribue à la croissance et à l'hypertrophie cellulaire.

Il a été suggéré que l'oxyde nitrique (NO), une substance vasoactive, contribue à la régulation des réponses induites par plusieurs hormones comme l'EGF, le PDGF et l'ET-1 en exerçant un effet anti-mitogénique et anti-prolifératif *in vitro*. Cependant, le mécanisme par lequel NO antagonise l'effet de ET-1 est à ce jour inconnu. Le but de cette étude est donc d'évaluer si la génération de NO pourrait modifier les composantes de la voie de signalisation de ET-1. L'effet de NO a été déterminé en mesurant les niveaux de phosphorylation de ERK1/2, PKB et Pyk2 à l'aide d'immunobavardage. Le traitement des cellules A-10 avec S-nitroso-N-acetylpenicillamine (SNAP), un donneur de NO, a permis d'atténuer la phosphorylation de ERK1/2, PKB et Pyk2 induite par ET-1. Comme NO médie principalement son action par la voie de GMPc/guanylate cyclase soluble, on a donc étudié le role du 8-Br-GMPc, un analogue du GMPc non-métabolisable et perméable à la cellule. Un effet similaire à celui de SNAP a été observé. En outre, ODQ, un inhibiteur de l'activité de la guanylate cyclase, a renversé l'effet de NO sur la phosphorylation des composantes de signalisation stimulées par ET-1. SNAP a permis aussi de diminuer la synthèse protéique induite par ET-1. Finalement, les résultats démontrent que NO atténue sélectivement la phosphorylation de ERK1/2, PKB et Pyk2 de la voie de signalisation de ET-1 par l'intermédiaire du GMPc, ce qui antagonise les effets de ET-1 soit la croissance et la prolifération cellulaire.

Mots clés: ET-1, ROS, NO, MAPKs, ERK1/2, PKB, Pyk2, A-10 cells.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1
INTRODUCTION

CHAPTER 2
ARTICLE 1

CHAPTER 3
ARTICLE 2

CHAPTER 5
CONCLUSION

LIST OF ABBREVIATIONS

A II : Angiotensin II

ACE : Angiotensin converting enzyme

ATF-2 : Activation transcription factor 2

BH_4 : 6(R)-tetra-hydro-L-biopterin

CaM : Calmodulin

cAMP : Cyclic adenosine monophosphate

cGMP : Cyclic guanosine monophosphate

DG : Diacylglycerol

DNA : Deoxyribonucleic acid

ECE : Endothelin converting enzyme

EDCF : Endothelium-derived contracting factors

EDHF : Endothelium-derived hyperpolarizing factors

EDRF : Endothelium-derived relaxing factors

EGF : Epidermal growth factor

ERK : Extracellular signal-regulated kinases

ET : Endothelin

$FADH^{\cdot}$: Flavin adenine dinucleotide, radical form

FMN : Flavin mononucleotide

GC : Guanylate cyclase

GDP : guanosine diphosphate

GPCR : G-protein-coupled receptors

Grb2 : Growth factor binding protein 2

GTP : Guanosine triphosphate

IP_3 : Inositol triphosphate

JNK/SAPK : c-Jun NH2-terminal kinase/stress-activated protein kinase

kDa : Kilodaltons

mRNA : Messenger ribonucleic acid

MAPK : Mitogen-activated protein kinases

MEK/MKK: MAPK kinase

MEKK/MKKK: MAPK kinase kinase

$NADP^+$: Nicotinamide adenine dinucleotide, phosphate,oxidized form

NADPH : Nicotinamide adenine dinucleotide, phosphate,reduced form

NO : Nitric oxide

NOS : Nitric Oxide Synthase

P130Cas : Crk-associated substrate, protein of 130 kDa

PDE : Phosphodiesterase E

PDGF : Platelet derived growth factor

PDK : PI(3,4,5)P_3 dependent protein kinase

PI : Phosphatidylinositol

PIP_2 : Phosphatidylinositol-4,5-bisphosphate

PI3-K : Phosphatidylinositol 3-kinase

PKB : Protein kinase B

PKC : Protein kinase C

PKG : Protein kinase G

PLC : Phospholipase C

Pyk2 : Protein tyrosine kinase 2

ROS : Reactive oxygen species

SDS-PAGE : Sodium dodecyl sulfate - polyacrylamide gel

Shc : Src homology

SOS : Son of the sevenless

Src : Sarcoma rous virous

ACKNOWLEDGEMENTS

With a sense of humble compliance I feel great honor to express sincere gratitude to my supervisor Dr. Ashok K. Srivastava for molding me capable enough to understand the subject, conveying the knowledge and skill to accomplish this study. It was his constant inspiration, kind guidance, healthy criticism and suggestions that I am able to present this work; I shall always remain grateful to his guidance, advice and help.

I express my unfathomable sense of reverence to Dr. Madhu B. Anand-Srivastava rendering her valuable suggestions and encouragement in the completion of this study.

My immense gratitude are due to my colleagues, especially to Mohammed Mehdi and Dr. Nihar R. Pandey for the help, suggestions and healthy critics throughout. I would like to thank Dr. Yuan Li and Dr. Shehla Hashim for the needful technical support.

Words are inadequate to express my sincere feelings and indebtedness for my family without their moral support and encouragement it would have been impossible for me to carry out this study.

Last, but not the least, I wish to record my heartfelt feelings for immense cooperation of a special person my husband, Dori Dagher, who supported me throughout during this critical time.

I dedicate this thesis to my late mother.

CHAPTER 1

INTRODUCTION

1.1 Endothelin

Endothelin, one of the most potent vasoconstrictors, was discovered by Yanagisawa and co-workers (1) in 1988. It was characterized and cloned from porcine aortic endothelial cells (1) and exerts inotropic and mitogenic properties, influences homeostasis of salt and water and stimulates the renin-angiotensin-aldosterone and sympathetic nervous systems (2,3,4). In fact, the overall effect of the actions of endothelin is usually to increase vascular tone and blood pressure. Endothelin also play an important role in the pathophysiology of cardiac, vascular and renal diseases associated with regional or systemic vasoconstriction (5) such as hypertension and atherosclerosis.

1.2 Structures of endothelins

Endothelin (ET) is a 21 aminoacid peptide which exists in at least three isoforms: ET-1, ET-2 and ET-3 (6,7). All ET isopeptides share a common structure: two disulfide bonds (Cys^{1}-Cys^{15} and Cys^{3}-Cys^{11}), a cluster of three polar charged side chains on aminoacid residues 8-10 and a hydrophobic C-terminus (residues 16-21) containing the aromatic indole side chain at Trp^{21}. ET-2 contains two aminoacid substitutions (Trp^{6}-Leu^{7}) and shares 90% sequence homology with ET-1. ET-3 contains six aminoacid substitutions (Thr^{2}, Phe^{4}-Thr^{5}-Tyr^{6}-Lys^{7} and Tyr^{14}) and shares 71 % sequence homology with ET-1 and ET-2 (8,9). The hydrophobic C-terminus of ET is essential for its

bioactivity, as well as the loop configuration (9). ETs share close sequence homology (~ 67%) and similar bioactivities with the sarafotoxins, a group of peptide toxins isolated from venom of some scorpions and snakes (8). The disulfide bonds, polar side chains and hydrophobic C-terminus of ETs are largely conserved in sarafotoxins (9) (**Fig. 1.1**).

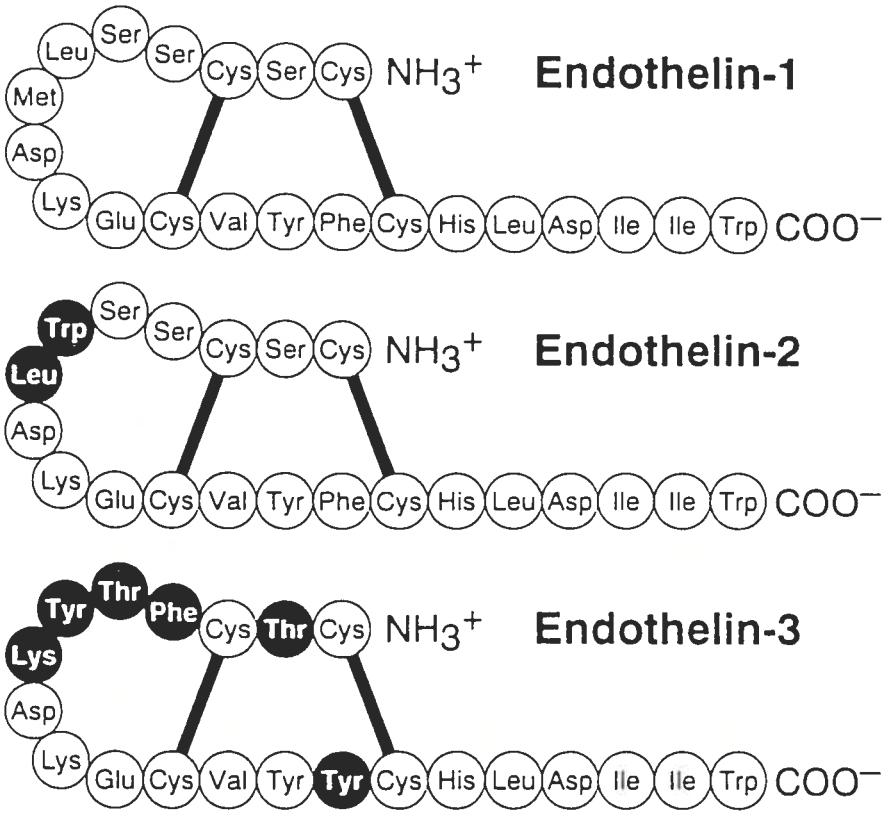


Figure 1.1 : Structures of endothelins. Dark circles indicate where aminoacids differ from those of endothelin-1. (Based on ref. 181)

1.3 Molecular genetics and regulation of generation of endothelin

Each member of the endothelin family possesses a separate gene that encodes a specific precursor for the mature isoform (5). The 5' upstream promoter region of the genes contains binding sites for activating protein 1 and nuclear factor 1, which are involved in transcriptional induction of mRNA for endothelin-1 by angiotensin II and transforming growth factor β (5,10). The 3' untranslated region (3'-UTR) of the mRNA contains adenine-uracil-rich (AU-rich) sequences that regulate the stability of preproendothelin-1 mRNA (5). Generation of endothelin-1 is induced by many stimuli, including vasoactive hormones, growth factors, hypoxia, shear stress, lipoproteins, free radicals, endotoxin and cyclosporin (11) (**Fig. 1.2**). Production of endothelin-1 is inhibited by stimuli that act to increase intracellular level of cyclic guanosine monophophate (cGMP), including endothelium-derived nitric oxide, nitrovasodilators, natriuretic peptides, heparin and prostaglandins (11).

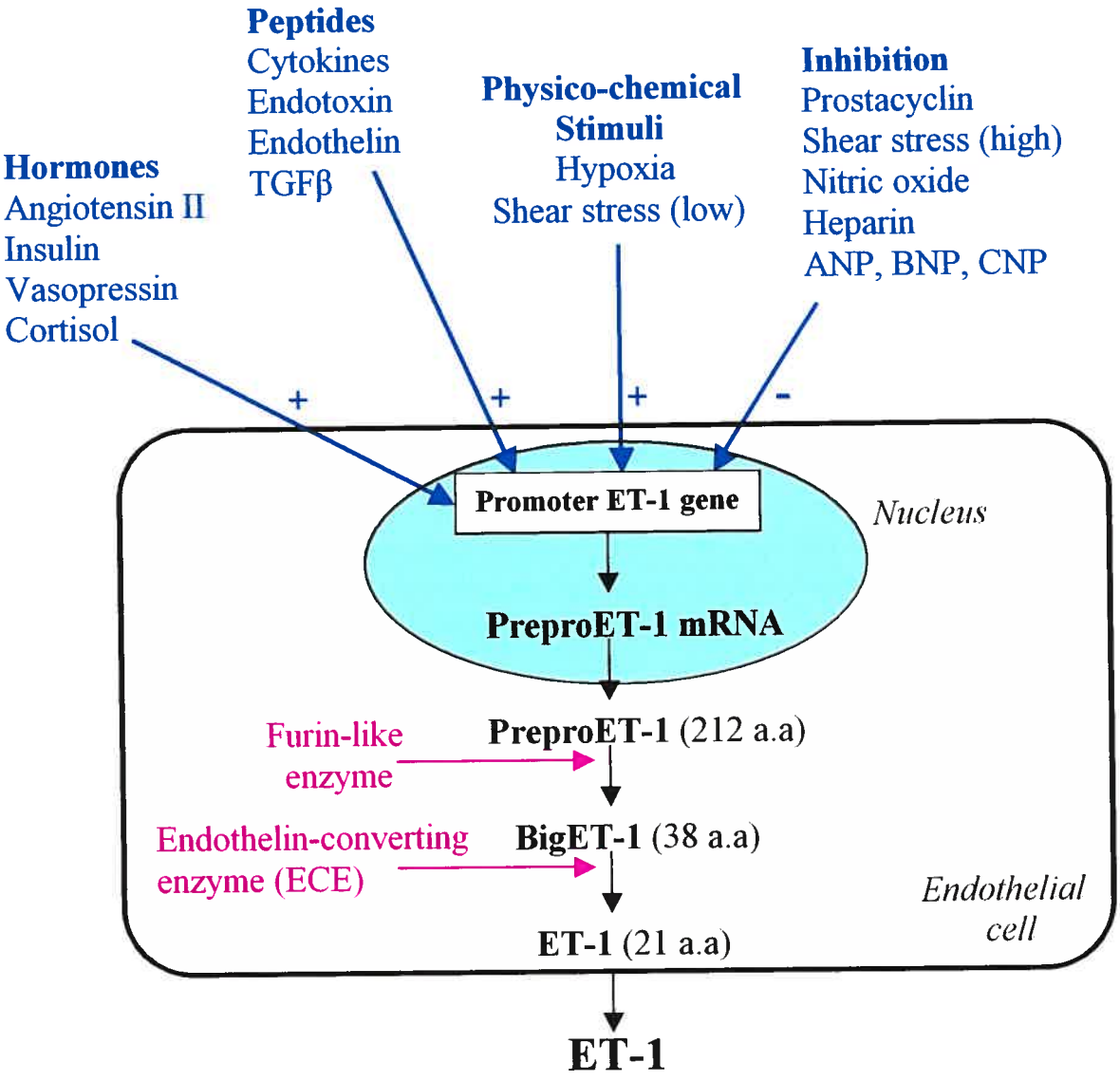


Figure 1.2 : Regulation of ET-1 synthesis and its pathway of generation. ANP = atrial natriuretic peptide; BNP = brain natriuretic peptide; CNP = C-type natriuretic peptides; a.a = aminoacids. (Based on ref. 5)

1.4 Biosynthetic pathway

The initial product of the human endothelin-1 gene is preproendothelin-1, a 212 aminoacid immature peptide (**Fig. 1.2**). Preproendothelin-1 is proteolytically cleaved by a furin-like enzyme to form biologically inactive intermediate, a 38-aminoacid peptide termed big endothelin-1 (1,5). A protease called, endothelin-converting enzyme (ECE), then cleaves Trp^{21}-Val^{22} of big endothelin-1 to form the mature 21-aminoacid ET-1 peptide (1,5,13). Processing of big ET-1 to ET-1 is essential for its biological activity (1,5,14). Endothelin is secreted by a constitutive pathway, but evidence also suggested that in some cells endothelin can be secreted by a pathway via secretory granules (15). The pathway of secretion involves the rough endoplasmic reticulum, golgi cisternae, golgi small exocytic vesicles directly beneath the plasma membrane (15).

1.5 Sites of generation

Endothelial cells are the major site of generation of endothelin-1, this tightly correlates with the high expression levels of mRNA for preproendothelin-1 and the presence of intracellular converting enzyme in these cells (6,8,12). Human aortic vascular smooth muscle cells express mRNA for endothelin-1, but its production is 100 fold less than that in endothelial cells (5). Also, endothelin-1 is produced by the airway epithelial cells, macrophages, fibroblasts, cardiomyocytes, posterior pituitary and central nervous system

(6,8,11). Endothelin-2 is produced by the kidney and intestinal epithelial cells, and endothelin-3 by brain neurons and renal tubular epithelial cells (6,8).

1.6 Plasma concentrations of endothelins

Plasma concentrations of ET-1 is in the range 1-10 pmol/L in healthy subjects (5,6). Plasma ET-2 and ET-3 are found at even lower concentrations (6). Therefore, under normal physiological conditions, endothelins are not circulating hormones; rather they act as autocrine and paracrine factors at multiple sites in the body (6).

1.7 Biological actions of endothelin-1

When injected *in vivo*, ET is the most potent vasoconstrictor agent yet identified (1,9) particularly in the brain (16), renal (17) and pulmonary vasculature (18,19). Intraventricular injection of ET-1 induces a transient increase in arterial pressure, respiratory rate and renal sympathetic nerve activity followed by a long-term depression of these parameters (6,20). While, intravenous bolus administration of ET-1 in different species leads to a short-lived decrease in vascular resistance followed by the long-term increase, implicating a balancing act of dilator and pressor functions for endothelins (6). ET-1 exerts a positive chronotropic (21) and inotropic (22) action on human heart, and also mediate cardiac hypertrophy (23) and remodeling in congestive

heart failure through its mitogenic properties (24). ET-1 has also mitogenic effects on smooth muscle cells (25), fibroblasts (26), macrophages (27), mesangial cells (31), and increases as well cell proliferation (28,29,30,31).

1.8 Endothelin receptors

Endothelins exert their biological actions through the activation of two receptor subtypes, ET-A and ET-B (14,32). Both receptors belong to a large family of transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs) (33). They contain seven transmembrane domains of 22-26 hydrophobic aminoacids in their ~400-aminoacid sequences (14,32). Their N-terminal region is extracellular and their C-terminal region is intracellular (7). Type A receptors exist mainly in vascular smooth muscle cells but is also found in cardiomyocytes, fibroblasts, hepatocytes, adipocytes, osteoblasts and brain neurons (22,32,34) and present higher affinities for ET-1 and ET-2 than for ET-3 (6,14). Type B receptors exist predominantly in endothelial cells and smooth muscle cells but is also found in cardiomyocytes, hepatocytes, fibroblasts, osteoblasts, different epithelial cells and neurons (22,32,34) and have equal subnanomolar affinities for all endothelin peptides (6,14). Therefore, ET-1 binding to ET-A and ET-B receptors on smooth muscle produces vasoconstriction, cell growth and cell adhesion (14,35). The binding of ET-1 to endothelial ET-B receptors stimulates the release of NO and prostacyclin which

prevents apoptosis, inhibits ECE-1 expression in endothelial cells and plays an important role in ET-1 clearance (14,35).

1.9 Activation of the phosphoinositide cascade by ET-1

Following the binding of ET-1 to its receptor, the hormone-receptor complex activates Gq-protein which is the best characterized signaling interaction with ET-A receptor (6,7). As with all heterotrimeric G-proteins, Gq consists of an α-subunit (αq, or related α-subunit, such as α11), a member of the β-subunit family as well as a member of the γ-subunit family and is associated to the membrane (36) (**Fig. 1.3**). In the inactive Gq heterotrimer, αq is ligated to GDP. Exchange of GDP for GTP on αq leads to the dissociation of αq(GTP) and βγ and both remain associated with the membrane (33). Their dissociation leads to activation of phosphoinositide-specific phospholipase C β (PLC β) (37,38), which then hydrolyzes the membrane phospholipid, phosphatidylinositol-4',5'-bisphosphate [PtdIns(4,5)P_2] to two second messengers: hydrophobic diacylglycerol (DAG), which remains in the plane of the membrane, and soluble inositol-1',4',5'-trisphosphate [Ins(1,4,5)P_3] (37,38). Ins(1,4,5)P_3 diffuses into the cytoplasm and activates some calcium channels of the sarcoplasmic reticulum, which leads to an increase of Ca^{2+} levels in the sarcoplasma and cell contraction (7,8). DAG together with Ca^{2+} activates the phosphatidylserine-dependent protein kinase, protein kinase C (PKC) (39) (**Fig. 1.3**).

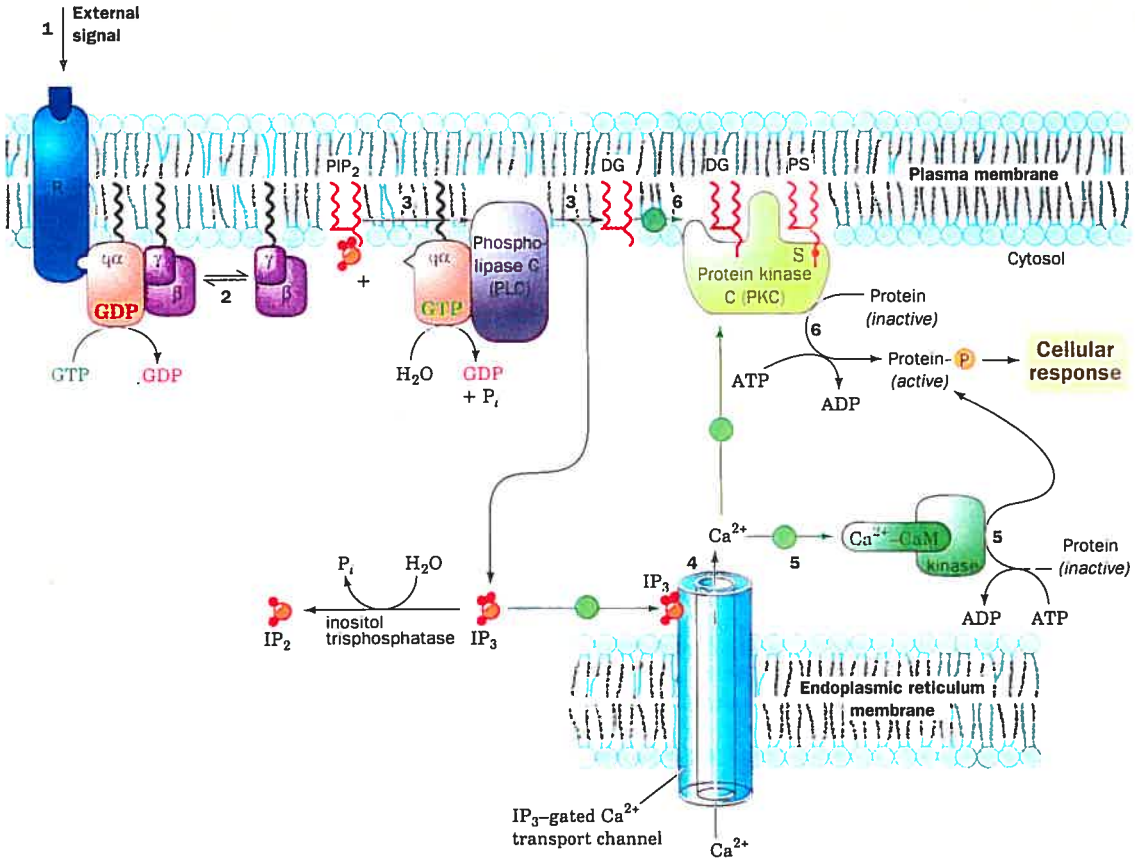


Figure 1.3 : Special case of Gq-protein-coupled receptor in intracellular signaling. **(1)** The binding of an agonist to a surface receptor, R, activates phospholipase C through the intermediacy of what is shown here as **(2)** a Gq-protein. Phospholipase C catalyzes the hydrolysis of PIP_2 to IP_3 and DG **(3)**. The water-soluble IP_3 stimulates the release of Ca^{2+} sequestered in the endoplasmic reticulum **(4)** which in turn activates numerous cellular processes through the intermediacy of calmodulin and its homologs **(5)**. The non-polar DG remains associated with the membrane, where it activates protein kinase C to phosphorylate and thereby modulate the activities of a number of cellular proteins **(6)**. This latter activation process also requires the presence of the membrane lipid phosphatidylserine (PS) and Ca^{2+}. PIP_2 = phosphatidylinositol bisphosphate; IP_3 = inositol triphosphate; DG = diacylglycerol. The green circles illustrated in the figure do not apply to the special case of Gq-protein-coupled receptor. (Based on ref. 134)

PKC is a family of serine/threonine kinases that is subdivided into three groups. The classical or conventionnal PKCs which require DAG and Ca^{2+} include the isoenzymes α, β and γ (40). Novel PKC (nPKC) where the activities are DAG-dependent though probably Ca^{2+}-independent include the isoenzymes δ, ε, η, θ, μ and ν and atypical PKC (aPKC) are independent of DAG and Ca^{2+} and include ζ and λ isoenzymes (39).

1.10 Mechanisms of ET-1-induced activation of MAPK cascade

The next process associated with exposure to ET-1 is the activation of a member of the small GTP-binding protein family, Ras which involves exchange of GDP for GTP (41). Once activated, Ras-bound to membrane recruit the first member of the mitogen-activated protein kinases (MAPK) called Raf or MAPKKK (42,43). Raf phosphorylates MEK/MAPKK at specific serine/threonine residues, which in turn, phosphorylates ERK1/2 (MAPK42/44) on threonine and tyrosine residues (42,43). MAPK are serine/threonine protein kinases, which are also activated in response to a variety of external stimuli such as growth factors, hormones and stress (23,42,43). In a variety of cell types, activation of ERK1/2 leads to the phosphorylation of downstream cytosolic regulatory proteins, such as $p90^{rsk}$ which phosphorylates ribosomal proteins and participates in protein synthesis (44) (**Fig. 1.4**). Also, ERK1/2 migrates from the cytosol to the nucleus and phosphorylates many transcription factors which lead to activation of genes involved in growth and differentiation (181) (**Fig. 1.4**).

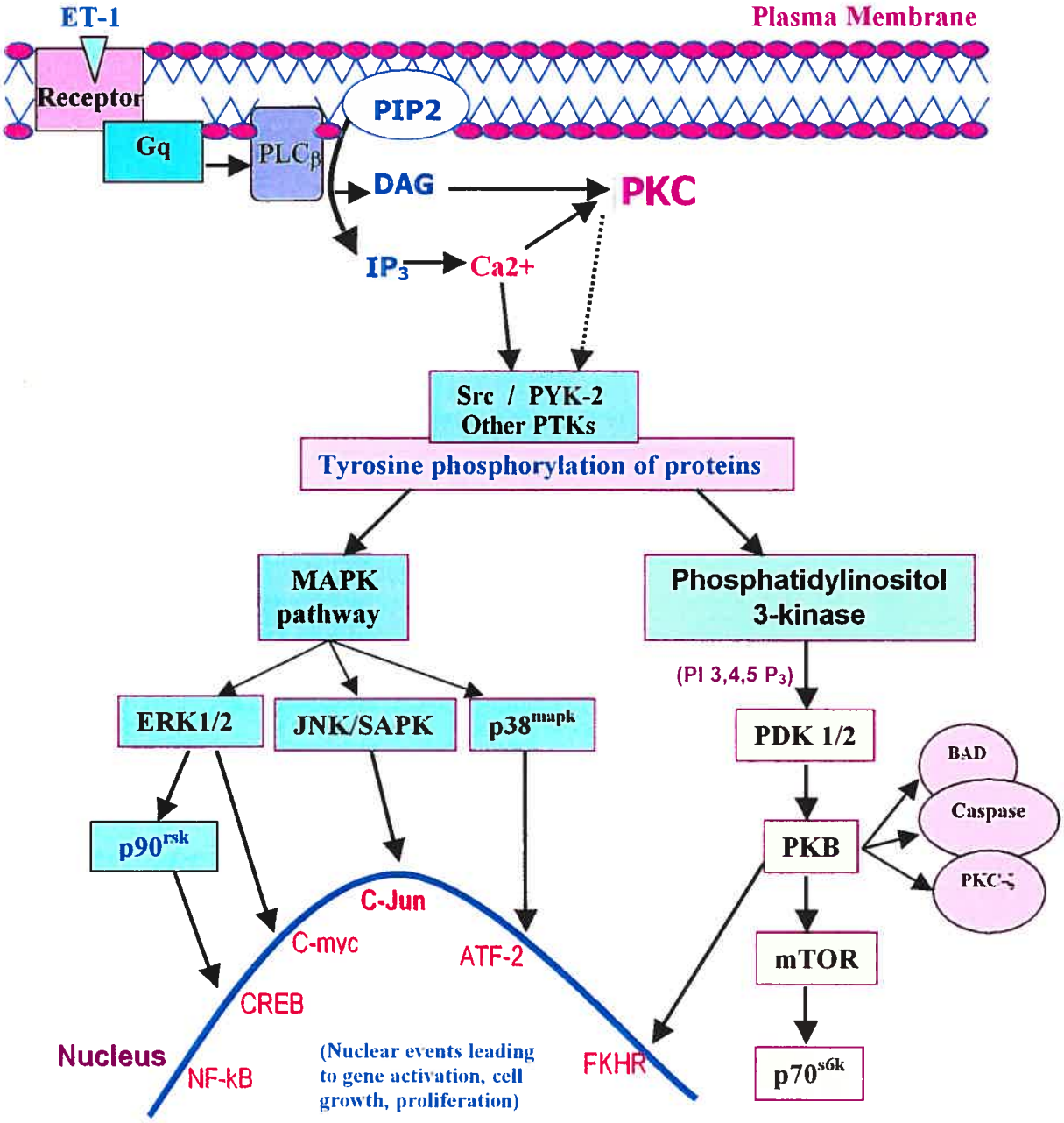


Figure 1.4 : Schematic model showing key steps in ET-1-induced activation of MAPK and PI-3K/PKB signaling. PTKs = protein tyrosine kinases.

Several reports have demonstrated that ET-1 activates ERK1/2 signaling pathway in many cell types including cardiomyocytes (45), fibroblasts (28), glomerular mesangial cells (31) and vascular smooth muscle cells (VSMC) (46). Activation of the ERK1/2 cascade is the best characterized. Although, JNK and p38mapk are most strongly activated by cytotoxic cellular stress and are better classified as stress-activated protein kinases (SAPK). ET-1 also activates JNK (c-Jun-NH_2 terminal kinase) and p38mapk cascades to a lesser degree than ERK in cardiomyocytes (45,47), VSMC (48) as well as in mesangial cells (49). In addition, several other small G-protein families have been stimulated including Rho, Rab and Ran (41). ET-1 activates members of the Rho family in cardiomyocytes (50) and fibroblasts (51) which are positive regulators of p38mapk pathway (52). The remaining small G-proteins and their expression characteristics have not been investigated systematically.

There is some evidence supporting the involvement of PKC in Ras activation in many cell types including cardiomyocytes (53) and rat myometrial cells (40). The nature of the connection between the two processes is obscure and other pathways may operate. A possible role of a calcium-regulated cytoplasmic proline-rich tyrosine kinase 2, Pyk2 (also known as related adhesion focal tyrosine kinase (RAFTK), focal adhesion kinase-2 (FAK-2) and cell adhesion kinase β (CAK β), calcium-dependent tyrosine kinase (CADTK)), in the activation of MAPK has been suggested in primary astrocytes (54,55) and rat kidney mesangial cells (56). In several other cell types, the Ca^{2+}- and PKC-

dependent Pyk2 activation (40,57) has been shown to link GPCRs to upstream regulators of ERK1/2 MAPKs, such as Src, Shc, Grb2, son of the sevenless (SOS) and the Ras guanosine nucleotide exchange factor (27,56,58,59). Previous data had shown that ET-1-induced association of Pyk2 through the binding of its autophosphorylated Tyr-402 to the SH2 (Src-homology 2) domain of c-Src lead to c-Src activation in many cell types including mesangial cells (56,58) and cardiomyocytes (47,56,60). Activated c-Src bound to Pyk2 might directly phosphorylate adjacent cellular proteins, such as p130Cas. Once tyrosine-phosphorylated, p130Cas has been shown to act as docking protein to recruit its effectors able to activate JNK (61,62) in cardiomyocytes (47).

Evidence suggested that ET-1 mediates EGF receptor (EGFR) transactivation which predominantly contributes to ERK activation, while Pyk2 contributed less in cardiomyocytes (60). Both kinases activation are mediated through PKC signaling (60). Conversely, a recent report demonstrated that ET-1-induced JNK activation is preferentially regulated by Pyk2, c-Src and the p130Cas/Crk complex but not by EGFR (47). However, the regulation of transactivation of EGFR is significantly different among cell types. In VSMC, EGFR transactivation has been shown to mediate the angiotensin II-induced ERK activation (63). ET-1-induced transactivation of EGFR contributes to the activation of Shc adapter molecule, leading to its interaction with Grb2 (64), which could then associate with SOS. Shc-associated Grb2/SOS induces exchange of GDP for GTP on Ras (59) and ultimately activate ERK signaling

(60,65). However, the involvement of Pyk2 in ET-1-induced signaling in VSMCs has not been yet defined.

1.11 Phosphatidylinositol-3 kinase cascade

Another effector of Ras species, phosphatidylinositol-3 kinase (PI-3K) is an enzyme that is implicated in a myriad of cellular processes. PI-3K activity has been linked to cell growth and transformation, differentiation, motility and survival (66,67). The PI-3K group of lipid kinases catalyze the transfer of phosphate from ATP to the 3'position of the inositol ring of the membrane-localized phosphoinositides. PI-3K phosphorylates at least three substrates: phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns-4-P) and phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) and generates the reaction products: PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 respectively (68). These phospholipids act as second messengers to activate several proteins like PDK, PKB/Akt and $p70^{S6}K$ (67,68).

1.11.1 Classification of PI-3Ks

PI-3Ks are divided into three classes based on their structure and mechanism of regulation (69). Class I PI-3Ks generate PtdIns-3-P, PtdIns-3,4-P_2, PtdIns-3,4,5-P_3 and are activated by receptor tyrosine kinases and G-protein-coupled receptors (70). Class II PI-3Ks generate PtdIns-3-P and PtdIns-3,4-P_2 and possess a lipid binding domain, whereas, Class III PI-3Ks generate PtdIns-3-P only (70). PtdIns-3-P is constitutively present in all cells and its levels do not change following stimulation (70).

1.11.1.1 Class I PI-3Ks

Class I PI-3Ks are family of heterodimeric proteins, each of which consists of a catalytic subunit of 110-120 kDa and a regulatory subunit of 85 kDa (66). Three mammalian catalytic PI-3Ks sharing 42-48% aminoacid sequence identity have been cloned and are designated p110α, p110β, p110δ. Each of these proteins interacts with the p85 regulatory subunits at the N-terminal region, and contains a domain that binds to the small G-protein Ras, a «PIK domain» homologous to a region found in other phosphoinositide kinases and a C-terminal catalytic domain (66,68). The catalytic p110 subunit possesses both intrinsic kinase serine/threonine and phosphoinositide kinase activities (69,71).

Two isoforms of p85, p85α and p85β, have been purified and cloned (71,72). P85 subunits do not possess any known enzymatic activity but are composed of several domains with homology to those found in other docking proteins. P85α and p85β contain an N-terminal Src-homology 3 (SH3) domain, two or three proline-rich segments, a region of homology to GTPase-activating proteins for the rho family of small G proteins (rho-GAPs) and two Src-homology 2 (SH2) domains (71). The inter-SH2 domain located between the two SH2 domains is necessary and sufficient for interaction with the N-terminal of p110 catalytic subunits (71,72).

1.11.2 Protein kinase B signaling pathway

Several targets of PI-3K have been identified, however, most widely studied target is protein kinase B (PKB), also known as Akt (a product of akt proto-oncogene). PKB is a serine/threonine kinase and three isoforms have been identified in mammalian system: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (67,73,74). They are activated by dual phosphorylation on threonine (Thr^{308}) and serine (Ser^{473}) residues (73). All family members contain a central kinase domain with specificity for serine or threonine residues in substrate proteins (73,74). N-terminal of PKB possesses a pleckstrin homology (PH) domain that binds phospholipids. A short glycine-rich region that bridges the PH domain to the catalytic domain follows the PH domain. The C-terminus of PKB is hydrophobic and possesses a proline-rich domain (75).

The lipid products of PI-3K bind with high affinity and specificity to the PH-domain of PKB with a preference of PtdIns-3,4-P_2 over PtdIns-3,4,5-P_3 (76). This binding induces translocation of PKB to the plasma membrane where phosphorylation of Thr308 by PtdIns-3,4,5-P_3 dependent protein kinase-1 (PDK-1) and Ser473 by the hypothetical site PDK-2 is required for the activation of PKB (76). Phosphorylation of both sites is mitogen- and PI-3K-dependent (76). Several different targets of PKB have been identified and include members of the apoptotic cascade such as Bad (73,75), caspase (77) and glycogen synthase kinase 3 (GSK-3) (78) (**Fig. 1.4**).

In mesangial cells, ET-1 receptor activation has been shown to stimulate PI-3K phosphorylation through Ras (59). Also, in rabbit internal carotid artery vascular smooth muscle cells (ICA VSMCs), PI-3K appeared to be involved in ET-1-induced Pyk2 tyrosine phosphorylation (79). Conversely, studies using angiotensin II (AII), a vasoactive peptide with similar effects to ET-1, have suggested that Pyk2 regulates PI-3K cascade specifically via interaction of Pyk2 with p130Cas which lead to their association with PI-3K in VSMC (65). Recently, ET-1 has been shown to slightly increase PKB phosphorylation in cardiomyocytes (80). However, no direct activation of PKB in response to ET-1 has been demonstrated in VSMC.

1.12 Role of endothelin in cardiovascular diseases

1.12.1 Endothelin in human hypertension

The hallmark of hypertension is an increase in peripheral vascular resistance which is considered to be related to an increase in tone of resistance arteries as well as to structural changes or vascular remodeling of the blood vessels (81,82). Several forms of hypertension are mediated by high endothelin (ET) levels in the circulation or by alterations in response to ET at the receptor level (82,83). Besides the abilities of ET to increase vascular tone, it also induces hypertrophy in smooth muscle cells and functions as mitogen as well (81,84,85) (**Fig. 1.5**).

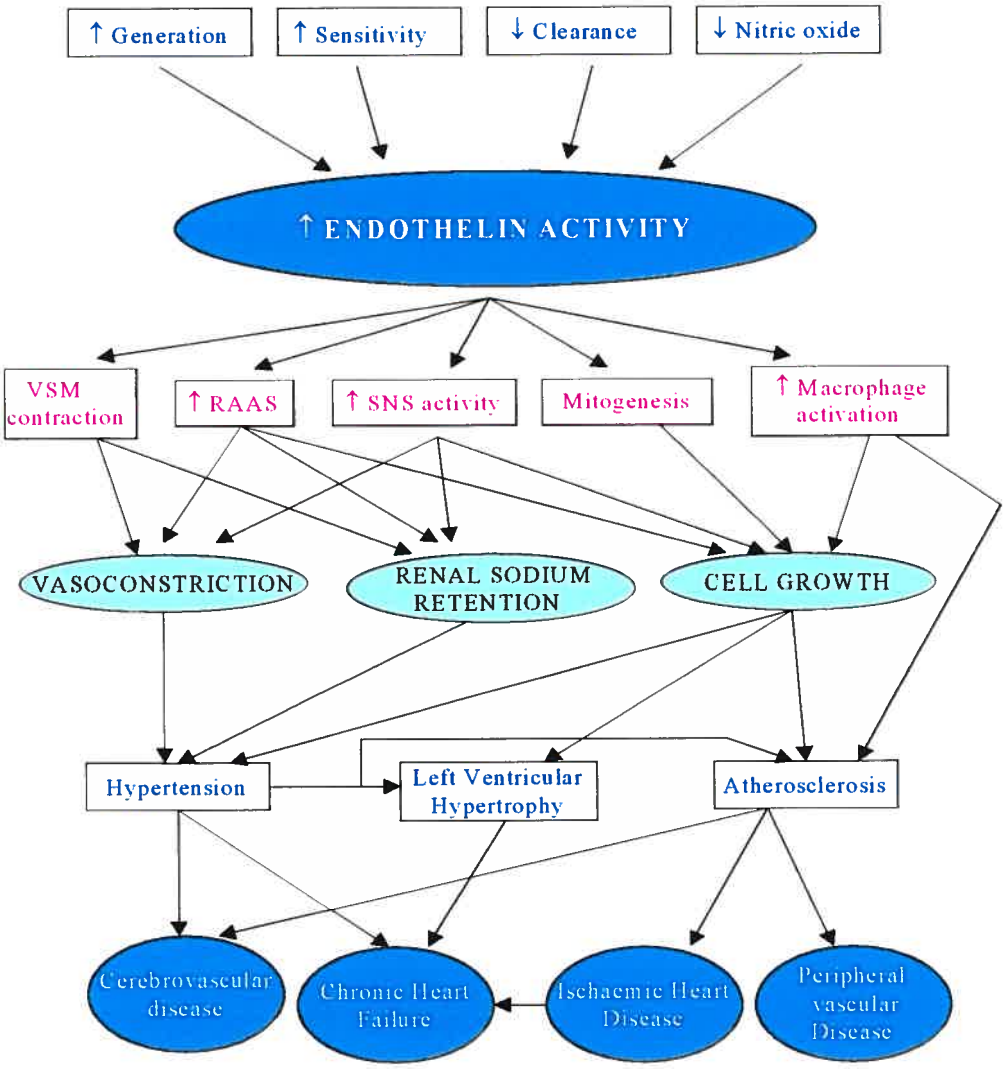


Figure 1.5 : Potential pathways of endothelin-1 in the pathophysiology of hypertension or its complications. VSM, vascular smooth muscle; RAAS, renin-angiotensin-aldosterone system; SNS, sympathetic nervous system. (Based on ref. 5)

Enhancement of generation of endothelin-1 (ET-1) plays a role in hypertrophic remodeling of arteries in moderately to severely hypertensive patients (83). Also, this enhancement of ET-1 generation contributes to elevate blood pressure and may explain the reduced responsiveness of arteries to ET-1 through downregulation of ET receptors. Recently, it has been shown that salt-sensitive hypertensive patients often have low plasma renin activity and their endothelin in plasma responds in an exaggerated fashion with an increase after sodium depletion, in association with enhanced plasma catecholamines (86). This suggests a relationship of the sympathetic system, sodium sensitivity and reactivity of the endothelin system that may contribute to blood pressure elevation in these subjects (81,82,87).

Proliferative effects of ET have been demonstrated in vascular smooth muscle cells (81,84,88) as well as in renal cells such as mesangial cells (81,88,89). Thus, the capacity of endothelin to regulate contractile responses and proliferation of vascular smooth muscle and its capacity to profoundly affect renal function make it a primary candidate as a mediator of hypertension.

1.12.2 Endothelin in experimental hypertension

Most hypertensive animal models have normal or only slightly increased plasma endothelin levels (90). Several studies have reported that in deoxycorticosterone acetate (DOCA)-salt and aldosterone-salt hypertensive rats (91,92), DOCA-salt treated spontaneously hypertensive rats (SHR) (93), Dahl salt-sensitive rats (94), one-kidney-one-clip Goldblatt hypertensive rats (95) and stroke-prone SHR (96), there is overexpression of preproET-1 mRNA in the endothelium (97). In SHR (91,93,98), endothelin doesn't seem to play an important role, although increased vasoconstrictor response to ET-1 has occasionally been reported in SHR (99).

Elevated vessel ET mRNA mediate structural effects such as vascular hypertrophy due to its growth promoting properties (82). DOCA-salt hypertensive rat arteries show severe vascular hypertrophy with prominent medial thickening (100) and overexpression of the ET-1 gene (101). However, in SHR, little vascular hypertrophy and no ET-1 gene expression are reported (102). Overall, it seems that in the DOCA-salt hypertensive rat and in rats with malignant hypertension, ET plays a more important role than in other models. This suggests that all animal hypertensive models are not the same. The different hypertensive diseases have different etiologies in which endothelin plays different roles, but in more severe forms of hypertension, such as malignant, ET plays a clear central role.

1.12.3 Endothelin in atherosclerosis

Atherosclerosis involves injury of endothelial cells, inflammation with macrophage and monocyte infiltration of the vessel wall, release of cytokines and growth factors, migration of smooth muscle cells to the intima, and lipid accumulation in foam cells (82). Evidence has recently accrued suggesting involvement of ET-1 in these processes leading to atherosclerosis development and progression (82). ET-1 is chemoattractant for monocytes and macrophages and acts as a comitogen for vascular smooth muscle cells together with growth factors (103,104). Plasma and tissue ET are elevated in proportion to the extension of atherosclerosis in patients with advanced disease (105). ET-B receptors are upregulated in atherosclerotic human coronary arteries (106). However, no change in ET-A and ET-B proportions in the media of coronary arteries was detected (107). Rossi et al. (108) showed that atherosclerotic and hypertensive individuals exhibited increased immunoreactive ET in the arteries. Many components of human atherosclerotic lesions such as endothelial cells, macrophages, and smooth muscle cells express ET-1 (109). Mechanisms whereby increased ET-1 may contribute to atherosclerosis include stimulation of migration of smooth muscle cells (110) into the intima of vessels, activation of inflammation in the vessel wall by stimulating cytokines and by increasing oxidative stress (111).

1.13 Reactive oxygen species and its implications in ET-1 signaling

During the last few years, evidence has accumulated to suggest that the generation of reactive oxygen species (ROS) play a crucial role in the development and the progression of vascular dysfunction (112). Under oxidative stress conditions, excessive endogenous formation of ROS overcomes cellular antioxidant defence mechanisms, which results in ROS-initiated modification of lipids, proteins, carbohydrates and DNA (112). ROS are very small, rapidly diffusible, highly reactive molecules and include hydroxyl radicals ($OH^{\cdot}$), superoxide anion ($O_2^{\cdot-}$) and non-radical derivative such as hydrogen peroxide (H_2O_2) (**Fig. 1.6**). Endogenously, the main source of ROS is the mitochondria which converts 1-2 % of consumed molecular oxygen into superoxide anion (113). In VSMCs and endothelial cells, NADH/NADPH oxidases represent the most important source of $O_2^{\cdot-}$ (114). NADPH oxidase catalyzes the NADPH-dependent reduction of oxygen to $O_2^{\cdot-}$, which is converted to H_2O_2 either by a protonation reaction or by the action of superoxide dismutase (SOD). H_2O_2 is reduced to H_2O by catalase or glutathion peroxidase. Under certain conditions and in presence of metals, H_2O_2 can generate the extremely active $OH^{\cdot}$ via Fenton or Haber-Weiss reaction (115) (**Fig. 1.6**).

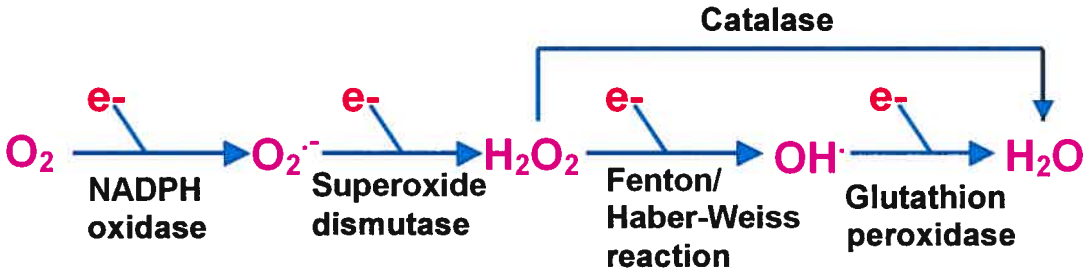


Figure 1.6 : Key steps in the production of reactive oxygen species.

NADPH oxidase is a multicomponent enzyme. Several isoforms have been found in the vascular wall (114). The plasma membrane-associated flavocytochrome b558 consists of two subunits: gp91phox and p22phox (114), and is the key catalytic component responsible for the transfer of electrons from NADPH to molecular oxygen (116). P47phox, p67phox and a small GTP-binding protein, Rac, are the cytosolic components that translocate from the cytosol to the membrane during NADPH oxidase assembly (117). ET-1 has been shown to activate NADPH oxidase, thereby increasing $O_2^{\cdot-}$ levels in endothelial cells (118) and stimulates $O_2^{\cdot-}$ production in pulmonary smooth muscle cells (119). Recent findings also suggest that ET-1 can increase $O_2^{\cdot-}$ levels via activation of NADPH oxidase in DOCA-salt rats (120).Whereas, growth factors such as AII and PDGF have been shown to generate ROS in VSMCs (114,121).

Increased ROS generation has been associated with a variety of cardiovascular pathologies (122) including hypertension (123) and atherosclerosis (124). Pathogenesis of cardiovascular diseases by activating

ROS are thought to participate in the cellular signaling pathways responsible for promoting cell growth (125) and proliferation (30,126,127). It has been demonstrated that ET-1 induces JNK and p38mapk activation through ROS generation but not ERK1/2 (30). These findings are consistent with those of Fei et al. (128), who demonstrated that JNK activation but not ERK1/2 activation by ET-1 was significantly inhibited by antioxidants in rat smooth muscle cells. Conversely, a recent study demonstrated the involvement of ROS in ET-1-induced activation of ERK1/2 pathway as well as JNK and p38mapk in cardiac fibroblasts (127). However, the contribution of ROS in ET-1-induced ERK1/2 activation is controversial and remain to be clarify in VSMC. In addition, ROS have been shown to regulate PKB signaling pathway in human hepatoma cell (126) as well as in VSMC (115). But whether ROS stimulate PKB activity in response to ET-1 has not been yet elucidated. More evidence have suggested that Pyk2 is activated in presence of H_2O_2 (129). However, a possible role of ROS in mediating the ET-1 response on Pyk2 and whether NADPH oxidase is involved in the generation of ROS by ET-1 are questions that still need to be answered.

1.14 Nitric oxide

Nitric oxide (NO) is a free radical that was previously described as a non-prostaglandin, endothelium-derived relaxing factor (EDRF) (14,130,131) and is involved in the regulation of a large number of biological processes (14,130).

Initial efforts to understand the role of NO in the nervous, cardiovascular and immune systems expanded to numerous cellular events and pathologies including apoptosis, inflammation, kidney function, diabetes, oxidative stress and aging (131,132).

1.14.1 Formation of nitric oxide

NO is formed from the aminoacid, $_{L}$-arginine, in an oxidative reaction that consumes molecular oxygen and reducing equivalents in the form of NADPH (131,132,133) (**Fig. 1.7**). Reaction products are NO, $NADP^{+}$ and citrulline. Since NO is a signaling hydrophobic molecule small enough to pass across the target-cell plasma membrane, NO cannot be stored and released as needed (130,134). NO is produced by the enzyme nitric oxide synthase (NOS), by the deamination of $_{L}$-arginine. NOS is an enzyme requiring FAD, FMN, heme, Ca^{2+}, calmodulin and 6(R)-tetra-hydro-L-biopterin (BH_4) as cofactors (133) (**Fig. 1.7**). NO acts locally because it has a short half-life (5-10 seconds) in the extracellular space before it is converted to nitrates and nitrites by oxygen and water (130,131,134).

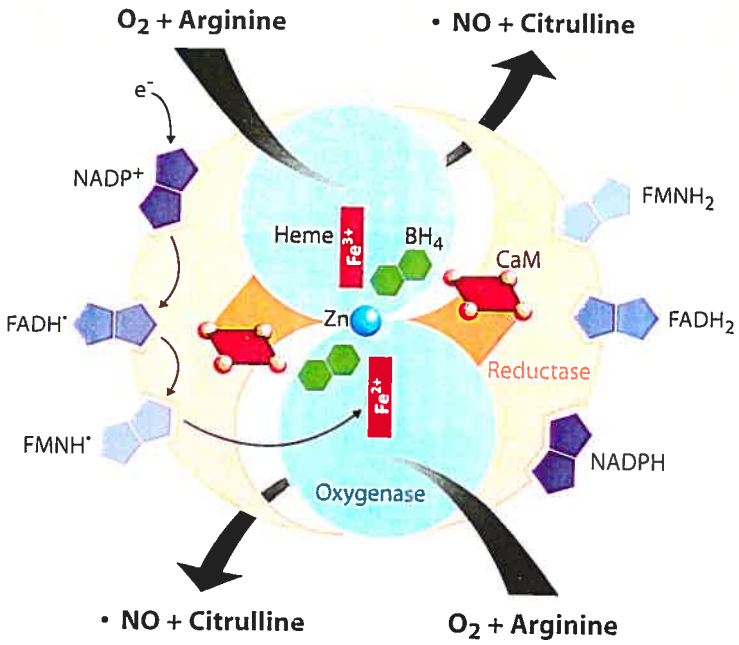


Figure 1.7 : The nitric oxide synthase (NOS) reaction. CaM = calmodulin; Zn = Zinc. (Based on ref. 135)

1.14.2 NOS isoforms

Three distinct NOS enzymes, each a product of a unique gene, have been identified and characterized (130,131,135,136). The neuronal form (nNOS or NOS-1) is a Ca^{2+}-dependent enzyme found in neuronal tissue and skeletal muscle. Four splice variants of full lengh nNOS (nNOSα) have been identified recently (nNOSβ, nNOSγ, nNOSμ and nNOS-2). The second isoform of NOS (iNOS or NOS-2) is inducible in a variety of cells and tissues in response to cytokine or endotoxin activation. The third form, first found in vascular

endothelial cells (eNOS or NOS-3), is also Ca^{2+}-dependent, but differs from the neuronal form by its smaller size. eNOS is myristoylated and palmitoylated at the N-terminus. Those modifications are required to localize to the plasmalemmal caveolae of endothelial cells. Human enzymes exhibit approximately 51-57 % homology at the aminoacid level (130,135). Structurally, all NOS isozymes consist of a carboxy-terminal reductase domain which binds the flavin cofactors. A Ca^{2+}/calmodulin binding domain lies in the center followed by an oxygenase domain where binding of heme, O_2, BH_4 and arginine substrate take place (135).

1.14.3 Nitric oxide function

NO cellular signaling involves the regulated synthesis of NO by eNOS in the vascular endothelium, diffusion of NO into adjacent smooth muscle cell and activation of the soluble isoform of guanylate cyclase (sGC) (137). When NO binds to the pentacoordinate ferrous heme of the sGC that appears to be uniquely tuned to interact with NO, conformational changes occur in the enzyme, stimulating the reaction (138). NO causes relaxation of the smooth muscle by mediating the formation of cGMP that acts as a second messenger and activates the cGMP-dependent protein kinases (protein kinase G) (137,139), which in turn, facilitates the phosphorylation of various proteins as well as the reduction of intracellular calcium concentrations by different mechanisms (140). Moreover, NO also targets many proteins either by nitrosylation of thiol

residues, nitration of tyrosine or oxidizing DNA and proteins (140). Increasing evidence indicates also that NO may inactivate NADPH oxidase by inhibiting its assembling process, thus reducing the ROS levels (141). In higher concentrations, NO can react rapidly with superoxide (O_2^-) to form peroxynitrite ($ONOO^-$), a potent oxidant with the potential to disrupt protein structures by nitrating the protein tyrosine residues (142). Although NO signaling is complex as a result of its interactions with ROS, heme groups on proteins, sulfhydryl groups, and other cellular targets, the activation of guanylate cyclase remains the most important pathway in mediating NO function (137).

1.14.4 Guanylate cyclase

Guanylate cyclase is an enzyme that catalyses the conversion of the guanosine triphosphate (GTP) to 3'-5'-guanosine monophosphate (cGMP). The guanylate cyclase is found in many cellular compartments (140). Two major forms of guanylate cyclase are known, the particular guanylate cyclase and the soluble guanylate cyclase. It is generally conceded that activation of soluble guanylate cyclase (sGC) is the principal intracellular event that initiates relaxation (143,144). The activity of the sGC is regulated by nitrovasodilators, oxidation products of fatty acid and free radicals (134,145). sGC is a heterodimer of two subunits α and β. Each subunit is divided in three different domains: the heme-binding domain, the catalytic domain and the dimerization domain (145).

N-terminal of each subunit contains heme as a prosthetic group which serves as a site for NO binding (145). sGC lacking the heme moiety, is not able to be activated by NO (145). Heme is attached to the protein portion of the enzyme by an imidazole axial ligand and binding of the heme is specific to the β subunit of the N-terminal region (146,147). C-terminal of each subunit possesses a catalytic domain with a high homology sequence between the monomers (146,147). Coexpression of the catalytic domain of both subunits is necessary for GC activity. There is the dimerization domain between both domains described above that mediates the association of the heterodimer which is essential for the catalytic subunit (146,147). NO binding to the heme of the sGC results in the formation of a complex penta-coordinate heme-nitrosyl that breaks the axial histidine link (147). This conformational change exposes the catalytic site to GTP, leading to the activation of the enzyme and conversion of GTP to cGMP by sGC in the presence of Mg^{2+} or Mn^{2+} ions (137) (**Fig. 1.8**).

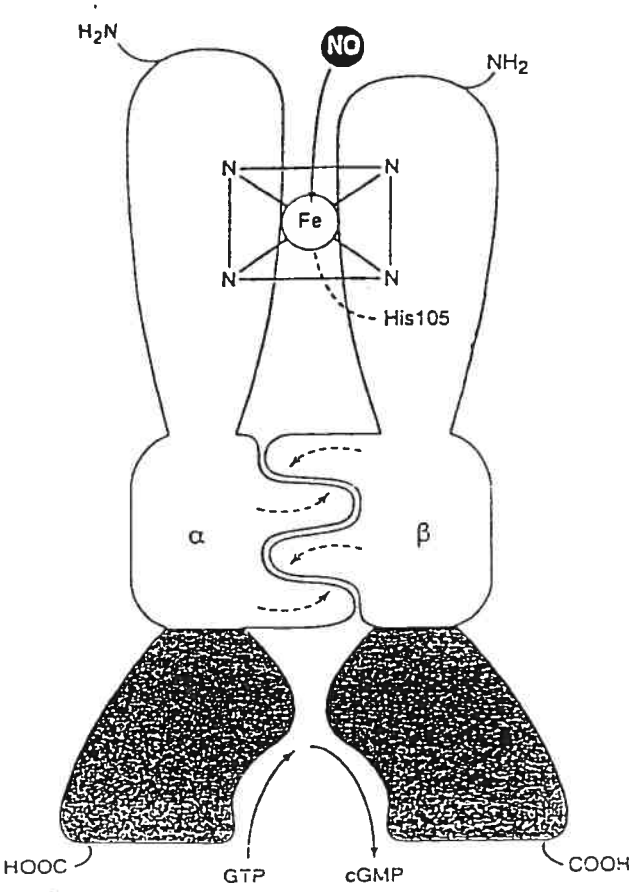


Figure 1.8 : Schematic representation of a soluble guanylate cyclase α/β heterodimer.

1.14.5 Regulation of cGMP production

In most tissues, the intracellular concentration of cGMP is determined by the rate of formation which is regulated by agonist-induced stimulation of a cyclase and hydrolysis of cGMP by a related group of phosphodiesterase E (PDE) (148) (**Fig. 1.9**). There are at least seven known distinct mammalian PDE families. Each one differs from each other in

biochemical and physical properties, responses to specific effectors, inhibitors and regulatory control mechanisms (148). Type V PDE has been isolated from a number of tissues including human platelets (149), trachea (150) and VSMC (151) and is commonly referred to as cGMP-specific PDE. PDE V is characterized by selectively hydrolyzing only cGMP, independently of Ca^{2+}/calmodulin. Inhibitors of PDE V such as A02131-1 have vasodilating and anti-aggregating properties, which may protect the vascular wall against arteriosclerotic changes (149).

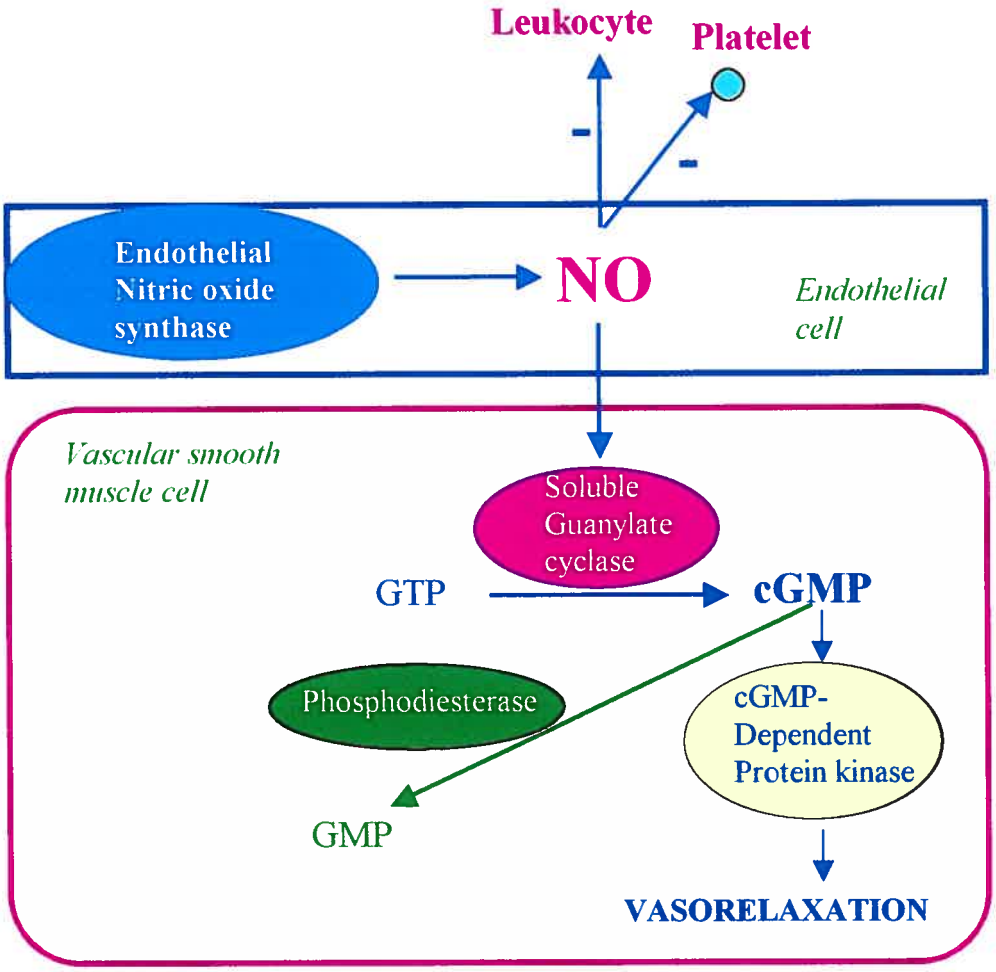


Figure 1.9 : The nitric oxide/cGMP signal transduction. (Based on ref. 130)

1.14.6 NO in signal transduction

The endothelium serves as the principal physiological source of NO in blood vessels (152). As evidenced, NO contributes to the regulation of several hormone-mediated responses (153,154,155). In addition to its vasodilating effect, NO can also inhibit atherogenesis (130), thrombocyte aggregation (156) and VSMC proliferation (148,152) and migration (157). There is also increasing body of evidence suggesting that NO is opposed to the physiological and pathophysiological effects (14,137) of growth factors and vasoactive peptides such as EGF (148), PDGF (158) and bFGF (157). This is probably achieved by inhibiting one or several of the signaling events induced by these factors (130,152,157,159,160). According to several studies, mitogens such as ET-1 stimulate the synthesis of DNA and cell proliferation by activating the phosphorylation cascade of MAPK (28,161,162,163). The potential mechanism that could modulate VSMC proliferation is the release of NO by the endothelium either via a cGMP-dependent (137,148) or a cGMP-independent mechanisms (164,165,166,169). In cardiomyocytes, ET-1-induced protein synthesis (170,171) has also been shown to be inhibited by NO (167). Furthermore, NO was recently found to suppress the AII-induced activation of three major MAPKs, ERK1/2, p38mapk and JNK (168) as well as Pyk2 (155) in cardiac fibroblasts. However, it is not known whether NO similar to its effect on growth factor and AII-induced responses can also modulate signaling events triggered by ET-1 receptor activation in VSMC. In smooth muscle-derived A7r5

cells, NO has been shown to regulate PDGF-induced activation of PKB (154). These data implicate the PKB signaling cascade as an important mitogenic pathway that is subjected to modulation by NO in VSMC (154). However, the role of NO/cGMP in modulating PKB signaling pathway in response to ET-1 has not yet been investigated in any cell type.

1.15 Objectives of the present study

As described above, ROS play an essential role in propagating the signals of several growth factors such as EGF, PDGF and AII. The contribution of ROS in ET-1-induced MAPK activation remain controversial and there are no reports documenting the activation of PKB as well as Pyk2 by ET-1 in VSMC. Therefore, we have undertaken this study to elucidate a role of ROS on key components of ET-1 signaling system as well as protein synthesis in VSMC, and have examined whether ROS generation contributes towards their response.

Furthermore, since NO is an important modulator of intracellular signaling system by many growth factors such as PDGF and EGF in VSMC as well as AII in cardiac fibroblasts. We have also investigated a possible role of NO on ET-1-sensitive signaling systems in VSMC. We first elucidated the role of NO on key components of ET-1 signaling system, ERK1/2, PKB and Pyk-2 as well as protein synthesis in VSMC. We then used 8-Bromo-cGMP, a cyclic GMP analogue and ODQ, an inhibitor of sGC, to examine whether NO is acting

through a cGMP-dependent mechanism. These studies have utilized standard protocols of cell biology such as cell culture, SDS-PAGE, western bloting as well as radioisotopes.

CHAPTER 2

ARTICLE 1

Reactive oxygen species mediate Endothelin-1-induced activation of ERK½, PKB and Pyk2 and protein synthesis in vascular smooth muscle cells

Reactive oxygen species mediate Endothelin-1-induced activation of ERK½, PKB and Pyk2 and protein synthesis in vascular smooth muscle cells

Grace Bou Daou and Ashok K. Srivastava

Centre de recherche, Centre hospitalier de l'Université de Montréal - Hôtel-Dieu, Department of Physiology, Université of Montréal, Montreal, Quebec, Canada

Running title: Role of ROS in ET-1-induced activation of ERK ½, PKB, Pyk2 and protein synthesis

Address for correspondence:

Ashok K. Srivastava, Ph.D
Centre de recherche
CHUM, Hôtel-Dieu
3840, rue St. Urbain
Montréal (Québec) H2W 1T7
Tel: 514-890-8000 ext.12917
Fax: 514-412-7152
[redacted]

Abstract

Reactive oxygen species (ROS) have been shown to mediate the effect of several growth factors such as angiotensin II (AII), epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Endothelin-1 (ET-1) is an important growth factor for vascular smooth muscle cells (VSMC) which is believed to contribute to the pathogenesis of vascular abnormalities such as atherosclerosis, hypertension and cardiac hypertrophy. However, a possible role of ROS generation in mediating the ET-1 response on ERK1/2 and PKB, key components of growth promoting and proliferative signaling pathways, has not been examined in detail. Therefore, the aim of the present study was to investigate the involvement of ROS in ET-1-mediated activation of ERK1/2 and PKB as well as Pyk2 in A-10 VSMCs. Pyk2 is a non-receptor protein tyrosine kinase and an upstream regulator of MAPK signaling. ET-1 stimulated the phosphorylation of ERK1/2, PKB and Pyk2 in a dose and time-dependent fashion with maximum response being elicited at 10 nM which peaked at 5 min. Treatment of VSMC with ET-1 resulted in an increase in the generation of ROS that could be blocked by diphenyleneiodonium (DPI), an inhibitor of NADPH oxidase. Furthermore, DPI pretreatment of cells prior to stimulation with ET-1, attenuated ET-1 enhanced phosphorylation of ERK1/2, PKB and Pyk2. N-acetylcysteine (NAC), another ROS scavenger, also exhibited a similar response. Moreover, DPI also caused a decrease in the protein synthesis stimulated by ET-1. These results demonstrate that ROS is a critical mediator of

ET-1-induced signaling events linked to hypertrophic and growth promoting pathways in VSMC.

Key words: ROS, ET-1, MAPK, ERK1/2, PKB, Pyk2, A-10 cells.

Introduction

Endothelin-1 (ET-1), a 21-amino acid peptide hormone, exhibits vasoconstrictor (1) and mitogenic (2) properties. These effects of ET-1 are elicited through the activation of 2 receptor subtypes, ET_A and ET_B, which belong to a family of heptahelical G-protein-coupled receptors (GPCRs) (3-5). ET-1 receptor activation is coupled to multiple signaling pathways, such as phospholipases C and D (6), Ca^{2+} (7), mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun-NH_2-terminal kinase (JNK), and p38MAPK (8-11) as well as phosphatidylinositol 3-kinase (12). Extensive studies, carried out predominantly in cardiomyocytes (6, 8, 28) and glomerular mesangial cells (11, 12), have implicated both receptor and non-receptor protein tyrosine kinases (PTKs) in transducing ET-1-evoked signaling responses (11, 15-18). Various PTKs activated by ET-1 include c-Src (13-15), epidermal growth factor (EGF) (17) and a Ca^{2+}-dependent PTK, Pyk2 (11, 17).

Recent experiments have indicated that reactive oxygen species (ROS) play an essential role in propagating the signals of several growth factors, peptide hormones and cytokines, such as platelet-derived growth factor (19), EGF (20), angiotensin II (AII) (21), insulin (22), interleukin-1 (23) and tumor necrosis factor-α (24). Increased ROS generation has been linked to the pathogenesis of several cardiovascular diseases, such as hypertension, atherosclerosis, restenosis and congestive heart failure (29-31). ET-1 has also

been shown to augment ROS production in various cell types (25-27), and a role of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the ET-1-induced elevation of vascular ROS production has been suggested recently (32).

Although it has been reported that ROS mediate ET-1-stimulated activation of ERK1/2 and JNK (27), to the best of our knowledge, a detailed investigation into the contribution of ROS in other ET-1-evoked growth-promoting signaling pathways has not been carried out. Moreover, despite the fact that ET-1-induced vascular smooth muscle cell (VSMC) proliferation (26, 33) and fibrogenesis (15) may contribute to vasculature remodeling leading to vascular disease, not much information is available on ET-1-evoked signaling in these cells. Therefore, in the present studies, we have investigated the effect of ET-1 on ERK1/2, protein kinase B (PKB) and Pyk2, key regulators of the proliferative signaling pathway in A-10 VSMCs, and examined whether ROS generation contributes to their activation.

Materials and Methods

Materials

ET-1 was purchased from Peninsula Laboratories (Belmont, CA, USA), and *N*-acetyl-$_L$-cysteine (NAC), diphenyleneiodonium (DPI) and bis-N-methylacridinium nitrate (lucigenin), from Sigma (St. Louis, MO, USA). Monoclonal phospho-specific-Tyr^{204}-ERK1/2 antibody, polyclonal ERK1/2 antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polyclonal phospho-specific-Ser^{473}-PKB and total PKB as well as phospho-specific-Tyr^{402}-Pyk2 and total Pyk2 antibodies were procured from New England Biolabs (Beverly, MA, USA). The enhanced chemiluminescence (ECL) detection system kit and L-(4,5-^{3}H) leucine were from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada).

Methods

Cell culture

VSMC derived from embryonic rat thoracic aorta A-10 cells were maintained in culture with DMEM containing 10% fetal bovine serum at 37^{0}C in a humidified atmosphere of 5% CO_2, as described earlier (34). The cells were grown to 80-90% confluence in 60-mm plates and incubated in serum-free DMEM 20 h prior to the treatments.

Cell lysis and Western blotting

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. The cell lysates were centrifuged at 12,000g for 10 min at 4^0C. Protein concentrations were measured by Bradford assay. Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel (SDS-PAGE), transferred to PVDF membranes (Millipore, MA, USA) and incubated with respective primary antibodies (monoclonal phospho-specific-Tyr^{204}-ERK1/2 antibody (1:2,000), polyclonal phospho-specific-Ser^{473}-PKB antibody (1:4,000), phospho-specific-Tyr^{402}-Pyk2 antibody (1:1,000)). The antigen-antibody complex was detected by a horseradish peroxidase-conjugated second antibody (1:4,000), and protein bands were visualized by ECL. The intensity of specific bands was quantified by NIH Image software as described previously (35).

Measurement of ROS generation

ROS production was measured by the lucigenin method (21) with minor modifications (36). Briefly, the cells were preincubated in the absence or

presence of 10 μM DPI in DMEM for 30 min, then treated with ET-1 (10 nM for 5 min). They were trypsinized, collected by centrifugation, and the pellet was washed in modified Krebs buffer containing NaCl (130 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (1.5 mM), K_2HPO_4 (1 mM), and HEPES (20 mM), pH 7.4. After washing, the cells were resuspended in Krebs buffer, and the cell concentration was adjusted to 1×10^7 in 900 μl buffer. To measure ROS production, the cell suspension was transferred to plastic tubes and assessed in a luminometer (LB 9507, Berthold, Wildbad, Germany). Measurement was started by an injection of 100 μl lucigenin (final concentration 5×10^{-4} M) at time zero. Photon emission was counted every 5 min for up to 20 min. The emission in relative light units was corrected for nonspecific luminescence in the absence of cells. Modified Krebs buffer was used as a control (blank). Solutions containing DPI in the absence of cells did not display any significant interference in the lucigenin assay.

Measurement of [^{3}H]leucine incorporation

A-10 cells were treated for 20 h with endothelin-1 (10 nM; Belmont, CA, USA). Protein synthesis was assessed by the addition of 2 μCi/mL of [^{3}H]leucine (ICN Biomedicals, Inc., Costa Mesa, CA, USA) for a period of 20 h. To assess the role of reactive oxygen species (ROS), cells were pretreated for 30 min with DPI (5 μM; Sigma, St-Louis, MO, USA), a specific inhibitor of flavoprotein, NADH/NAD(P)H oxidase (32,37). Following the completion of

the experimental protocol, A-10 cells were washed twice with cold PBS, and 1 ml of cold 5% trichloroacetic acid was added for 30 min to precipitate protein. The precipitates were subsequently washed twice with cold water and resuspended in 500 μl of 0.4 M NaOH. Aliquots were counted in a scintillation counter.

Statistics

Statistical analysis was performed by one-way, repeated-measures analysis of variance (ANOVA) followed by a Fisher *post hoc* test. All data are reported as means ± SE. The differences between means were considered significant at $P < 0.05$.

Results

Effect of ET-1 on ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs

Our initial experiments were aimed at analyzing the effect of ET-1 on the phosphorylation of 3 key elements of the signaling pathway, namely, ERK1/2, PKB and Pyk2. Their activation was assessed by using phospho-specific antibodies against each of these kinases. As shown in Figure 1, ET-1 concentration-dependently enhanced the phosphorylation of ERK1/2 (Fig. 1A), PKB (Fig. 1B) and Pyk2 (Fig. 1C). In each case, maximum phosphorylation occurred at ET-1 concentrations of 1-10 nM. Next, we analyzed the time-dependence of the ET-1 response at 10 nM. As seen in Figure 2, ET-1 treatment of A-10 cells rapidly increased the phosphorylation of ERK1/2 (Fig. 2A), PKB (Fig. 2B) and Pyk2 (Fig. 2C). A significant increment was detected within 1 min of treatment and peaked at about 5 min, then gradually declined to submaximal levels at 30 to 60 min. No alteration in the total amount of ERK1/2, PKB or Pyk2 was observed under these experimental conditions.

Effect of DPI on ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2 in A-10 VSMCs

To investigate whether ROS generation was involved in ET-1-induced activation of ERK1/2, PKB and Pyk2, we utilized DPI, a frequently-employed inhibitor of the flavoprotein NADH/NAD(P)H oxidase (32,37). As depicted in Figure 3, DPI pretreatment concentration-dependently inhibited ET-1-induced phosphorylation of ERK1/2 (Fig. 3A), PKB (Fig. 3B) and Pyk2 (Fig. 3C).

However, among the 3 kinases, Pyk2 appeared to be most sensitive to inhibition by DPI, which elicited a significant reduction in phosphorylation at 1 μM (Fig. 3C). At this DPI concentration, ERK1/2 phosphorylation was not significantly affected (Fig. 3A), and PKB phosphorylation was suppressed only slightly (Fig. 3B), whereas 10 μM DPI pretreatment was found to block ET-1-induced phosphorylation of all the kinases by about 80 % (Fig. 3).

Effects of ET-1 and DPI on ROS generation in A-10 VSMCs

The results described above suggested that ROS generation by a DPI-inhibitable NADPH oxidase might contribute to ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. Therefore, we evaluated the effect of ET-1 on ROS generation in A-10 cells by the lucigenin chemiluminescence method. As illustrated in Figure 4, stimulation with 10 nM ET-1 for 5 min evoked a significant increase of ROS production in A-10 cells. However, their treatment with DPI prior to stimulation with ET-1 almost completely blocked ET-1-evoked ROS production. These results revealed that in A-10 cells, ET-1 caused an increase in ROS production which was suppressed by DPI. DPI alone had no significant effect on the basal production of ROS (data not shown).

Effect of NAC on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs

To further confirm a role of ROS in ET-1-induced responses, we tested the effect of NAC, a thiol-containing agent, on ERK1/2, PKB and Pyk2

phosphorylation by ET-1. VSMCs were preincubated with NAC for 6 h and then stimulated with ET-1 for 5 min. As shown in Figure 5, NAC treatment decreased ERK1/2, PKB and Pyk2 phosphorylation induced by ET-1. As with DPI (Fig. 3), attenuation of Pyk2 phosphorylation by NAC was more potent compared to ERK1/2 and PKB phosphorylation by ET-1.

Effect of DPI on protein synthesis induced by ET-1 in A-10 VSMCs

Since increased protein synthesis is one of the physiological consequences of ET-1 receptor activation, we evaluated whether ET-1-induced protein synthesis in A-10 VSMCs is also regulated by ROS generation. As presented in Figure 6, treatment of A-10 cells with ET-1 heightened [^{3}H]leucine incorporation into protein. ET-1 caused more than a 3-fold increment of protein synthesis, which was almost completely attenuated in cells pretreated with DPI. However, DPI did not significantly affect basal [^{3}H]leucine incorporation.

Discussion

In the present studies, we have demonstrated that ET-1 stimulated ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs. Although earlier investigations in cardiomyocytes (6,8,28) and glomerular mesangial cells (11,12) have reported ET-1-induced activation of ERK1/2 (8-10) and Pyk2 (11,17), similar work in VSMC has not been conducted. Moreover, an effect of ET-1 on PKB phosphorylation has not been examined previously. Thus, our data represent a first demonstration of ET-1-induced ERK1/2, PKB and Pyk2 activation in VSMCs. These signaling components are believed to be among the key players mediating the growth, proliferation, hypertrophy, migration and survival responses of the cells (38). Activation of ERK1/2 signaling pathways has been suggested to be important for ET-1-induced proliferation of VSMCs (39) and myometrial cells (14) as well as cell cycle progression of NIH 3T3 cells (40).

Pyk2 has been shown to serve as an upstream regulator of ERK1/2 and PKB activation by AII in VSMCs (41), but ET-1-induced ERK1/2 phosphorylation in kidney mesangial cells (12) and neonatal cardiomyocytes (17) appears to be Pyk2-independent. Thus, it is possible that Pyk2 regulates ERK1/2 phosphorylation in a ligand-specific manner. The time-course of Pyk2 and ERK1/2 phosphorylation in A-10 VSMCs (Fig. 2) showed almost an identical pattern, and did not provide any evidence for a potential temporal relationship between the 2 kinases. Thus, more detailed kinetic analysis with

pharmacological and genetic tools will be required to test the role of Pyk2 in mediating ET-1-induced responses in VSMCs. ET-1 was recently demontrated to exert an anti-apoptotic effect in cardiomyocytes (42), but its influence on PKB activation was not investigated in these studies. Since PKB is believed to exert an anti-apoptotic action through the phosphorylation of BAD and caspases (38), it may be postulated that ET-1-induced activation of PKB contributes to this response.

Another important finding of the present work is that DPI, an inhibitor of NADPH oxidase, blocked ET-1-evoked enhancement of ERK1/2, PKB and Pyk2 phosphorylation. We further demonstrated that ET-1 caused an increase in ROS generation, which was blocked in cells pretreated with DPI. These observations are consistent with a role of ROS as a mediator of the ET-1 response in A-10 VSMCs. However, in some earlier studies, DPI pretreatment was shown to specifically inhibit ET-1-induced JNK and p38MAPK phosphorylation without affecting ERK1/2 phosphorylation in VSMCs isolated from the adult rat aorta (27,39). These data argue against a role for ROS generation in ET-1-induced ERK1/2 phosphorylation in adult rat VSMCs.

In contrast to studies in VSMCs, ROS involvement in ET-1-stimulated ERK1/2 phosphorylation in cardiac fibroblasts has been suggested (43). In addition, divergent effects of DPI on AII-induced ERK1/2 phosphorylation in VSMCs have also been noted. For example, an inhibitory action of DPI on AII-

stimulated ERK1/2 phosphorylation was demonstrated by Frank et al. (37), whereas Viedt et al. (44) and Touyz et al. (45) failed to detect any influence of DPI on ERK1/2 phosphorylation. The reasons for these conflicting data remain unclear, but differences in the DPI dose used or the time of DPI pretreatment as well as the cell types or culture conditions might have contributed to these varied responses. Our data on NAC, a free radical scavenger, which almost completely attenuated the effect of ET-1 on ERK1/2, PKB and Pyk2 phosphorylation provided additional support for the involvement of ROS generation in mediating the action of ET-1 in A-10 VSMCs.

We have also demonstrated that ET-1 treatment stimulated the rate of protein synthesis in VSMCs. Although ET-1 has been shown to enhance protein synthesis (46) via the ERK1/2 signaling pathway (28), no substantial work into its effect on protein synthesis in VSMCs has been conducted. Moreover, a clear role of ERK1/2 in the process in VSMCs has not been established. However, since DPI pretreatment blocks both ERK1/2 phosphorylation and protein synthesis in A-10 VSMCs, an involvement of this pathway in mediating this response may be hypothesized.

In conclusion, we have demonstrated that ET-1-induced activation of DPI-sensitive NADPH oxidase contributed to ROS generation in A-10 VSMCs. ROS generation was also involved in the enhanced phosphorylation and activation of ERK1/2, PKB and Pyk2 as well as protein synthesis in A-10 VSMCs. From

these data, we conclude that ROS plays a critical role in triggering ET-1-induced signaling pathways linked to growth-promoting responses.

Figure legends

Figure 1. ET-1-induced dose-responses of ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were treated without or with the indicated ET-1 concentrations for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr^{204}-ERK1/2 antibodies (A), phospho-specific-Ser^{473}-PKB antibodies (B) and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the middle panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (bottom panels of each section). Top panels represent average data quantified by densitometric scanning of immunoblots. Values are the means ± SE of at least 3 independent experiments and are expressed as fold increase over basal phosphorylation. (A) $*P< 0.002$ vs control. (B) $*P< 0.02$ vs control. (C) $*P< 0.006$ vs control.

Figure 2. Time-course of ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were treated without or with ET-1 (10 nM) for the indicated time periods. Cell lysates were immunoblotted by phospho-specific-Tyr^{204}-ERK1/2 antibodies (A), phospho-specific-Ser^{473}-PKB antibodies (B) and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the middle panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (bottom panels of each section). Top panels represent average data quantified by densitometric scanning of immunoblots. Values are the means ± SE of at least 3 independent experiments

and are expressed as fold increase over basal phosphorylation. (A) *P< 0.007 vs control. (B) *P< 0.03 vs control. (C) *P< 0.02 vs control.

Figure 3. Dose-dependent effect of the NADPH oxidase inhibitor DPI on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with the indicated DPI concentrations for 30 min, followed by 10 nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr^{204}-ERK1/2 antibodies (A), phospho-specific-Ser^{473}-PKB antibodies (B) and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the middle panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (bottom panels of each section). Top panels represent average data quantified by densitometric scanning of immunoblots. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage phosphorylation where phosphorylation observed with ET-1 alone is defined as 100%. (A) *P< 0.0001 vs control, †P< 0.0001 vs ET-1. (B) *P< 0.0001 vs control, †P< 0.02 vs ET-1. (C) *P< 0.02 vs control, †P< 0.04 vs ET-1.

Figure 4. Effect of DPI on ET-1-induced ROS generation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with DPI (10 μM) for 30 min before the addition of ET-1 (10 nM) for 5 min. The cells were then trypsinized and collected by centrifugation for the assessment of ROS generation as described in Materials and Methods. Relative light unit

measurements were compared 5 min after the addition of lucigenin. Non-treated cells were considered as controls. Values are the means ± SE of 3 independent experiments; *P< 0.02 vs control, †P< 0.02 vs ET-1 .

Figure 5: Effect of the superoxide scavenger NAC on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with NAC (20 mM) for 6 h, followed by 10 nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr^{204}-ERK1/2 antibodies (A), phospho-specific-Ser^{473}-PKB antibodies (B) and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the middle panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (bottom panels of each section). Top panels represent average data quantified by densitometric scanning of immunoblots. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage phosphorylation where phosphorylation observed with ET-1 alone is defined as 100%. (A) *P< 0.0001 vs control, †P< 0.005 vs ET-1. (B) *P< 0.0001 vs control, †P< 0.009 vs ET-1. (C) *P< 0.0008 vs control, †P< 0.04 vs ET-1.

Figure 6: Effect of DPI on ET-1-induced [^{3}H]leucine incorporation into proteins. Serum-starved quiescent A-10 cells were pretreated with DPI (5 μM) for 30 min in the absence (control) or presence of ET-1 (10 nM); then, the cells were labeled to equilibrium with [^{3}H]leucine for 20 h as described in Materials and Methods. Values are the means ± SE of 3 independent experiments and are

expressed as a percentage of change in [^{3}H]leucine incorporation over basal values. *P< 0.0002 vs control, †P< 0.0002 vs ET-1.

FIGURE 1

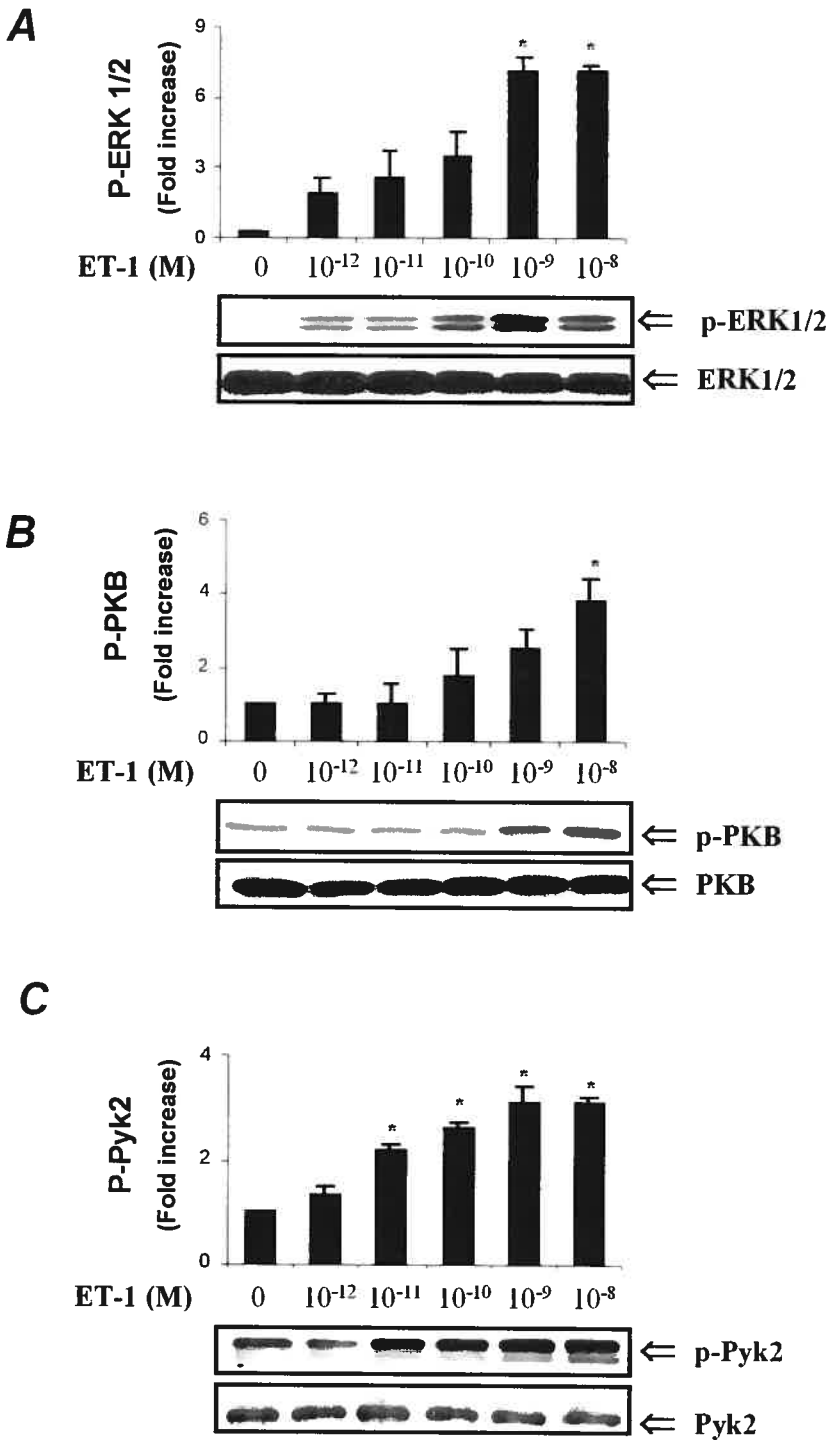

A
P-ERK 1/2
(Fold increase)
9
6
3
0
ET-1 (M)
0
10-12
10-11
10-10
10-9
10-8
p-ERK1/2
ERK1/2
B
P-PKB
(Fold increase)
6
4
2
0
ET-1 (M)
0
10-12
10-11
10-10
10-9
10-8
p-PKB
PKB
C
P-Pyk2
(Fold increase)
4
2
0
ET-1 (M)
0
10-12
10-11
10-10
10-9
10-8
p-Pyk2
Pyk2

FIGURE 2

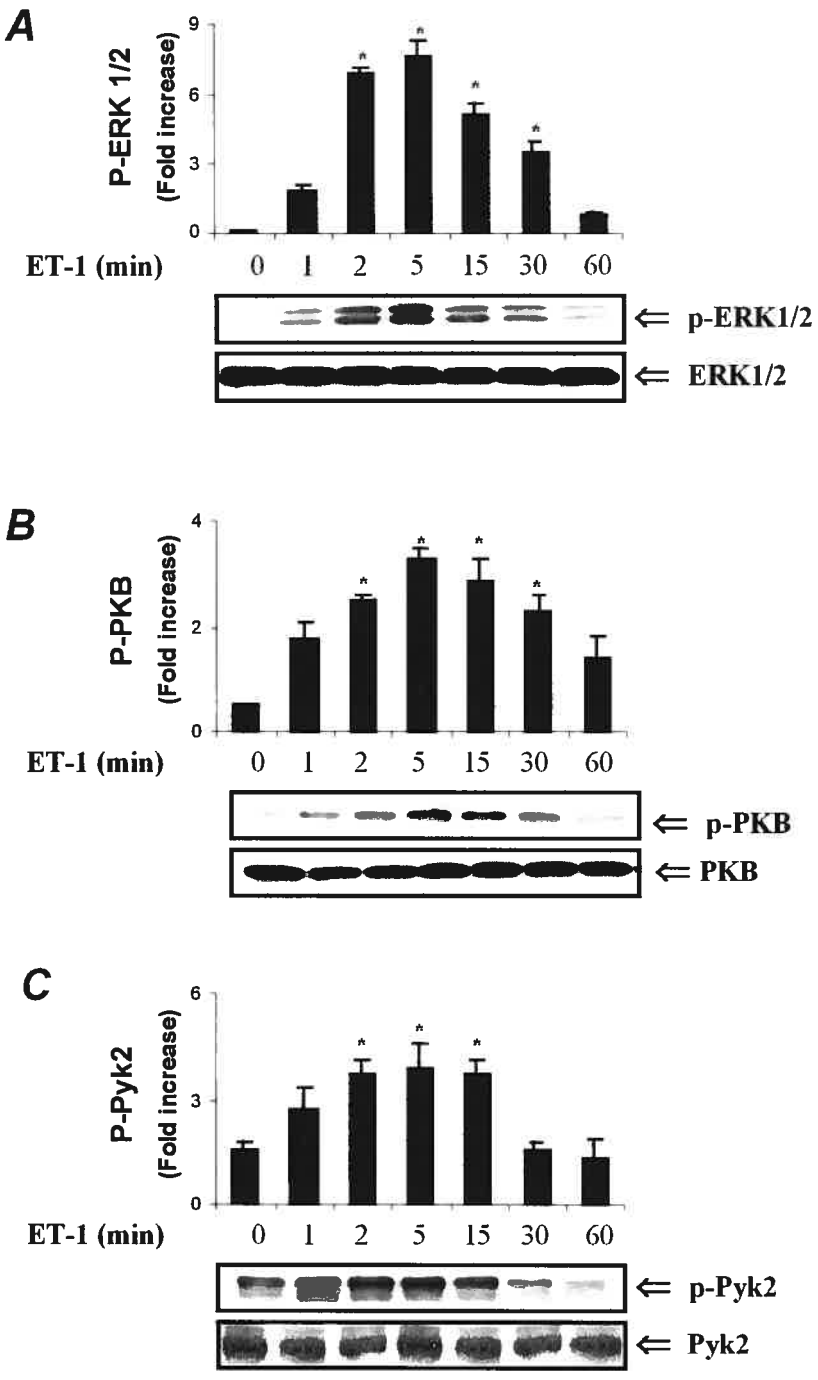

A
P-ERK 1/2
(Fold increase)
9
6
3
0
ET-1 (min)
0 1 2 5 15 30 60
⇐ p-ERK1/2
⇐ ERK1/2
B
P-PKB
(Fold increase)
4
2
0
ET-1 (min)
0 1 2 5 15 30 60
⇐ p-PKB
⇐ PKB
C
P-Pyk2
(Fold increase)
6
3
0
ET-1 (min)
0 1 2 5 15 30 60
⇐ p-Pyk2
⇐ Pyk2

FIGURE 3

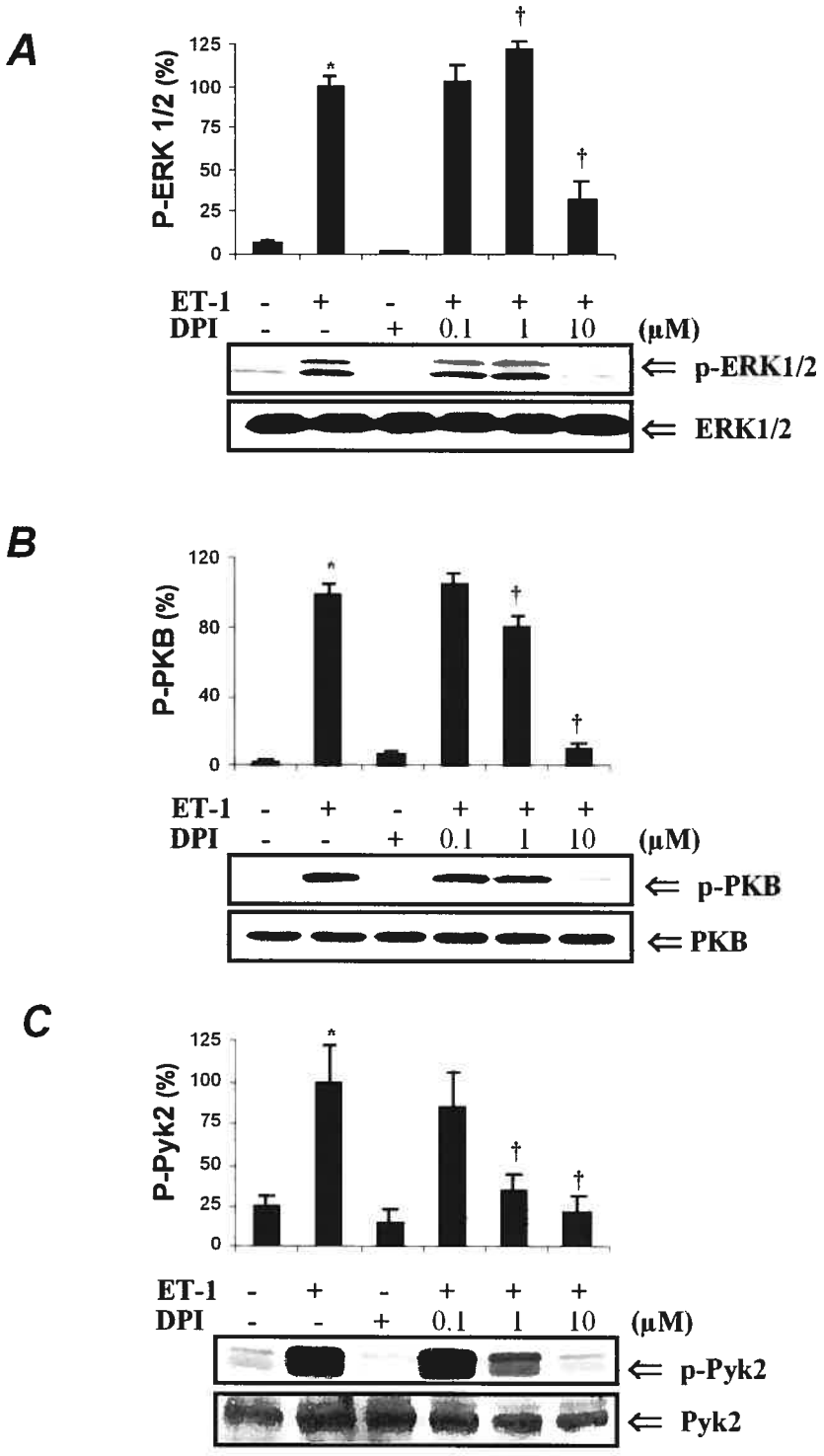

A
P-ERK 1/2 (%)
125
100
75
50
25
0
*
†
†
ET-1 - + - + + +
DPI - - + 0.1 1 10 (μM)
⇐ p-ERK1/2
⇐ ERK1/2
B
P-PKB (%)
120
80
40
0
*
†
†
ET-1 - + - + + +
DPI - - + 0.1 1 10 (μM)
⇐ p-PKB
⇐ PKB
C
P-Pyk2 (%)
125
100
75
50
25
0
*
†
†
ET-1 - + - + + +
DPI - - + 0.1 1 10 (μM)
⇐ p-Pyk2
⇐ Pyk2

FIGURE 4

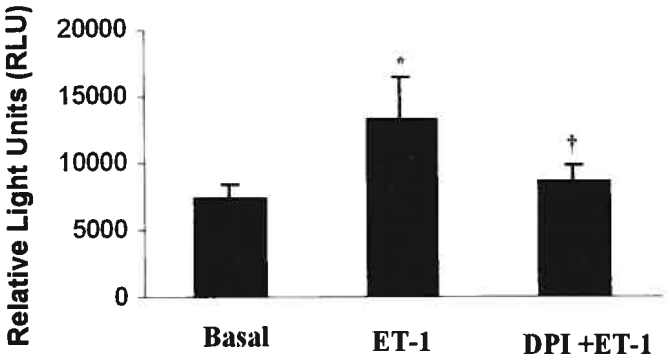

Relative Light Units (RLU)
20000
15000
10000
5000
0
*
†
Basal
ET-1
DPI +ET-1

FIGURE 5

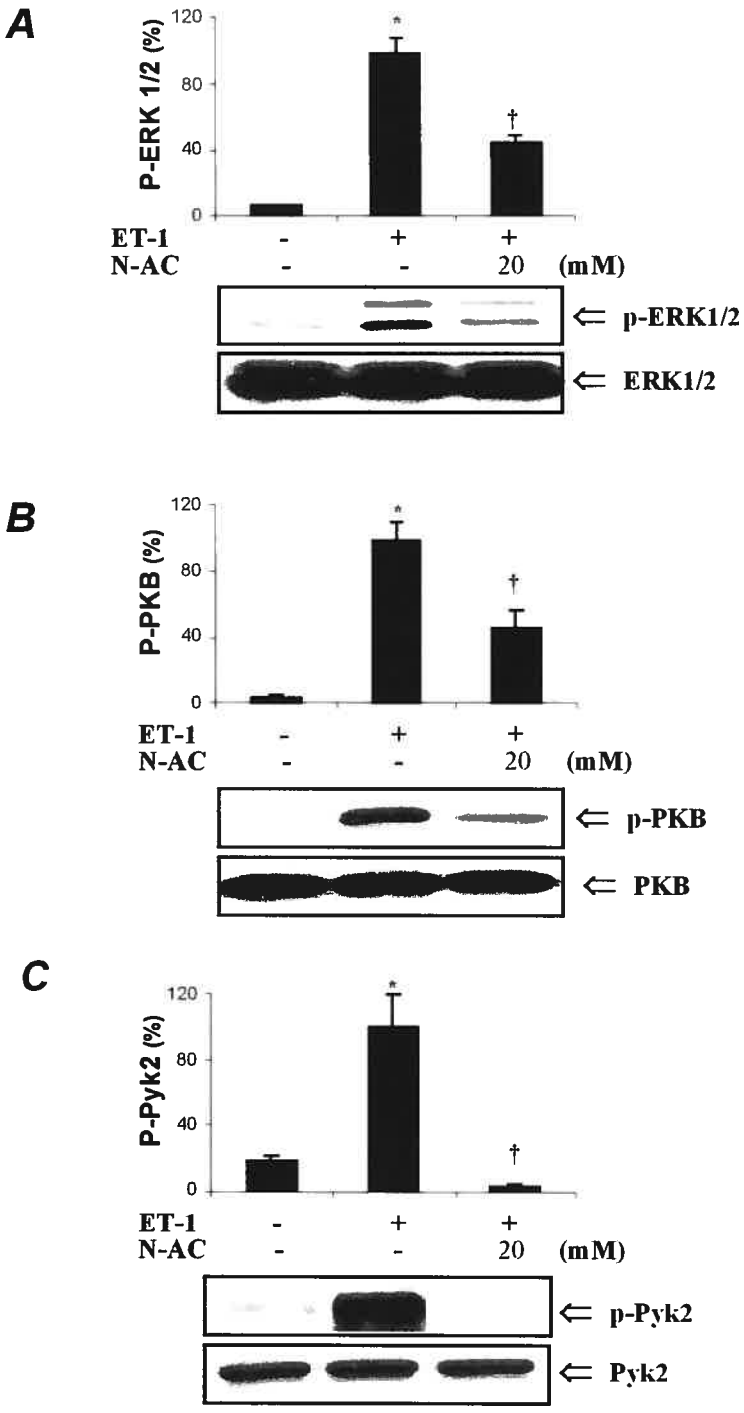
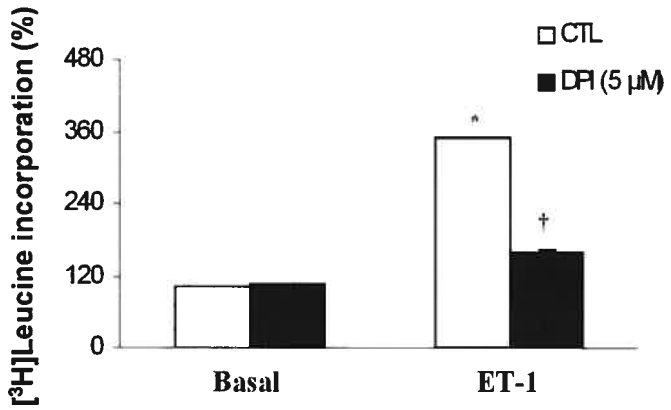

A
P-ERK 1/2 (%)
120
80
40
0
*
†
ET-1 - + +
N-AC - - 20 (mM)
⇐ p-ERK1/2
⇐ ERK1/2
B
P-PKB (%)
120
80
40
0
*
†
ET-1 - + +
N-AC - - 20 (mM)
⇐ p-PKB
⇐ PKB
C
P-Pyk2 (%)
120
80
40
0
*
†
ET-1 - + +
N-AC - - 20 (mM)
⇐ p-Pyk2
⇐ Pyk2

FIGURE 6


[³H]Leucine incorporation (%)
480
360
240
120
0
CTL
DPI (5 µM)
*
†
Basal
ET-1

References

1. Yanagisawa M, Kurihara H, Kimura S et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411-415, 1998.

2. Battistini B, Chailler P, D'Orleans-Juste P, Briere N and Sirois P. Growth regulatory properties of endothelins. *Peptides* 14: 385-399, 1993.

3. Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K and Masaki T. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 348: 732-735, 1990.

4. Arai H, Hori S, Aramori I, Okhubo H and Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 348: 730-32, 1990.

5. Kedzierski RM and Yanagisawa M. Endothelin system: the double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol* 41: 851-76, 2001.

6. Clerk A and Sugden P. Regulation of phospholipase C and D in rat ventricular myocytes: stimulation by endothelin-1, bradykinin and phenylephrine. *J Mol Cell Cardiol* 29: 1593-1604, 1997.

7. Pollock DM, Keith TL and Highsmith RF. Endothelin receptors and calcium signaling. *FASEB J* 9: 1196-1204, 1995.

8. Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A, Lazou A, Marshall CJ, Parker PJ and Sugden PH. Endothelin-1 and fibroblasts growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J Biol Chem* 269: 1110-1119, 1994.

9. Yamboliev IA, Hruby A and Gerthoffer WT. Endothelin-1 activates MAP kinases and c-Jun in pulmonary artery smooth muscle. *Pulm Pharmacol Ther* 11: 205-208, 1998.

10. Yoshizumi M, Kim S, Kagami S, Hamaguchi A, Tsuchiya K, Houchi H, Iwao H, Kido H and Tamaki T. Effect of endothelin-1 (1-31) on extracellular signal-regulated kinase and proliferation of human coronary artery smooth muscle cells. *Br J Pharmacol* 125: 1019-1027, 1998.

11. Sorokin A, Kozlowski P, Graves L and Philip A. Protein tyrosine kinase Pyk2 mediates endothelin-induced p38MAPK activation in glomerular mesangial cells. *J Biol Chem* 276: 21521-21528, 2001.

12. Foschi M, Chari S, Dunn MJ and Sorokin A. Biphasic activation of p21ras by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase. *EMBO J* 16: 6439-6451, 1997.

13. Bisotto S and Fixman ED. Src-family tyrosine kinases, phosphoinositide 3-kinase and Gab1 regulate extracellular signal-regulated kinase 1 activation induced by the type A endothelin-1 G-protein-coupled receptor. *Biochem J* 360: 77-85, 2001.

14. Robin P, Boulven I, Desmyter C, Harbon S, Leiber D. ET-1 stimulates ERK signaling pathway through sequential activation of PKC and Src in rat myometrial cells. *Am J Physiol Cell Physiol* 283: C251-60, 2002.

15. Flamant M, Tharaux PL, Placier S, Henrion D, Coffman T, Chatziantoniou C and Dussaule JC. Epidermal growth factor trans-activation mediates the tonic and fibrogenic effects of endothelin in the aortic wall of transgenic mice. *FASEB* J 17: 327-329, 2003.

16. Yamauchi J, Miyamoto Y, Kokubu H, Nishii H, Okamoto M, Sugawara Y, Hirasawa A, Tsujimoto G and Itoh H. Endothelin suppresses cell migration via the JNK signaling pathway in a manner dependent upon Src kinase, Rac1 and Cdc42. *FEBS Lett* 527: 284-288, 2002.

17. Kodama H, Fukuda K, Takahashi T and Sano M. Role of EGF receptor and Pyk2 in endothelin-1-induced ERK activation in rat cardiomyocytes. *J Mol Cell Cardiol* 34: 139-150, 2002.

18. Kodama H, Fukuda K, Takahashi E, Tahara S et al. Selective involvement of p130Cas/Crk/Pyk2/c-Src in ET-1-induced JNK activation. *Hypertension* 41: 1372-9, 2003.

19. Sundaresan M, Yu ZX, Ferrans VJ, Irani K and Finkel T. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. *Science* 270: 296-299, 1995.

20. Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB and Rhee SG. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* 272: 217-221, 1997.

21. Griendling KK, Minieri CA, Ollerenshaw JD and Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74: 1141-1148, 1994.

22. Mahadev K, Wu X, Zilbering A, Zhu L, Lawrence JT and Goldstein BJ. Hydrogen peroxide generated during cellular insulin stimulation is integral

to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes. *J Biol Chem* 276: 48662-48669, 2001.

23. Lo YY, and Cruz TF. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J Biol Chem* 270: 11727-11730, 1995.

24. Lo YY, Wong JM and Cruz TF. Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J Biol Chem* 271: 15703-15707, 1996.

25. Cheng TH, Shih NL, Chen SY, Wang DL and Chen JJ. Reactive oxygen species modulate endothelin-I-induced c-fos gene expression in cardiomyocytes. *Cardiovasc Res* 41: 654-662, 1999.

26. Wedgwood S, Dettman RW and Black SM. ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol* 281: L1058-L1067, 2001.

27. Fei J, Viedt C, Soto U, Elsing C, Jahn L and Kreuzer J. Endothelin-1 and smooth muscle cells: induction of jun amino-terminal kinase through an oxygen radical-sensitive mechanism. *Arterioscler Thromb Vasc Biol* 20: 1244-1249, 2000.

28. Sugden PH. An overview of endothelin signaling in the cardiac myocyte. *J Mol Cell Cardiol* 35: 871-886, 2003.

29. Singal PK, Khaper N, Farahmand F and Bello-Klein A. Oxidative stress in congestive heart failure. *Curr Cardiol Rep* 2: 206-211, 2000.

30. Griendling KK, Sorescu D and Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86: 494-501, 2000.

31. Dhalla NS, Temsah RW and Netticaden T. Role of oxidative stress in cardiovascular disease. *J Hypertens* 18: 655-673, 2000.

32. Li L, Fink GD, Watts SW, Northcott CA, Galligan JJ, Pagano PJ and Chen AF. Endothelin-1 increases vascular superoxide via endothelin(A)-NADPH oxidase pathway in low-renin hypertension. *Circulation* 107: 1053-1058, 2003.

33. Yang Z, Krasnici N and Luscher TF. Endothelin-1 potentiates human smooth muscle cell growth to PDGF: effects of ETA and ETB receptor blockade. *Circulation* 100: 5-8, 1999.

34. Srivastava AK and Pandey SK. Stimulation of mitogen-activated protein kinases ERK-1 and ERK-2 by H_2O_2 in vascular smooth muscle cells. In: Takeda N, Nagano M and Dhalla NS, eds. The hypertrophied heart. 197-206, 2000. Boston, Kluwer Academic Publishers.

35. Pandey SK, Theberge JF, Bernier M and Srivastava AK. Phosphatidylinositol 3-kinase requirement in activation of the ras/C-raf-1/MEK/ERK and p70(s6k) signaling cascade by the insulinomimetic agent vanadyl sulfate. *Biochemistry* 38: 14667-14675, 1999.

36. Hsieh TJ, Zhang SL, Filep JG, Tang SS, Ingelfinger JR and Chan JS. High glucose stimulates angiotensinogen gene expression via reactive oxygen species generation in rat kidney proximal tubular cells. *Endocrinology* 143: 2975-2985, 2002.

37. Frank GD, Eguchi S, Yamakawa T, Tanaka S, Inagami T and Motley ED. Involvement of reactive oxygen species in the activation of tyrosine kinase and extracellular signal-regulated kinase by angiotensin II. *Endocrinology* 141: 3120-3126, 2000.

38. Blanc A, Pandey NR and Srivastava AK. Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H2O2 in vascular smooth muscle

cells: potential involvement in vascular disease. *Int J Mol Med* 11: 229-234, 2003.

39. Kyaw M, Yoshizumi M, Tsuchiya K, Kirima K, Suzaki Y, Abe S, Hasegawa T and Tamaki T. Antioxidants inhibit endothelin-1 (1-31)-induced proliferation of vascular smooth muscle cells via the inhibition of mitogen-activated protein (MAP) kinase and activator protein-1 (AP-1). *Biochem Pharmacol* 64: 1521-1531, 2002.

40. Suzuki E, Nagata D, Kakoki M, Hayakawa H, Goto A, Omata M and Hirata Y. Molecular mechanisms of endothelin-1-induced cell-cycle progression: involvement of extracellular signal-regulated kinase, protein kinase C, and phosphatidylinositol 3-kinase at distinct points. *Circ Res* 84: 611-619, 1999.

41. Rocic P, Govindarajan G, Sabri A and Lucchesi PA. A role for PYK2 in regulation of ERK1/2 MAP kinases and PI 3-kinase by ANG II in vascular smooth muscle. *Am J Physiol Cell Physiol* 280: C90-C99, 2001.

42. Ogata Y, Takahashi M, Ueno S, Takeuchi K, Okada T, Mano H et al. Antiapoptotic effect of endothelin-1 in rat cardiomyocytes in vitro. *Hypertension* 41: 1156-1163, 2003.

43. Cheng CM, Hong HJ, Liu JC, Shih NL et al. Crucial role of extracellular signal-regulated kinase pathway in reactive oxygen species-mediated endothelin-1 gene expression induced by endothelin-1 in rat cardiac fibroblasts. *Mol Pharmacol* 63: 1002-1011, 2003.

44. Viedt C, Soto U, Krieger-Brauer HI, Fei J, Elsing C et al. Differential activation of mitogen-activated protein kinases in smooth muscle cells by angiotensin II: involvement of p22phox and reactive oxygen species. *Arterioscler Thromb Vasc Biol* 20: 940-948, 2000.

45. Touyz RM, Cruzado M, Tabet F, Yao G, Salomon S and Schiffrin EL. Redox-dependent MAP kinase signaling by Ang II in vascular smooth muscle cells: role of receptor tyrosine kinase transactivation. *Can J Physiol Pharmacol 81*: 159-167, 2003.

46. Sugden PH, Fuller SJ, Mynett JR, Hatchett RJ, Bogoyevitch MA and Sugden MC. Stimulation of adult rat ventricular myocyte protein synthesis and phosphoinositide hydrolysis by the endothelins. *Biochim Biophys Acta* 1175: 327-32, 1993.

CHAPTER 3

ARTICLE 2

Nitric oxide attenuates Endothelin-1-induced activation of ERK1/2, PKB and Pyk2 in vascular smooth muscle cells by a cGMP-dependent pathway

Nitric oxide attenuates Endothelin-1-induced activation of ERK1/2, PKB and Pyk2 in vascular smooth muscle cells by a cGMP-dependent pathway

Grace Bou Daou and Ashok K. Srivastava

Centre de recherche, Centre hospitalier de l'Université de Montréal - Hôtel-Dieu, Department of Physiology, Université of Montréal, Montreal, Quebec, Canada

Running title: Role of NO in ET-1-induced activation of ERK ½, PKB, Pyk2 and protein synthesis

Address for correspondence:

Ashok K. Srivastava, Ph.D
Centre de recherche
CHUM, Hôtel-Dieu
3840, rue St. Urbain
Montréal (Québec) H2W 1T7
Tel: 514-890-8000 ext.12917
Fax: 514-412-7152
[redacted]

Abstract

Nitric oxide (NO) is an important free radical that has been shown to contribute to the regulation of several hormone-mediated responses including EGF, PDGF as well as endothelin-1 (ET-1) and exerts an anti-mitogenic and anti-proliferative effect *in vitro*. ET-1 is a vasoactive peptide implicated in the pathogenesis of vascular abnormalities such as hypertension and atherosclerosis. However, the mechanism by which NO antagonizes ET-1 effect remains unknown. Therefore, the aim of this study was to determine if NO generation would modify ET-1-induced signaling pathways involved in cellular growth and proliferation in A-10 VSMC. NO effect has been evaluated by measuring phosphorylation levels of ERK1/2, PKB and Pyk2 by immunoblot. Treatment of A-10 cells with S-nitroso-N-acetylpenicillamine (SNAP), a NO donor, attenuated the ET-1-enhanced phosphorylation of ERK1/2, PKB and Pyk2. Since, NO mediates principally its effect through a cyclic GMP/soluble guanylate cyclase pathway, we investigated the role of 8-Br-cGMP, a non-metabolizable and cell permeable analogue of cGMP, which exhibited a similar effect as SNAP on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. Furthermore, ODQ, an inhibitor of guanylate cyclase activity, reversed the inhibitory effect of NO on ET-1-induced responses. SNAP also appeared to decrease the protein synthesis induced by ET-1. Taken together, these data demonstrate that NO attenuated ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2 and also antagonized the growth-promoting, proliferative as well as hypertrophic effects of ET-1.

Key words: NO, ET-1, MAPKs, ERK1/2, PKB, Pyk2, A-10 cells

Introduction

The endothelium is the major source of endothelin-1 (ET-1) production (1,2). ET-1 is a 21-aminoacid peptide and is considered as a potent vasoconstrictor (3). It also exhibits mitogenic activity in vascular smooth muscle cells (VSMC) (4,5,6), suggesting a possible role for ET-1 in the pathogenesis of many diseases, such as atherosclerosis (7), hypertension (8) and restenosis after angioplasty (9).

ET-1 exerts its effects through heteromeric G-protein-coupled receptor (GPCR) that is linked to multiple signaling pathways which include phospholipases C and D (10), Ca^{2+} (11), mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun-NH_2-terminal kinase (JNK) and p38mapk (12,13,14,15) and phosphatidylinositol 3-kinase (PI-3K) (16). Most of the studies related to ET-1 signaling have been conducted in cardiomyocytes (10,12,17) and in glomerular mesangial cells (15,16). Activation of receptor and non-receptor protein tyrosine kinases (PTKs) in transducing ET-1-induced signaling responses have been demonstrated (15,18,19,20,21). PTKs activated by ET-1 include epidermal growth factor (EGF) (21), c-Src (18,20,22) and a Ca^{2+}-dependent PTK, Pyk2 (15,20). Of particular interest, ET-1 mediates Pyk2 activation which contributes to ERK1/2 (20) and JNK (21) signaling in cardiomyocytes and p38mapk (15) in mesangial cells.

Nitric oxide (NO) is an important free radical that has been suggested to contribute to the regulation of several hormone-mediated responses (22). NO mediates relaxation principally through the stimulation of soluble guanylyl cyclase, leading to enhanced production of intracellular cGMP, which in turn, activates cGMP-dependent protein kinases (23). NO can also influence cellular events by a cGMP-independent mechanism (24,25) and is also able to react with superoxide anion to form the reactive peroxynitrite radical (26), a potent oxidant with the potential to disrupt protein structures by nitrating the protein tyrosine residues (27). In addition to its vasodilating effect, NO has been suggested to antagonize the physiological and pathophysiological effects of several growth factors such as EGF (28), angiotensin II (AII) (29) as well as ET-1 (30). This is probably achieved by inhibiting one or several of the tyrosine kinases implicated in the signaling events induced by these factors. Extensive studies using AII, have shown that NO suppressed the activation of ERK1/2, p38mapk and JNK (31) as well as Pyk2 (29) in cardiac fibroblasts.

However, to our knowledge, a possible contribution of NO on ET-1-induced intracellular transduction events has not been investigated in VSMC. Therefore, in the present studies, we have examined the effect of NO on ET-1-stimulated phosphorylation of ERK1/2, PKB and Pyk2 which are key mediators of growth promoting, proliferative, migratory, survival and death responses. In addition, we have examined whether NO is acting via a cGMP-dependent mechanism in the A-10 VSMCs. Our study demonstrated that NO donor, S-

nitroso-N-acetylpenicillamine (SNAP), attenuated ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation via cGMP dependent pathway. These results demonstrate that NO may contribute to the anti-mitogenic and anti-proliferative effects by antagonizing growth promoting and proliferative signaling events in VSMC.

Materials and Methods

Materials

ET-1 was purchased from Peninsula Laboratories (Belmont, CA, USA), and S-nitroso-N-acetylpenicillamine (SNAP), 8-Bromo-guanosine 3', 5'-cyclic monophosphate (8-Br-cGMP) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), from Calbiochem (San Diego, CA, USA). Monoclonal phospho-specific-Tyr^{204}-ERK1/2 antibody, polyclonal ERK1/2 antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The phospho-specific-Ser^{473}-PKB and total PKB as well as phospho-specific-Tyr^{402}-Pyk2 and total Pyk2 antibodies were procured from New England Biolabs (Beverly, MA, USA). The enhanced chemiluminescence (ECL) detection system kit and L-(4,5-^{3}H) leucine were from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada).

Methods

Cell culture

VSMC derived from embryonic rat thoracic aorta A-10 cells were maintained in culture with DMEM containing 10% fetal bovine serum at 37^{0}C in a humidified atmosphere of 5% CO_2 as described earlier (32). The cells were grown to 80-90% confluence in 60-mm plates and incubated in serum-free DMEM 20 h prior to the treatments.

Cell lysis and Western blotting

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. The cell lysates were centrifuged at 12,000g for 10 min at 4^0C. Protein concentrations were measured by Bradford assay. Equal amounts of protein were subjected to 10% SDS-polyacrylamide gel (SDS-PAGE), transferred to PVDF membranes (Millipore, MA, USA) and incubated with respective primary antibodies (monoclonal phospho-specific-Tyr^{204}-ERK1/2 antibody (1:2,000), polyclonal phospho-specific-Ser^{473}-PKB antibody (1:4,000), phospho-specific-Tyr^{402}-Pyk2 antibody (1:1,000)). The antigen-antibody complex was detected by a horseradish peroxidase-conjugated second antibody (1:4000), and protein bands were visualized by ECL. The intensity of specific bands was quantified by NIH Image software as described previously (33).

Measurement of [^{3}H]leucine Incorporation

A-10 cells were treated for 20 h with endothelin-1 (10 nM; Belmont, CA, USA). Protein synthesis was assessed by the addition of 2 µCi/mL of

[^{3}H]leucine (ICN Biomedicals, Inc., Costa Mesa, CA, USA) for a period of 20 h. To assess the role of nitric oxide (NO), cells were pretreated for 30 min with S-nitroso-N-acetylpenicillamine (SNAP) (300 μM; Calbiochem, San Diego, CA, USA) which spontaneously generates NO. Following the completion of the experimental protocol, A-10 cells were washed twice with cold PBS, and 1 ml of cold 5% trichloroacetic acid was added for 30 min to precipitate protein. The precipitates were subsequently washed twice with cold water and resuspended in 500 μl of 0.4 M NaOH. Aliquots were counted in a scintillation counter.

Statistics

Statistical analysis was performed by one-way, repeated-measures analysis of variance (ANOVA) followed by a Fisher *post hoc* test. All data are reported as means ± SE. The differences between means were considered significant at $P < 0.05$.

Results

SNAP inhibited ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2 in A-10 VSMCs

In order to determine if the anti-mitogenic and anti-proliferative effects of NO are mediated by its ability to attenuate growth-promoting signaling pathway in VSMC, we examined the effect of S-nitroso-N-acetylpenicillamine (SNAP), which spontaneously generates NO, on ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2. As shown in Figure 1, pretreatment of A-10 VSMC with SNAP for 15 min dose-dependently attenuated ET-1-induced phosphorylation of all the 3 protein kinases. Among the 3 kinases, PKB appeared to be most sensitive to the inhibitory effect of SNAP and exhibited almost complete attenuation in ET-1-stimulated phosphorylation at 10 μM (Fig. 1B). In contrast, ET-1-enhanced phosphorylation of ERK1/2 and Pyk2 was inhibited significantly only by 300 μM SNAP.

8-Br-cGMP inhibited ET-1-induced phosphorylation of ERK1/2, PKB in A-10 VSMCs

Since SNAP-induced production of NO would cause the elevation of cGMP, we evaluated the possibility that the effect of SNAP on ET-1-induced responses was mediated by a mechanism involving cGMP. We tested this possibility by pretreating the cells with 8-Br-cGMP, a non-metabolizable and cell permeable analogue of cGMP. As shown in Figure 2, ERK1/2 and PKB

phosphorylation induced by ET-1 was decreased in a dose-dependent manner with a significant reduction observed at 10 μM.

ODQ reversed the inhibitory effect of SNAP on ET-1-induced ERK1/2, PKB and Pyk2 in A-10 VSMCs

Since NO stimulates cGMP production by activating a soluble form of guanylate cyclase, we wished to determine the contribution of this enzyme in SNAP-induced attenuation of ET-1 response. To validate this possibility, we used ODQ, a selective inhibitor of the soluble guanylate cyclase, which prevents the generation of cGMP from GTP. Cells for these experiments, were preincubated with ODQ for 15 min, then with 300 μM SNAP for 15 min and finally stimulated with 10 nM ET-1 for 5 min. As shown in Figure 3, pretreatment with 1 μM ODQ had no significant effect but 10 μM ODQ completely reversed the inhibition induced by SNAP on ET-1-stimulated ERK1/2, PKB and Pyk2 phosphorylation (Fig. 3).

SNAP inhibited ET-1-stimulated [^{3}H]leucine incorporation into proteins

Activation of ERK1/2, PKB and Pyk2 signaling has been implicated in mediating the hypertrophic response of ET-1 (17), therefore, we next examined whether there was correlation between the response of SNAP and ET-1-induced protein synthesis. As shown in Figure 4, ET-1 increased [^{3}H]leucine incorporation by about 150% as compared to control. However, pretreatment with SNAP caused a significant decrease of ET-1 induced [^{3}H]leucine

incorporation. SNAP alone did not significantly affect the basal [^{3}H]leucine uptake.

Discussion

Nitric oxide (NO), a gaseous biological molecule and a major vasodilator released from the endothelial cells, has been identified to regulate cellular functions. In addition to the relaxation of smooth muscle cells, NO can also inhibit atherogenesis, thrombocyte aggregation as well as cell proliferation and migration. NO donors have been found to attenuate EGF (28), PDGF (34) and angiotensin II (AII) (35) stimulated proliferation of VSMC and cardiac fibroblasts. It has been suggested that NO might exert these effects by modifying the growth-promoting signaling events. The support for this notion is provided from studies in which NO was shown to attenuate Ras/ERK1/2 signaling in response to EGF(28) and AII (31) as well as PKB in response to PDGF (34). NO was also shown to attenuate AII-stimulated Pyk2 phosphorylation in cardiac fibroblasts (29). Despite the fact that a potential cross-talk between ET-1 and NO exists, and that NO is believed to counteract the effects of ET-1, not much information on the ability of NO to modify ET-1-induced signaling in VSMC is available. Therefore, in the present studies, we have investigated if NO generation would modify ET-1-induced signaling pathways involved in cell growth and proliferation.

First, a time and dose-dependent phosphorylation of ERK1/2, PKB and Pyk2 by ET-1 were established (data not shown). Our data indicated that addition of the NO donor, SNAP decreased the phosphorylation level of ERK1/2, PKB and Pyk2 involved in ET-1 signaling pathway (Fig. 1). These

results are similar to previous studies in which AII-induced phosphorylation of ERK1/2 and Pyk2 was blocked by SNAP (29,31). However, our work represents the first study demonstrating a role that NO antagonizes ET-1-induced signaling in VSMC. Pyk2 has also been implicated in AII and ET-1-induced MAPK activation in cardiac fibroblasts and VSMC. Thus, it is possible that Pyk2 serves as an upstream mediator of ET-1-signaling in A-10 VSMC.

We have also demonstrated a role of cGMP in mediating the attenuating effect of NO on ET-1 signaling pathway by using 8-Br-cGMP. The results showed that 8-Br-cGMP decreased ERK1/2 and PKB phosphorylation induced by ET-1 (Fig. 2) and it thus mimicked the inhibitory effect of SNAP. We further evaluated an involvement of soluble guanylate cyclase by using a specific inhibitor, ODQ, and demonstrated that it could reverse the inhibitory effect of SNAP on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation (Fig. 3). It has been previously shown that ODQ can block SNAP-induced elevations in cGMP levels in rat aortic VSMC (36), endothelial cells (37) and cardiomyocytes (38). Finally, we also provide evidence showing that ET-1-stimulated protein synthesis, a hallmark of hypertrophic response is also attenuated by NO donor, SNAP (Fig. 4).

Taken together, we demonstrate that NO inhibits the ET-1-stimulated increase of ERK1/2, PKB and Pyk2 activation in A-10 VSMC. Since ERK1/2, PKB and Pyk2 plays a crucial role by mediating VSMC growth and

hypertrophy, it may be suggested that the ability of NO to attenuate these pathways may serve as a potential mechanism by which NO counteracts the biological responses of ET-1.

Figure legends

Figure 1. Dose-dependent effect of the NO donor, SNAP on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with the indicated SNAP concentrations for 15 min followed by 10 nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr^{204}-ERK1/2 antibodies (A), phospho-specific-Ser^{473}-PKB antibodies (B) and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the middle panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (bottom panels of each section). Top panels represent average data quantified by densitometric scanning of immunoblots. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage phosphorylation where phosphorylation observed with ET-1 alone is defined as 100%. (A) $*P< 0.0001$ vs control, $\dagger P< 0.0001$ vs ET-1. (B) $*P< 0.0001$ vs control, $\dagger P< 0.0001$ vs ET-1. (C) $*P< 0.002$ vs control, $\dagger P< 0.0003$ vs ET-1.

Figure 2. Effect of a stable analogue of cGMP, 8-Br-cGMP on ET-1-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with the indicated 8-Br-cGMP concentrations for 15 min followed by 10 nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr^{204}-ERK1/2 antibodies (A) and phospho-specific-Ser^{473}-PKB antibodies (B), as shown in the middle panels of each section. Blots were also analyzed for total ERK1/2 and PKB (bottom

panels of each section). Top panels represent average data quantified by densitometric scanning of immunoblots. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage phosphorylation where phosphorylation observed with ET-1 alone is defined as 100%. (A) *P< 0.003 vs control, †P< 0.005 vs ET-1. (B) *P< 0.0001 vs control, †P< 0.0003 vs ET-1.

Figure 3. Effect of the inhibitor of the soluble guanylate cyclase, ODQ on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated without or with the indicated ODQ concentrations for 15 min before addition of 300 μM SNAP for 15 min followed by 10 nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Tyr^{204}-ERK1/2 antibodies (A), phospho-specific-Ser^{473}-PKB antibodies (B) and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the middle panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (bottom panels of each section). Top panels represent average data quantified by densitometric scanning of immunoblots. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage phosphorylation where phosphorylation observed with ET-1 alone is defined as 100%. (A) *P< 0.0002 vs control, †P< 0.007 vs ET-1, ‡ P< 0.002 vs SNAP + ET-1. (B) *P< 0.0002 vs control, †P< 0.0006 vs ET-1, ‡ P< 0.003 vs SNAP + ET-1. (C) *P< 0.0005 vs control, †P< 0.002 vs ET-1, ‡ P< 0.02 vs SNAP + ET-1.

Figure 4. Effect of SNAP on ET-1-induced [^{3}H]leucine incorporation into proteins. Serum-starved quiescent A-10 cells were pretreated with SNAP (300 μM) for 30 min in absence (control) or in presence of ET-1 (10 nM), then the cells were labeled to equilibrium with [^{3}H]leucine for 20 h as described in Materials and Methods. Values are the means ± SE of 3 independent experiments and are expressed as percentage of change in [^{3}H]leucine incorporated over the basal values. *P< 0.04 vs control, †P< 0.02 vs SNAP and SNAP+ET-1.

FIGURE 1

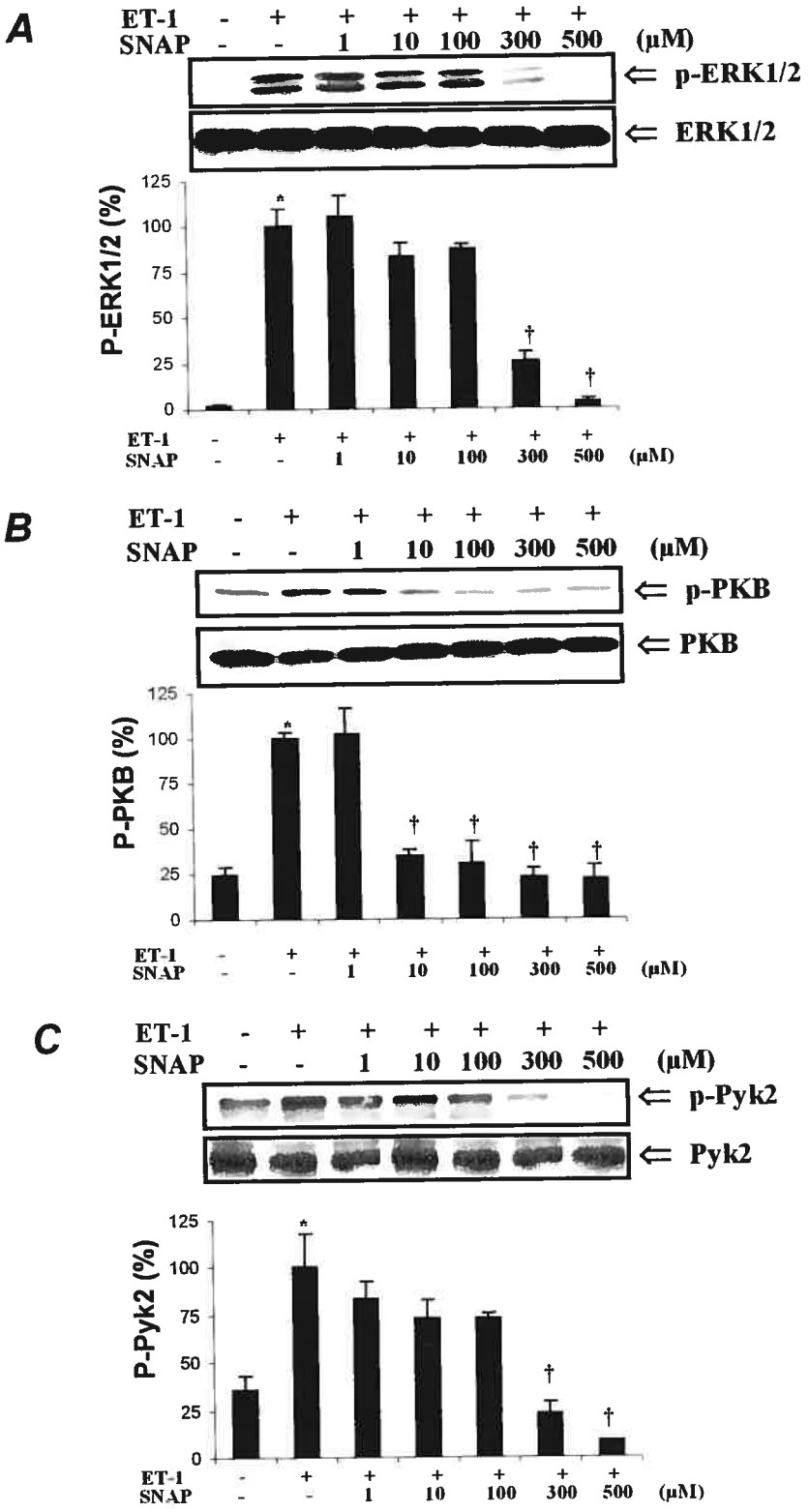

A
ET-1 - + + + + + +
SNAP - - 1 10 100 300 500 (μM)
⇐ p-ERK1/2
⇐ ERK1/2
P-ERK1/2 (%)
125
100
75
50
25
0
B
ET-1 - + + + + + +
SNAP - - 1 10 100 300 500 (μM)
⇐ p-PKB
⇐ PKB
P-PKB (%)
C
ET-1 - + + + + + +
SNAP - - 1 10 100 300 500 (μM)
⇐ p-Pyk2
⇐ Pyk2
P-Pyk2 (%)

FIGURE 2

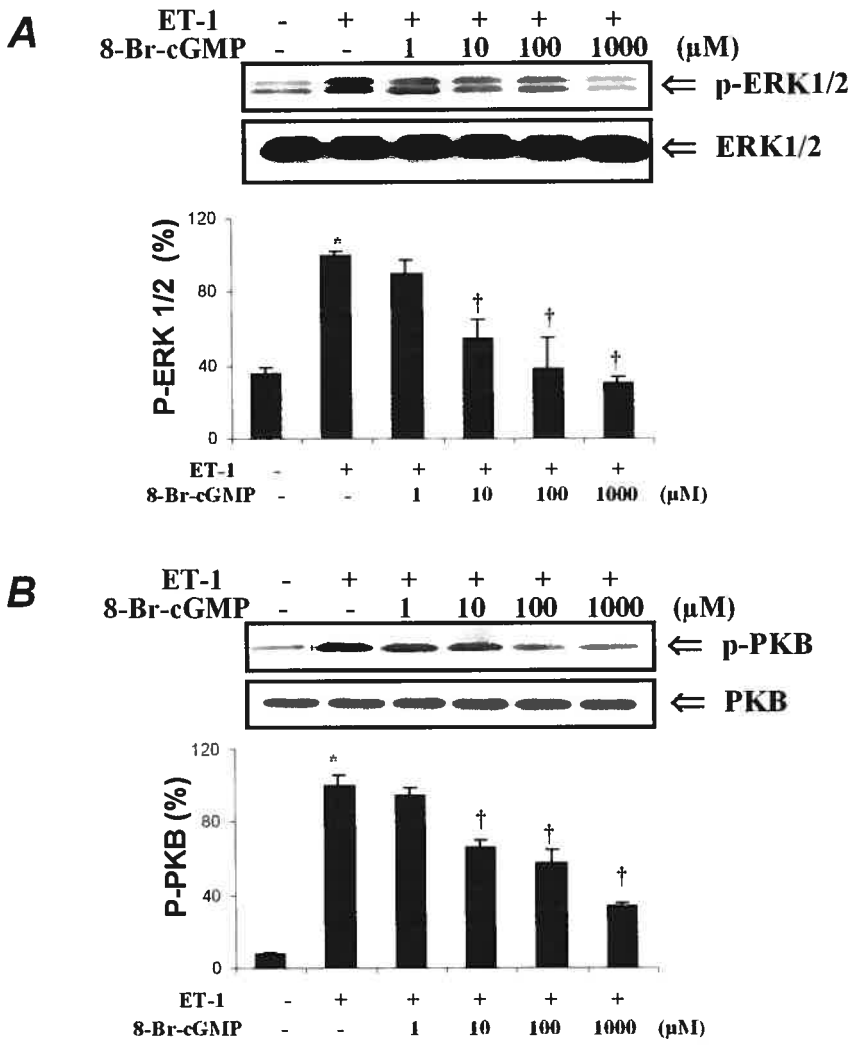

A
ET-1 - + + + + +
8-Br-cGMP - - 1 10 100 1000 (μM)
⇐ p-ERK1/2
⇐ ERK1/2
P-ERK 1/2 (%)
120
80
40
0
*
†
ET-1 - + + + + +
8-Br-cGMP - - 1 10 100 1000 (μM)
B
ET-1 - + + + + +
8-Br-cGMP - - 1 10 100 1000 (μM)
⇐ p-PKB
⇐ PKB
P-PKB (%)
120
80
40
0
*
†
ET-1 - + + + + +
8-Br-cGMP - - 1 10 100 1000 (μM)

FIGURE 3

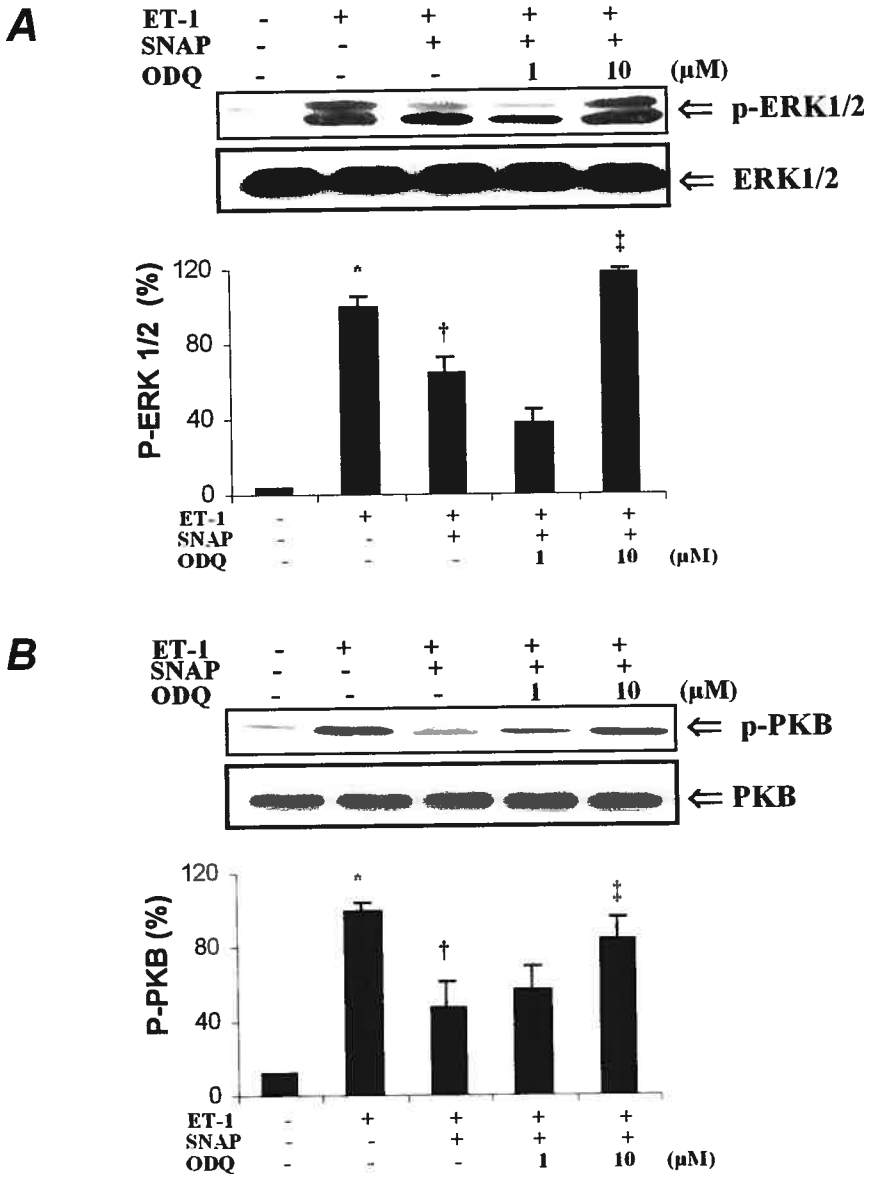

A
ET-1 - + + + +
SNAP - - + + +
ODQ - - - 1 10 (μM)
⇐ p-ERK1/2
⇐ ERK1/2
P-ERK 1/2 (%)
120
80
40
0
*
†
‡
ET-1 - + + + +
SNAP - - + + +
ODQ - - - 1 10 (μM)
B
ET-1 - + + + +
SNAP - - + + +
ODQ - - - 1 10 (μM)
⇐ p-PKB
⇐ PKB
P-PKB (%)
120
80
40
0
*
†
‡
ET-1 - + + + +
SNAP - - + + +
ODQ - - - 1 10 (μM)

FIGURE 3

C

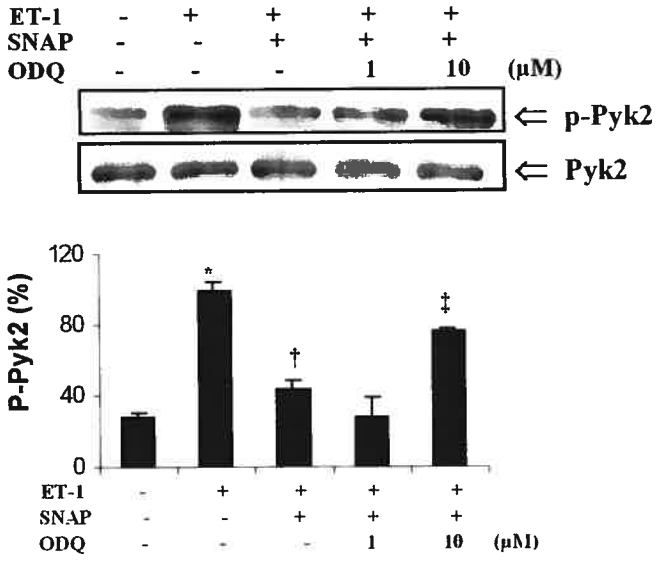
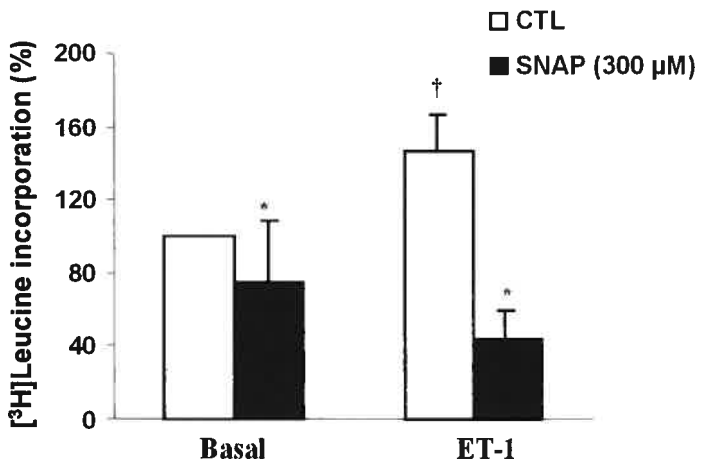

ET-1 - + + + +
SNAP - - + + +
ODQ - - - 1 10 (μM)
⇐ p-Pyk2
⇐ Pyk2
P-Pyk2 (%)
120
80
40
0
*
†
‡
ET-1 - + + + +
SNAP - - + + +
ODQ - - - 1 10 (μM)

FIGURE 4


CTL
SNAP (300 µM)
[³H]Leucine incorporation (%)
200
160
120
80
40
0
†
*
*
Basal
ET-1

References

1. Kedzierski RM and Yanagisawa M. Endothelin system: the double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol* 41: 851-76, 2001.

2. Goraca A. New views on the role of endothelin. *Endocrine regulations* 36: 161-167, 2002.

3. Webb DJ. Endothelin: from molecule to man. *Br J Clin Pharmacol* 44: 9-20, 1997.

4. Battistini B, Chailler P, D'Orleans-Juste P, Brière N and Sirois P. Growth regulatory properties of endothelins. *Peptides* 14: 385-99, 1993.

5. Iwasaki H, Eguchi S, Ueno H, Marumo F and Hirata Y. Endothelin-mediated vascular growth requires p42/p44 mitogen-activated protein kinase and p70 S6 kinase cascades via transactivation of epidermal growth factor receptor. *Endocrinology* 140: 4659-68, 1999.

6. Kawanabe Y, Hashimoto N and Masaki T. Extracellular Ca^{2+} influx and endothelin-1-induced intracellular mitogenic cascades in rabbit internal carotid artery vascular smooth muscle cells. *J Cardiovasc Pharmacol* 40: 307-14, 2002.

7. Mathew V, Hasadai D and Lerman A. The role of endothelin in coronary atherosclerosis. *Mayo Clinic Proc* 71: 769-777, 1996.

8. Haynes WG and Webb DJ. Endothelin as a regulator of cardiovascular function in health and disease. *J Hypertens* 16:1081-1098, 1998.

9. Burke SE, Lubbers NL, Gagne GD, Wessale JL, Dayton BD, Wegner CD and Opgenorth TJ. Selective antagonism of the ET(A) receptor reduces neointimal hyperplasia after balloon-induced vascular injury in pigs. *J Cardiovasc Pharmacol* 30: 33-41, 1997.

10. Clerk A and Sugden PH. Regulation of phospholipases C and D in rat ventricular myocytes: stimulation by endothelin-1, bradykinin and phenylephrine. *J Mol Cell Cardiol* 29:1593-1604, 1997.

11. Pollock DM, Keith and highsmith RF. Endothelin receptors and calcium signaling. *FASEB J* 9: 1196-1204, 1995.

12. Bogoyevitch MA, Glennon PE, Anderson MB, Clerk A, Lazou A, Marshall J, Parker PJ and Sugden PH. Endothelin-1 and fibroblast growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes: the potential role of the cascade on the integration of two

signaling pathways leading to myocyte hypertrophy. *J Biol Chem* 269: 110-119, 1994.

13. Yamboliev IA, Hruby A and Gerthoffer WT. Endothelin-1 activates MAP kinases and c-Jun in pulmonary artery smooth muscle. *Pulm Pharmacol Ther* 11: 205-208, 1998.

14. Yoshizumi M, Kim S, Kagami S, Hamaguchi A, Tsuchiya K, Houchi H, Iwao H, Kido H and Tamaki T. Effect of endothelin-1 (1-31) on extracellular signal-regulated kinase and proliferation of human coronary artery smooth muscle cells. *Br J Pharmacol* 125: 1019-1027, 1998.

15. Sorokin A, Kozlowski P, Graves L and Philip A. Protein-tyrosine kinase Pyk2 mediates endothelin-induced p38 MAPK activation in glomerular mesangial cells. *J Biol Chem* 276: 21521-8, 2001.

16. Foschi M, Chari S, Dunn MJ and Sorokin A. Biphasic activation of p21ras by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase. *EMBO J* 16: 6439-6451, 1997.

17. Sugden PH. An overview of endothelin signaling in the cardiac myocyte. *J Mol Cell Cardiol* 35: 871-86, 2003.

18. Flamant M, Tharaux PL, Placier S, Henrion D, Coffman T, Chatziantoniou C and Dussaule JC. Epidermal growth factor receptor trans-activation mediates the tonic and fibrogenic effects of endothelin in the aortic wall of transgenic mice. *FASEB J* 17: 327-329, 2003.

19. Yamauchi J, Miyamoto Y, Kokubu H, Nishii H, Okamoto M, Sugawara Y, Hirasawa A, Tsujimoto G and Itoh H. Endothelin suppresses cell migration via the JNK signaling pathway in a manner dependent upon Src kinase, Rac1, and Cdc42. *FEBS Lett* 527: 284-288, 2002.

20. Kodama H, Fukuda K, Takahashi T, Sano M, Kato T, Tahara S, Hakuno D, Sato T, Manabe T, Konishi F and Ogawa S. Role of EGF receptor and Pyk2 in endothelin-1-induecd ERK activation in rat cardiomyocytes. *J Mol Cell Cardiol* 34: 139-150, 2002.

21. Kodama H, Fukuda K, Takahashi T, Tahara S, Tomita Y, Ieda M, Kimura K, Owada K.M, Vuori K and Ogawa S. Selective involvement of p130Cas/Crk/Pyk2/c-Src in endothelin-1-induced JNK activation. *Hypertension* 41:1372-1379, 2003.

22. Schmidt HHHW and Walter U : NO at work. *Cell* 78: 919-925, 1994.

23. Mayer B, Pfeiffer S, Schrammel A, Koesling D, Schmidt K and Brunner F. A new pathway of nitric oxide/cyclic GMP signaling involving S-nitrosoglutathione. *J Biol Chem* 273: 3264-3270, 1998.

24. Cornwell TL, Arnold E, Boerth NJ and Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol Cell Physiol* 267: C1405-C1413, 1994.

25. Ishida A, Sasaguri T, Kosaka C, Nojima H and Ogata J. Induction of the cyclic-dependent kinase inhibitor p21 (sdi1/Cip1/Waf1) by nitric oxide-generating vasodilator in vascular smooth muscle cells. *J Biol Chem* 272: 10050-10057, 1997.

26. Kelm M, Dahmann R, Wink D and Feelisch M. The nitric oxide/superoxide assay. Insights into the biological chemistry of the NO/O-2. Interaction. *J Biol Chem* 272: 9922-9932, 1997.

27. Wink DA, Look JA, Kim SY, Vodovotz Y, Pacelli R, Krishna MC, Russo A, Mitchell JB, Jourd'heuil D, Miles AM and Grisham MB. Superoxide modulates the oxidation and nitrosation of thiols by nitric oxide-derived reactive intermediates. Chemical aspects involved in the balance between oxidative and nitrosative stress. *J Biol Chem* 272: 11147-11151, 1997.

28. Yu SM, Hung LM and Lin CC. cGMP-elevating agents suppress proliferation of vascular smooth muscle cells by inhibiting the activation of epidermal growth factor signaling pathway. *Circulation* 95: 1269-77, 1997.

29. Wang D, Yu X and Brecher P. Nitric oxide inhibits AII-induced activation of the calcium-sensitive tyrosine kinase proline-rich tyrosine kinase 2 without affecting epidermal growth factor receptor transactivation. *J Biol Chem* 274: 24342-24348, 1999.

30. Alonso D and Radomski MW. The nitric oxide-endothelin-1 connection. *Heart failure reviews* 8: 107-115, 2003.

31. Wang D, Yu X and Brecher P. Nitric oxide and N-acetylcysteine inhibit the activation of mitogen-activated protein kinases by angiotensin II in rat cardiac fibroblasts. *J Biol Chem* 273: 33027-33034, 1998.

32. Srivastava AK and Pandey SK. Stimulation of mitogen-activated protein kinases ERK-1 and ERK-2 by H_2O_2 in vascular smooth muscle cells. In: Takeda N, Nagano M and Dhalla NS, eds. The hypertrophied heart. 197-206, 2000. Boston, Kluwer Academic Publishers.

33. Pandey SK, Theberge JF, Bernier M and Srivastava AK. Phosphatidylinositol 3-kinase requirement in activation of the ras/C-raf-1/MEK/ERK and p70(s6k) signaling cascade by the insulinomimetic agent vanadyl sulfate. *Biochemistry* 38: 14667-14675, 1999.

34. Sandirasegarane L, Charles R, Bourbon N and Kester M. NO regulates PDGF-induced activation of PKB but not ERK in A7r5 cells: implications for vascular growth arrest. *Am J Physiol Cell Physiol* 279: C225-C235, 2000.

35. Takizawa T, Gu M, Chobanian AV and Brecher P. Effect of nitric oxide on DNA replication induced by angiotensin II in rat cardiac fibroblasts. *Hypertension* 30: 1035-40, 1997.

36. Lahteenmaki T, Sievi E and Vapaatalo H. Inhibitory effects of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives on vascular smooth muscle cell mitogenesis and proliferation in vitro. *Br J Pharmacol* 125: 402-408, 1998.

37. Heller R, Polack T, Grabner R and Till U. Nitric oxide inhibits proliferation of human endothelial cells via a mechanism independent of cGMP. *Atherosclerosis* 144: 49-57, 1999.

38. Sandirasegarane L and Diamond J. The nitric oxide donors, SNAP and DEA/NO, exert a negative inotropic effect in rat cardiomyocytes which is independent of cyclic GMP elevation. *J Mol Cell Cardiol* 31: 799-808, 1999.

CHAPTER 4

GENERAL DISCUSSION

Endothelin-1 (ET-1) is the predominant isoform of the endothelin peptide family and is synthetized mainly in the vascular endothelium and smooth muscle cells. Binding of ET-1 to ET-A receptors on vascular smooth muscle cells (VSMC) causes vasoconstriction and stimulates cell growth and proliferation. ET-1 has been implicated in the pathogenesis of arterial hypertension, renal disorders and cardiovascular diseases. Based on recent experimental data, treatment with newly available ET-1 antagonists is likely to inhibit ET-1-induced funtional and structural alterations in the vasculature. However, a better knowledge of ET-1 signaling transduction pathways would be important for devising specific therapeutic agents directed against critical components of signaling systems implicated in vascular remodeling.

ET-1 elicits its effects through the stimulation of G-protein-coupled receptors (GPCR) which lead to the recruitment of two distinct signaling pathways : protein kinases of the Raf family and lipid kinases of the phosphatidylinositol 3-kinase (PI-3K) family. Both pathways are shown to be recruited by Ras, a small G-protein, which is activated by ET-1 (7,41,59). Raf is the first member of the mitogen-activated protein kinase cascades (MAPK). Several reports have demonstrated that ET-1 activates MAPK including extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun-NH_2-terminal kinase (JNK) and p38mapk (56,163,172,173) as well as PI-3K (59) in many cell types. Several targets of PI-3K have been identified, however, most widely studied target is protein kinase B (PKB). Recently, ET-1 has been shown to slightly

increase PKB phosphorylation in cardiomyocytes (80). Moreover, various receptor and non-receptor protein tyrosine kinases (PTKs) have been implicated in transducing ET-1-evoked signaling responses including c-Src, epidermal growth factor (EGF) receptor and a Ca^{2+}-dependent PTK, Pyk2. Extensive studies have been carried out predominantly in cardiomyocytes (7,172,174) and glomerular mesangial cells (56,59) and have suggested a role for Pyk2 in ET-1-induced activation of JNK (47) and p38mapk (56) signaling pathways. However, the involvement of Pyk2 in ERK1/2 signaling pathway appeared to be less predominant than for EGF receptor transactivation (60). We therefore investigated the effect of ET-1 on ERK1/2, PKB and Pyk2 signaling which are believed to be key players in mediating growth-promoting, proliferative, migratory, survival and death responses in the cell. In the present study, ET-1 showed an increase in ERK1/2 phosphorylation in A-10 VSMCs which is consistent with other studies done in VSMC (46,48). However, these studies are the first to report an activation of PKB and Pyk2 by ET-1 in VSMCs.

A-10 VSMCs were grown until they become confluent, then were starved in a medium without serum prior to the treatments. Serum-deprivation makes the cell quiescent and brings them to the stationary phase. This serum-deprivation doesn't change A-10 VSMCs morphology and they remain healthy without any sign of apoptosis. Moreover, the quantification of proteins following each experiment indicated the survival of the cells. Serum contains many growth

factors which activates several signaling molecules, its presence would have distorted the results by raising the basal phosphorylation levels of proteins.

Several studies have indicated that reactive oxygen species (ROS) play an essential role in propagating the signals of many growth factors, peptide hormones and cytokines such as EGF (175), angiotensin II (AII) (176) and tumor necrosis factor-α (TNF-α) (177). Increased ROS generation have been associated with a variety of cardiovascular pathologies including hypertension and atherosclerosis. ET-1 has been shown to increase ROS production in various cell types (30,125,128). Also, NADPH oxidase has been suggested to play a role in the increase of vascular ROS production by ET-1 in endothelial cells (118), in pulmonary smooth muscle cells (119) and in DOCA-salt rats (120). In VSMC and endothelial cells, NADH/NADPH oxidases represent the most important source of superoxide anions (112). ROS also function as intracellular messengers to modulate the signaling pathway and thus, regulate the transcriptional activity in the cell (125).

Conflicting results have been obtained on the role of ROS in mediating ET-1-induced signaling. Fei et al. (128) demonstrated that JNK activation but not ERK1/2 activation by ET-1 was significantly inhibited by antioxidants in rat smooth muscle cells. Similarly, Kyaw et al. (30) showed that ET-1 induces JNK and p38mapk activation but not ERK1/2 through ROS generation in VSMC. In contrast, a recent study demonstrated that ROS plays a role in ET-1-induced

activation of ERK1/2 pathway as well as JNK and p38mapk in cardiac fibroblasts (127). Thus, the contribution of ROS in ET-1-induced ERK1/2 activation is controversial and remains to be clarified in VSMC. In addition, a role of ROS to regulate PKB and Pyk2 signaling by ET-1 in VSMC has not been investigated.

Our results provided the first evidence that ROS mediates ERK1/2, PKB and Pyk2 signaling induced by ET-1 via NADPH oxidase activation. In this first part of the study, we examined the effect of DPI, a potent inhibitor of flavonoid-containing enzymes, such as NADPH oxidase. Experiments with the chemiluminescence reagent, lucigenin showed that stimulation with ET-1 induced generation of ROS in A-10 VSMC, while treatment with DPI caused a decrease of ROS generation in these cells (**Fig. 4, article 1**). Furthermore, DPI also inhibited ET-1 stimulation of ERK1/2, PKB and Pyk2 in A-10 VSMC, which suggests the involvement of NADPH oxidase for ROS formation (**Fig. 3, article 1**). To further confirm the role of ROS in A-10 VSMCs, N-acetylcysteine (NAC), a superoxide scavenger, was also used in these experiments and showed a decrease in ERK1/2, PKB and Pyk2 phosphorylation induced by ET-1 (**Fig. 5, article 1**). In the present study, we found that NAC and DPI inhibited the activation of ERK1/2 induced by ET-1 which is consisting with previous data on cardiac fibroblasts (127). Furthermore, we also showed that DPI could inhibit ET-1-induced protein synthesis of VSMCs. Increased protein synthesis and accumulation of proteins are the essential component of the hypertrophic

response observed with ET-1. Overall, our data reveal that ROS generation via flavonoid oxidases mediates ET-1-induced key components of the signaling pathway and it suggests that antioxidants can serve as attenuator of ET-1 action.

Nitric oxide (NO) is another important free radical that has been shown to contribute to the regulation of several hormone-mediated responses. NO has been found to attenuate EGF (148), PDGF (154) and angiotensin II (AII) (178) stimulated proliferation of VSMC and cardiac fibroblasts. It has been suggested that NO might exert these effects by modifying the growth-promoting signaling events. The support for this notion is provided from studies in which NO was shown to attenuate ras/ERK1/2 signaling in response to EGF(148) and AII (168) as well as PKB in response to PDGF (154). NO was also shown to attenuate AII-stimulated Pyk2 phosphorylation in cardiac fibroblasts (155). Despite the fact that a potential cross-talk between ET-1 and NO exists, and NO is believed to counteract the effects of ET-1, not much information on the ability of NO to modify ET-1-induced signaling in VSMC is available. Therefore, in the present studies, we have investigated if NO generation would modify ET-1-induced signaling pathways involved in cell growth and proliferation.

Our data indicated that addition of the NO donor, SNAP decreased the phosphorylation level of ERK1/2, PKB and Pyk2 involved in ET-1 signaling pathway. These results are similar to previous studies in which AII-induced phosphorylation of ERK1/2 and Pyk2 was blocked by SNAP. However, our

work represents the first study demonstrating that NO antagonizes ET-1-induced signaling in VSMC. Pyk2 has also been implicated in AII and ET-1-induced MAPK activation in cardiac fibroblasts and VSMC. Thus, it is possible that Pyk2 serves as an upstream mediator of MAPK cascade induced by ET-1 in A-10 VSMC.

We have also demonstrated a role of cGMP in mediating the attenuating effect of NO on ET-1 signaling pathway by using 8-Br-cGMP. The results showed that 8-Br-cGMP decreased ERK1/2 and PKB phosphorylation induced by ET-1 (**Fig. 2, article 2**) and it thus mimicked the inhibitory effect of SNAP. We further evaluated an involvement of soluble guanylate cyclase by using a specific inhibitor, ODQ, and demonstrated that it could reverse the inhibitory effect of SNAP on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. It has been previously shown that ODQ can block SNAP-induced elevation in cGMP levels in rat aortic VSMC (166), endothelial cells (179) and cardiomyocytes (180). Finally, we also provide evidence showing that ET-1-stimulated protein synthesis, a hallmark of hypertrophic response, is also attenuated by NO donor, SNAP.

Taken together, we demonstrate that NO inhibits the ET-1-stimulated increase in the phosphorylation state of ERK1/2, PKB and Pyk2 in A-10 VSMC. Since ERK1/2, PKB and Pyk2 plays a crucial role by mediating VSMC growth and hypertrophy, it may be suggested that the ability of NO to attenuate these

pathways may serve as a potential mechanism by which NO counteracts the biological responses of ET-1.

CHAPTER 5

CONCLUSION

The results presented here demonstrate for the first time the involvement of ROS in mediating ET-1 signaling components: ERK1/2, PKB and Pyk2 in VSMC, which were inhibited by antioxidants such as NAC and DPI. A role for NADPH oxidase has been suggested in ET-1-enhanced ROS production. Furthermore, ROS also contribute to increase protein synthesis induced by ET-1 in these cells. Given the fact that NO has opposite effects to ET-1, studies were expanded and demontrated a novel role for NO in regulating ERK1/2, PKB and Pyk2 activation induced by ET-1 in a cGMP-dependent mechanism. We reported that NO can also decrease ET-1-induced responses at the level of protein synthesis.

Thus, the findings of the present study may augment the significant role of antioxidants in ROS-mediated ET-1-induced signal transduction pathway and suggest a clinical application of antioxidants in the pathogenesis of vascular diseases. Moreover, the inhibitory effect of NO on the specific protein kinases as well as protein synthesis may offer a partial explanation for the antagonistic effects of NO on ET-1 action in VSMC, which contribute to the anti-mitogenic and anti-proliferative effects (**Fig. 5.10**). NO could be of benefit in regulating vascular remodeling of various diseases. However, NO is able to interfere with diverse signaling pathways in various cell types and it is suggested that a single site of action probably is not enough to explain all of the effects of this substance.

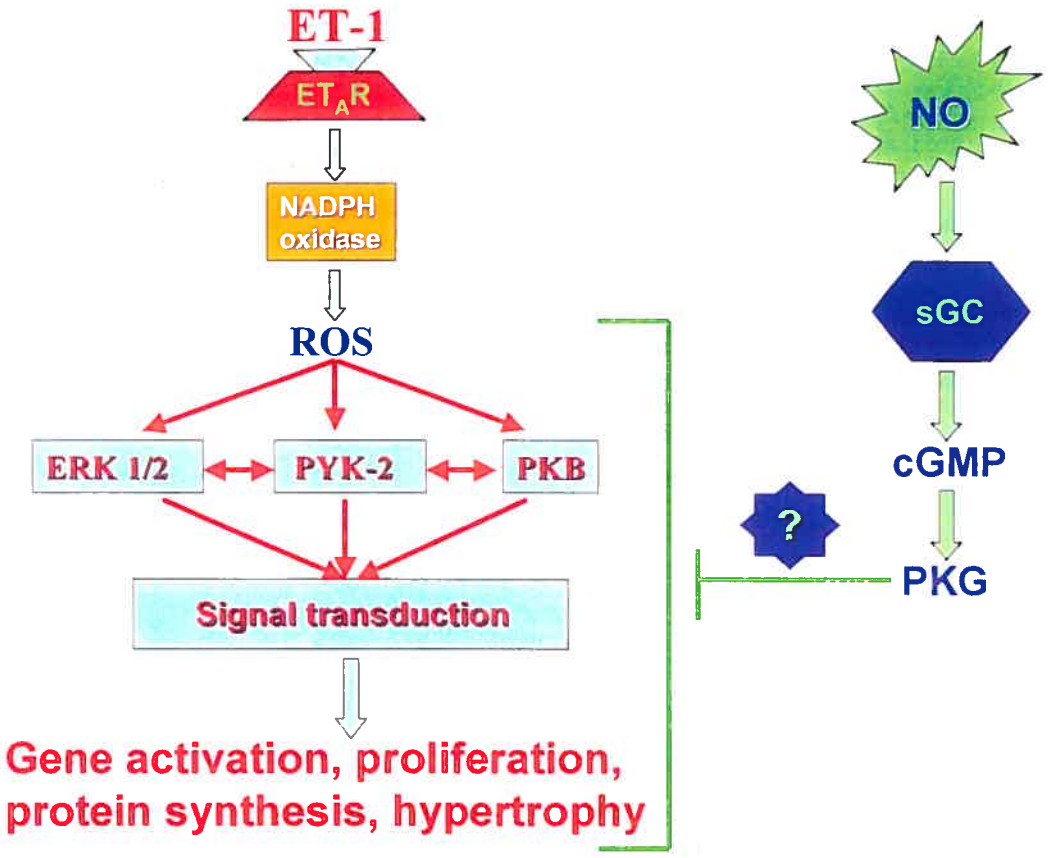


Figure 5.10 : Hypothetical model showing nitric oxide interaction with ET-1 signaling pathway.

CHAPTER 6

REFERENCES

1. Yanagisawa M, Kurihara H, Kimura S et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411-415, 1998.
2. Bobik A, Grooms A, Millar JA, Mitchell A and Grinpukel S. Growth factor activity of endothelin on vascular smooth muscle. *Am J Physiol* 258: C408-15, 1990.
3. Rabelink TJ, Kaasjager KA, Boer P, Stroes EG, Braam B and Koomans HA. Effects of endothelin-1 on renal function in humans: implications for physiology and pathophysiology. *Kidney Int* 46: 376-81, 1994.
4. Schiffrin EL. Endothelin: potential role in hypertension and vascular hypertrophy. *Hypertension* 25: 1135-43, 1995.
5. Haynes WG and Webb DJ. Endothelin as a regulator of cardiovascular function in health and disease. *J Hypertens* 16: 1081-1098, 1998.
6. Kedzierski RM and Yanagisawa M. Endothelin system: the double-edged sword in health and disease. *Annu Rev Pharmacol Toxicol* 41: 851-76, 2001.
7. Sugden PH. An overview of endothelin signaling in the cardiac myocyte. *J Mol Cell Cardiol* 35: 871-886, 2003.
8. Goraca A. New views on the role of endothelin. *Endocrine regulations* 36: 161-167, 2002.
9. Simonson MS and Dunn MJ. Endothelin: pathways of transmembrane signaling. *Hypertension* 15: I-5-12, 1990.
10. Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T et al. Cellular mechanisms of endothelin-1 release by angiotensin and vasopressin. *Hypertension* 18: 165-170, 1991.

11. Gray GA. Generation of endothelin. In: Gray GA, Webb DJ (editors): Molecular biology and pharmacology of the endothelins. Austin: RG Landes. pp. 13-32, 1995.

12. Webb DJ, Monge JC, Rabelink TJ and Yanagisawa M. Endothelin: new discoveries and rapid progress in the clinic. *Trends Pharmacol Sci* 19 : 5-8, 1998.

13. Xu D, Emoto N, Giaid A, Slaughter C, Kaw S, deWit D et al. ECE-1 : a membrane bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. *Cell* 78: 473-485, 1994.

14. Alonso D and Radomski MW. The nitric oxide-endothelin-1 connection. *Heart Failure Rev* 8: 107-115, 2003.

15. Rubanyi GM and Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology and pathology. *Pharmacol Rev* 46: 325-415, 1994.

16. Gulati A, Kumar A and Shahani BT. Cardiovascular effects of centrally administred endothelin-1 and its relationship to changes in cerebral blood flow. *Life Sci* 58: 437-45, 1996.

17. Evans RG, Bergstrom G, Cotterill E and Anderson WP. Renal haemodynamic effects of endothelin-1 and the ETA/ETB antagonist TAK-044 in anaesthetized rabbits. *J Hypertens* 16: 1897-905, 1998.

18. Adamicza A, Petak F, Asztalos T and Hantos Z. Effects of endothelin-1 on airway and parenchymal mechanics in guinea pigs. *Eur J Respir* 13: 767-74, 1999.

19. Hocher B, Schwarz A, Fagan KA, Thone-Reineke C, El-Hag K et al. Pulmonary fibrosis and chronic lung inflammation in endothelin-1 transgenic mice. *Am J Respir Cell Mol Biol* 23: 19-26, 2000.

20. Kuwaki T, Kurihara H, Cao WH, Kurihara Y, Unekawa M et al. Physiological role of brain endothelin in the central autonomic control: from neuron to knock out mouse. *Prog Neurobiol* 51: 549-79, 1997.

21. MacCarthy PA, Grocott-Mason R, Prendergast BD and Shah AM. Contrasting inotropic effects of endogenous endothelin in the normal and failing human heart: studies with an intracoronary ET(A) receptor antagonist. *Circulation* 101: 142-147, 2000.

22. Endoh M, Fujita S, Yang HT, Talukder MA, Maruya J and Norota I. Endothelin: receptor subtypes, signal transduction, regulation of Ca^{2+} transients, and contractility in rabbit ventricular myocardium. *Life Sci* 62: 1485-9, 1998.

23. Sugden PH. Signalling pathways in cardiac myocyte hypertrophy. *Ann Med* 33: 611-22, 2001.

24. Miyauchi T and Masaki T. Pathophysiology of endothelin in the cardiovascular system. *Annu Rev Physiol* 61: 391-415, 1999.

25. Fujitani Y, Ninomiya H, Okada T, Urada Y and Masaki T. Suppression of endothelin-1-induced mitogenic responses of human aortic smooth muscle cells by interleukin-1 beta. *J Clin Invest* 95: 2472-82, 1995.

26. MacNulty EE, Plevin R and Wakelam MJ. Stimulation of the hydrolysis of phosphatidylinositol-4,5-bisphosphate and phosphatidylcholine by

endothelin, a complete mitogen for Rat-1 fibroblasts. *Biochem J* 272: 761-66, 1990.

27. Ruetten H and Thiemermann C. Endothelin-1 stimulates the biosynthesis of tumor necrosis factor in macrophages: ET receptors, signal transduction and inhibition by dexamethasone. *J Physiol Pharmacol* 48 : 675-88, 1997.
28. Cheng CM, Hong HJ, Liu JC and Shih NL. Crucial of extracellular signal-regulated kinase pathway in reactive oxygen species-mediated endothelin-1 gene expression induced by endothelin-1 in rat cardiac fibroblasts. *Mol Pharmacol* 63: 1002-11, 2003.
29. Wu-Wong JR, Chiou WJ and Wang J. Extracellular signal-regulated kinases are involved in the antiapoptotic effect of endothelin-1. *J Pharmacol Exp Ther* 293: 514-521, 2000.
30. Kyaw M, Yoshizumi M, Tsuchiya K et al. Antioxidants inhibit endothelin-1 (1-31)-induced proliferation of vascular smooth muscle cells via the inhibition of mitogen-activated protein (MAP) kinase and activator protein-1 (AP-1). *Biochem Pharmacol* 64: 1521-31, 2002.
31. Sorokin A, Foschi M and Dunn MJ. Endothelin signalling and regulation of protein kinases in glomerular mesangial cells. *Clin Sci* 103: 132S-136S, 2002.
32. Arai H, Hori S, Aramori I, Okhubo H and Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. *Nature* 348: 730-32, 1990.

33. Pierce KL, Premont RT and Lefkowitz RJ. Seven-transmembrane receptors. *Nat Rev Mol Cell Biol* 3: 639-650, 2002.

34. Ruffolo R. Endothelin receptors from the gene to the human. Boca Raton, FL: CRC Press, 1995.

35. Gellai M, Fletcher T, Pullen M and Nambi P. Evidence for the existence of endothelin-B receptor subtypes and their physiological roles in the rat. *Am J Physiol* 27: R254-R261, 1996.

36. Gutkind JS. Cell growth control by G-protein-coupled receptors: from signal transduction to signal integration. *Oncogene* 17: 1331-1342, 1998.

37. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* 70: 281-312, 2001.

38. Fukami K. Structure, regulation, and function of phospholipase C enzymes. *J Biochem* 131: 293-9, 2002.

39. Newton AC. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem Rev* 101: 2353-64, 2001.

40. Robin P, Boulven I, Desmyter C, Harbon S and Leiber D. ET-1 stimulates ERK signaling pathway through sequential activation of PKC and Src in rat myometrial cells. *Am J Physiol Cell Physiol* 283: C251-60, 2002.

41. Takai Y, Sasaki T and Matozaki T. Small GTP-binding proteins. *Physiol Rev* 81: 153-208, 2001.

42. Seger R and Krebs EG. The MAPK signaling cascade. *FASEB J* 9: 726-735, 1995.

43. Su B and Karin M. Mitogen-activated protein kinase cascades and regulation of gene expression. *Curr Opin Immunol* 3: 402-11, 1996.

44. Frodin M and Gammeltoft S. Role and regulation of 90 kDa ribosomal S6 kinase (RSK) in signal transduction. *Mol Cell Endocrin* 151: 65-77, 1999.

45. Yue TL, Gu JL, Wang C, Reith AD et al. Extracellular signal-regulated kinase plays an essential role in hypertrophic agonists, endothelin-1 and phenylephrine-induced cardiomyocyte hypertrophy. *J Biol Chem* 275: 37895-901, 2000.

46. Brehm BR, Klaussner M and Wolf SC. Chronic elevated endothelin-1 concentrations regulate mitogen-activated protein kinases ERK1/2 in vascular smooth muscle cells. *Clin Sci* 48: 137S-140S, 2002.

47. Kodama H, Fukuda K, Takahashi E et al. Selective involvement of p130Cas/Crk/Pyk2/c-Src in ET-1-induced JNK activation. *Hypertension* 41: 1372-9, 2003.

48. Chevalier D, Thorin E and Allen BG. Simultaneous measurement of ERK, p38 and JNK MAPK cascades in vascular smooth muscle cells. *J Pharmacol Toxicol Methods* 44: 429-39, 2000.

49. Araki S, Haneda M, Togawa M and Kikkawa R. Endothelin-1 activates c-Jun-NH_2 terminal kinase in mesangial cells. *Kidney Int* 51: 631-9, 1997.

50. Clerk A, Pham FH, Fuller SJ, Sahai E and Aktories K. Regulation of mitogen-activated protein kinases in cardiac myocytes through the small G-protein, Rac-1. *Mol Cell Biol* 21: 1173-84, 2001.

51. Yee HF, Melton AC and Tran BN. RhoA/Rho-associated kinase mediates fibroblast. *Biochem Biophys Res Commun* 9: 1340-5, 2001.

52. Lewis TS, Shapiro PS and Ahn NG. Signal transduction through MAP kinase cascades. *Adv Cancer Res* 74: 49-139, 1998.

53. Chiloeches A, Paterson HF, Marais RM, Clerk A, Marshall CJ and Sugden PH. Regulation of Ras.GTP loading and Ras-Raf association in neonatal rat ventricular myocytes by G protein-coupled receptor agonists and phorbol ester. Activation of the extracellular signal-regulated kinase cascade by phorbol ester is mediated by Ras. *J Biol Chem* 274: 19762-70, 1999.

54. Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM, Plowman GD, Rudy B and Schlessinger J. Protein tyrosine kinase Pyk2 involved in Ca^{2+}-induced regulation of ion channel and MAP kinase functions. *Nature* 376: 737-745, 1995.

55. Cazaubon S, Chaverot N, Romero IA, Girault JA, Adamson P, Strosberg AD and Couraud PO: Growth factor activity of endothelin-1 in primary astrocytes mediated by adhesion-dependent and –independent pathways. *J Neurosci* 17: 6203-6212, 1997.

56. Sorokin A, Kozlowski P, Graves L and Philip A: Protein-tyrosine kinase Pyk2 mediates endothelin-induced p38MAPK activation in glomerular mesangial cells. *J Biol Chem* 276: 21521-21528, 2001.

57. Sabri A, Govindarajan G, Griffin TM, Byron KL, Samarel AM and Lucchesi PA: Calcium and protein kinase C depedent activation of the tyrosine kinase

Pyk2 by angiotensin II in vascular smooth muscle. *Circ Res* 83: 841-851, 1998.

58. Dikic I, Tokiwa G, Lev S, Courtneidge SA and Schlessinger J: A role for PYK2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature* 383: 547-550, 1996.

59. Foschi M, Chari S, Dunn MJ and Sorokin A: Biphasic activation of p21ras by endothelin-1 sequentially activates the ERK cascade and phosphatidylinositol 3-kinase. *EMBO J* 16: 6439-6451, 1997.

60. Kodama H, Fukuda K, Takahashi T and Sano M. Role of EGF receptor and Pyk2 in endothelin-1-induced ERK activation in rat cardiomyocytes. *J Mol Cell Cardiol* 34: 139-150, 2002.

61. Sakai R, Iwamatsu A, Hirano N, Ogawa S et al. A novel signaling molecule, p130, forms stable complexes in vivo with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner. *EMBO J* 13: 3748-3756, 1994.

62. Vuori K, Hirai H, Aizawa S and Ruoslahti E. Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Mol Cell Biol* 16: 2606-2613, 1996.

63. Eguchi S, Numguchi K, Iwasaki H and Matsumoto T. Calcium-dependent epidermal growth factor receptor transactivation mediates the angiotensin II-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *J Biol Chem* 273: 8890-8896, 1998.

64. Yamaguchi M, Tanaka T, Wakai M, Kitanaka A, Kamano H, Kubota Y, Ohnishi H, Takahara J and Irino S: Antisense Src expression inhibits

tyrosine phosphorylation of Shc and its association with Grb2 and Sos which leads to MAPkinase activation in U937 human leukemia cells. *Leukemia* 11: 497-503, 1997.

65. Rocic P, Govindarajan G, Sabri A and Lucchesi P: A role for PYK2 in regulation of ERK1/2 MAP kinases and PI-3Kinase by Ang II in vascular smooth muscle. *Am J Physiol Cell Physiol* 280: C90-C99, 2001.
66. Fruman DA, Meyers RE and Cantley LC. Phosphoinositide kinases. *Annu Rev Biochem* 67: 481-507, 1998.
67. Kandel ES and Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res* 253: 210-229, 1999.
68. Katso R, Okkenhaug K, Ahmadi K, White S, Timm SJ and Waterfield MD. Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu Rev Cell Dev Biol* 17: 615-75, 2001.
69. Rameh L.E and Cantley L.C. The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem* 274: 8347-8350, 1999.
70. Leevers SJ, Vanhaesebroeck B and Waterfield MD. Signaling through phosphoinositide 3-kinases: the take centre stage. *Curr Opin Cell Biol* 11: 219-225, 1999.
71. Vanhaesebroeck B and Waterfield MD. Signaling by distinct classes of phosphoinositide 3-kinases. *Exp Cell Res* 253: 239-254, 1999.

72. Chan Tang O, Rittenhouse Susan E and Tsichlis Philip N: Akt/PKB and other D_3 Phosphoinositide-regulated kinases. *Annu Rev Biochem* 68: 965-1014, 1999.

73. Cross TG, Scheel-Toellner D, Henriquez NV, Deacon E, Salmon M and Lord JM. Serine/threonine protein kinases and apoptosis. *Exp Cell Res* 256: 34-41, 2000.

74. Coffer PJ, Jin J and Woodgett JR. Protein kinase B (c-Akt): a multifunctional mediator of phosphoinositide 3-kinase. *Biochem J* 335: 1-13, 1998.

75. Datta SR, Brunet A and Greenberg ME. Cellular survival: a play in three Akts. *Genes and Dev* 13: 2905-2927, 1999.

76. Toker A. Protein kinases as mediators of phosphoinositide 3-kinase signaling. *Mol Pharmacol* 57: 652-658, 2000.

77. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke T, Stanbridge E, Frisch S and Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. *Science* 282: 1318-1321, 1998.

78. Cross DA, Aless DR, Cohen P, Andjelkovich M and Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378: 785-789, 1995.

79. Kawanabe Y, Hashimoto N and Masaki T. Involvements of voltage-independent Ca^{2+} channels and phosphoinositide 3-kinase in endothelin-1-induced Pyk2 tyrosine phosphorylation. *Mol Pharmacol* 63: 808-813, 2003.

80. Valks DM, Cook SA, Pham FH, Morrison PR, Clerk A and Sugden PH. Phenylephrine promotes phosphorylation of Bad in cardiac myocytes through the extracellular signal-regulated kinases 1/2 and protein kinase A. *J Mol Cell Cardiol* 34: 749-63, 2002.

81. Pinto-Sietsma SJ and Paul M. A role for endothelin in the pathogenesis of hypertension: fact or fiction? *Kidney Int* 67: S115-S121, 1998.

82. Schiffrin EL. Role of endothelin-1 in hypertension and vascular disease. *Am J Hypertens* 14: 83S-89S, 2001.

83. Schiffrin EL, Deng LY, Sventek P and Day R. Enhanced expression of endothelin-1 gene in resistance arteries in severe human essential hypertension. *J Hypertens* 15: 57-63, 1997.

84. Hirata Y, Takagi Y, Fukuda Y and Harumo F. Endothelin is a potent mitogen for rat vascular smooth muscle cells. *Artherosclerosis* 78: 225-228, 1989.

85. Bobik A, Grooms A, Millar JA, Mitchell A and Grinpukel S. Growth factor activity of endothelin on vascular smooth muscle. *Am J Physiol* 258: C408-C415, 1990.

86. Elijovich F, Laffer CL, Amador E, Gavras H, Bresnahan MR and Schiffrin EL. Regulation of Plasma Endothelin by Salt in Salt-Sensitive Hypertension. *Circulation* 103: 263-268, 2001.

87. Schiffrin EL. Endothelin: Potential role in hypertension and vascular hypertrophy. *Hypertension* 25: 1135-1143, 1995.

88. Luscher TF, Seo BG and Buhler FR. Potential role of endothelin in hypertension. Controversy on endothelin in hypertension. *Hypertension* 21: 752-7, 1993.

89. Badr KF, Murray JJ, Breyer MD, Takahashi K, Inagami T and Harris RC. Mesangial cell, glomerular and renal vascular responses to endothelin in the rat kidney. Elucidation of signal transduction pathways. *J Clin Invest* 83: 336-42, 1989.

90. Suzuki N, Miyauchi T, Tomobe Y, Matsumoto H, Goto K, Masaki T and Fujino M. Plasma concentrations of endothelin-1 in spontaneously hypertensive rats and DOCA-salt hypertensive rats. *Biochem Biophys Res Commun* 30: 941-7, 1990.

91. Larivière R, Thibault G and Schiffrin EL. Increased endothelin-1 content in blood vessels of deoxycorticosterone acetate-salt hypertensive but not in spontaneously hypertensive rats. *Hypertension* 21: 294-300, 1993.

92. Park JB and Schiffrin EL. ET-A antagonist prevents blood pressure elevation and vascular hypertrophy of resistance arteries in aldosterone-infused rats. *Hypertension* 36: 724, 2000.

93. Schiffrin EL, Larivière R, Li JS, Sventek P and Touyz RM. Deoxycorticosterone acetate plus salt induce overexpression of vascular endothelin-1 and severe vascular hypertrophy in spontaneously hypertensive rats. *Hypertension* 25: 769-773, 1995.

94. D'Uscio LV, Barton M, Shaw S, Moreau P and Luscher TF. Structure and function of small arteries in salt-induced hypertension: effects of chronic endothelin-subtype-A-receptor blockade. *Hypertension* 30: 905-911, 1997.

95. Sventek P, Turgeon A, Garcia R and Schiffrin EL. Vascular and cardiac overexpression of endothelin-1 gene in one-kidney, one clip Goldblatt hypertensive rats but only in the late phase of two-kidney one clip Goldblatt hypertension. *J Hypertens* 14: 57-64, 1996.

96. Sharifi AM, He G, Touyz RM and Schiffrin EL. Vascular endothelin-1 expression and effect of an endothelin ETA antagonist on structure and function of small arteries from stroke-prone spontaneously hypertensive rats. *J Cardiovasc Pharmacol* 31: S309-S312, 1998.

97. Day R, Larivière R and Schiffrin EL. In situ hybridization shows increased endothelin-1 mRNA levels in endothelial cells of blood vessels of deoxycorticosterone acetate-salt hypertensive rats. *Am J Hypertens* 8: 294-300, 1995.

98. Larivière R, Sventek P and Schiffrin EL. Expression of endothelin-1 gene in blood vessels of adult spontaneously hypertensive rats. *Life Sci* 56: 1889-1896, 1995.

99. Cargnelli G, Rossi G, Bova S and Pessina AC. In vitro vascular reactivity to endothelin: a comparison between young and old normotensive and hypertensive rats. *Clin Exp Hypertens A* 12: 1437-1451, 1990.

100. Deng LY and Schiffrin EL. Effects of endothelin on resistance arteries of DOCA-salt hypertensive rats. *Am J Physiol* 262: H1782-7, 1992.

101. Schiffrin EL, Larivière R, Li JS, Sventek P and Touyz RM. Endothelin-1 gene expression and vascular hypertrophy in DOCA-salt hypertension compared to spontaneously hypertensive rats. *Clin Exp Pharmacol Physiol* 22: S188-90, 1995.

102. Deng LY and Schiffrin EL. Effects of endothelin-1 and vasopressin on resistance arteries of spontaneously hypertensive rats. *Am J Physiol* 5: 817-822, 1992.

103. Hasdai D and Lerman A. The atherogenic potential of endothelin. *Coronary artery Dis* 6: 901-4, 1995.

104. Griendling KK and Alexander RW. Endothelial control of the cardiovascular system. *FASEB J* 10: 283-92, 1996.

105. Lerman A, Edwards BS, Hallett JW, Heublein DM, Sandberg SM and Burnett JC Jr. Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N Engl J Med* 325: 997-1001, 1991.

106. Dagassan PH, Breu V, Clozel M, Kunzli A, Vogt P, Turina M, Kiowski W and Clozel JP. Up-regulation of endothelin-B receptors in atherosclerotic human coronary arteries. *J Cardiovasc Pharmacol* 27: 147-53, 1996.

107. Bacon CR, Cary NR and Davenport AP. Endothelin peptide and receptors in human atherosclerotic coronary artery and aorta. *Circ Res* 79: 794-801, 1996.

108. Rossi GP, Colonna S, Pavan E, Albertin G, Della Rocca F, Gerosa G, Casarotto D et al. Endothelin-1 and its mRNA in the wall layers of human arteries ex vivo. *Circulation* 99: 1147-55, 1999.

109. Ihling C, Lippoldt A, Wessels S, Paul M, Schaefer HE and Zeiher AM. Endothelin-1-like immunoreactivity in human atherosclerotic coronary tissue: a detailed analysis of the cellular distribution of endothelin-1. *J Pathol* 179: 303-8, 1996.

110. Kohno M, Yokokawa K, Yasunari K, Kano H, Minami M and Yoshikawa J. Effect of the endothelin family of peptides on human coronary artery smooth-muscle cell migration. *J Cardiovasc Pharmacol* 31: S84-9, 1998.

111. Browatzki M, Schmidt J, Kubler W and Kranzhofer R. Endothelin-1 induces interleukin-6 release via activation of the transcription factor NF-kappaB in human vascular smooth muscle cells. *Basic Res Cardiol* 95: 98-105, 2000.

112. Lunec J and Winyard P. Reactive oxygen species: associated pathology. *J Int Fed Clin Chem* 10: 42-44, 1998.

113. Turrens JF. Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 17: 3-8, 1997.

114. Griendling KK and Ushio-Fukai M. NADH/NADPH oxidase: role in cardiovascular biology and disease. *Circ Res* 86: 494-501, 2000.

115. Blanc A, Pandey NR and Srivastava AK. Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H_2O_2 in vascular smooth muscle cells: potential involvement in vascular disease. *Int J Mol Med* 11: 229-234, 2003.

116. Koshkin V and Pick E. Generation of superoxide by purified and relipidated cytochrome b559 in the absence of cytosolic activators. *FEBS Lett* 327: 57-62, 1993.

117. Heyworth PG, Curnutte JT, Nauseef WM, Volpp BD, Pearson DW, Rosen H and Clark RA. Neutrophil nicotinamide adenine dinucleotide phosphate oxidase assembly. Translocation of p47-phox and p67-phox requires interaction between p47-phox and cytochrome b558. *J Clin Invest* 87: 352-356, 1991.

118. Duerrschmidt N, Wippich N, Goettsch W, Broemme HJ and Morawietz H. Endothelin-1 induces NAD(P)H oxidase in human endothelial cells. *Biochem Biophys Res Commun* 269: 713-717, 2000.

119. Wedgwood S, Dettman RW and Black SM. ET-1 stimulates pulmonary arterial smooth muscle cell proliferation via induction of reactive oxygen species. *Am J Physiol Lung Cell Mol Physiol* 281: L1058-L1067, 2001.

120. Li L, Fink GD, Watts SW, Northcott CA, Galligan JJ, Pagano PJ and Chen AF. Endothelin-1 increases vascular superoxide via endothelin(A)-NADPH oxidase pathway in low-renin hypertension. *Circulation* 107: 1053-1058, 2003.

121. Sundaresan M, Yu ZX, Ferrans VJ, Irani K and Finkel T. Requirement for generation of H2O2 for platelet-derived growth factor signal transduction. *Science* 270: 296-299, 1995.

122. Griendling KK and Alexander RW. Oxidative stress and cardiovascular disease. *Circulation* 96: 3264-5, 1997.

123. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK and Harrison DG. Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 97: 1916-23, 1996.

124. Warnholtz A, Nickening G, Schulz E, Macharzina R, Brasen JH, Skatchkov M, Heitzer T, Stasch JP, Griendling KK, Harrison DG, Bohm M, Meinertz T and Munzel T. Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: evidence for involvement of the renin-angiotensin system. *Circulation* 99: 2027-33, 1999.

125. Cheng TH, Shih NL, Chen SY, Wang DL and Chen JJ. Reactive oxygen species modulate endothelin-I-induced c-fos gene expression in cardiomyocytes. *Cardiovasc Res* 41: 654-662, 1999.

126. Liu SL, Lin X, Shi DY, Cheng J, Wu CQ and Zhang YD. Reactive oxygen species stimulated human hepatoma cell proliferation via cross-talk between PI-3K/PKB and JNK signaling pathways. *Arch Biochem Biophys* 406: 173-82, 2002.

127. Cheng CM, Hong HJ, Liu JC, Shih NL et al. Crucial role of extracellular signal-regulated kinase pathway in reactive oxygen species-mediated endothelin-1 gene expression induced by endothelin-1 in rat cardiac fibroblasts. *Mol Pharmacol* 63: 1002-1011, 2003.

128. Fei J, Viedt C, Soto U, Elsing C, Jahn L and Kreuzer J. Endothelin-1 and smooth muscle cells: induction of jun amino-terminal kinase through an

oxygen radical-sensitive mechanism. *Arterioscler Thromb Vasc Biol* 20: 1244-1249, 2000.

129. Frank GD, Motley ED, Inagami T and Eguchi S. PYK2/CAKbeta represents a redox-sensitive tyrosine kinase in vascular smooth muscle cells. *Biochem Biophys Res Commun* 270: 761-765, 2000.

130. Lloyd-Jones DM and Bloch KD. The vascular biology of nitric oxide and its role in atherogenesis. *Annu Rev Med* 47: 365-375, 1996.

131. Buchwalow IB, Podzuweit T, Bocker W, Samoilova VE, Thomas S, Wellner M et al. Vascular smooth muscle and nitric oxide synthase. *FASEB J* 16: 500-508, 2002.

132. Kurowska EM. Nitric oxide therapies in vascular diseases. *Curr Pharm Des* 8: 155-66, 2002.

133. Marletta MA. Nitric oxide synthase : aspects concerning structure and catalysis. *Cell* 78 : 927-930, 1994.

134. Voet D and Voet JG. *Biochemistry* (2nd ed.), John Wiley & Sons, Inc., Ch. 34: 1288-1291, 1995.

135. Raman CS, Li H, Martasek P, Kral V, Masters BS and Poulos TL. Crystal structure of constitutive endothelial nitric oxide synthase: a paradigm for pterin function involving a novel metal center. *Cell* 95: 939-950, 1998.

136. Mohaupt MG, Elzie JL, Ahn KY, Clapp WL, Wilcox CS and Kone BC. Differential expression and induction of mRNAs encoding two inducible nitric oxide synthases in rat kidney. *Kidney Int* 46: 653-65, 1994.

137. Lincoln TM, Dey N and Sellak H. cGMP-dependent protein kinase signaling mechanisms in smooth muscle: from the regulation of tone to gene expression. *J Appl Physiol* 91: 1421-30, 2001.

138. Traylor TG and Sharma VS. Why NO? *Biochemistry* 31: 2847-9, 1992.

139. Murad F. The nitric oxide-cyclic GMP signal transduction system for intracellular and intercellular communication. *Recent Prog Hormone Res* 49: 239-48, 1994.

140. Ortega Mateo A and Amaya Aleixandre de Artinano. Nitric oxide reactivity and mechanisms involved in its biological effects. *Pharmacol Res* 42: 421-427, 2000.

141. Fujii H, Ichimori K, Hoshiai K and Nakazawa H. Nitric oxide inactivates NADPH oxidase in pig neutrophils by inhibiting its assembling process. J Biol Chem 272: 32773-8, 1997.

142. Marletta MA and Spiering MM. Trace elements and nitric oxide function. *J Nutr* 133: 1431S-1433S, 2003.

143. Eigenthaler M, Lohmann SM, Walter U and Pilz RB. Signal transduction by cGMP-dependent protein kinases and their emerging roles in the regulation of cell adhesion and gene expression. *Rev Physiol Biochem Pharmacol* 135: 173-209, 1999.

144. Hofmann F, Ammendola A and Schlossmann J. Rising behind NO: cGMP-dependent protein kinases. *J Cell Sci* 113: 1671-1676, 2000.

145. Denninger JW and Marletta MA. Guanylate cyclase and the .NO/cGMP signaling pathway. *Biochim Biophys Acta* 1411: 334-350, 1999.

146. Koesling D, Harteneck C, Humbert P, Bosserhoff A, Frank R, Schultz G and Bohme E. The primary structure of the larger subunit of soluble guanylyl cyclase from bovine lung. Homology between the two subunits of the enzyme. *FEBS Lett* 266: 128-132, 1990.

147. Nakane M, Arai K, Saheki S, Kuno T, Buechler W and Murad F. Molecular cloning and expression of cDNAs coding for soluble guanylate cyclase from rat lung. *J Biol Chem* 265: 16841-16845, 1990.

148. Yu SM, Hung LM and Lin CC. cGMP-elevating agents suppress proliferation of vascular smooth muscle cells by inhibiting the activation of epidermal growth factor signaling pathway. *Circulation* 95: 1269-77, 1997.

149. Yu SM, Tsai SY, Kuo SC and Ou JT. Inhibition of platelet function by A02131-1, a novel inhibitor of cGMP-specific phosphodiesterase, in vitro and in vivo. *Blood* 87: 3758-3767, 1996.

150. Shahid M, van Amsterdam RG, de Boer J, Berge RE, Nicholson CD and Zaagsma J. The presence of five cyclic nucleotide phosphodiesterase isoenzyme activities in bovine tracheal smooth muscle and the functional effects of selective inhibitors. *Br J Pharmacol* 104: 471-477, 1991.

151. Yu SM, Cheng ZJ and Kuo SC. Endothelium-dependent relaxation of rat aorta by butein, a novel cyclic AMP-specific phosphodiesterase inhibitor. *Eur J Pharmacol* 280: 69-77, 1995.

152. D'souza FM, Sparks RL, Chen H, Kadowitz PJ and Jeter JR Jr. Mechanism of eNOS gene transfer inhibition of vascular smooth muscle cell proliferation. *Am J Physiol Cell Physiol* 284: C191-C199, 2003.

153. Schmidt HHHW and Walter U : NO at work. *Cell* 78: 919-925, 1994.

154. Sandirasegarane L, Charles R, Bourbon N and Kester M. NO regulates PDGF-induced activation of PKB but not ERK in A7r5 cells: implications for vascular growth arrest. *Am J Physiol Cell Physiol* 279: C225-C235, 2000.

155. Wang D, Yu X and Brecher P. Nitric oxide inhibits AII-induced activation of the calcium-sensitive tyrosine kinase proline-rich tyrosine kinase 2 without affecting epidermal growth factor receptor transactivation. *J Biol Chem* 274: 24342-24348, 1999.

156. Wever R, Stroes E and Rabelink TJ. Nitric oxide and hypercholesterolemia: a matter of oxidation and reduction? *Atherosclerosis* 137: S51-60, 1998.

157. Alfke H, Kleb B and Klose KJ. Nitric oxide inhibits the basic fibroblast growth factor-stimulated migration of bovine vascular smooth muscle cells in vitro. *VASA* 29: 99-102, 2000.

158. Failli P, DeFranco RM, Caligiuri A, Gentilini A, Romanelli RG, Marra F, Batignani G et al. Nitrovasodilators inhibit platelet-derived growth factor-induced proliferation and migration of activated human hepatic stellate cells. *Gastroenterology* 119: 479-492, 2000.

159. Ni CW, Wang DL, Lien SC, Cheng JJ, Chao YJ and Hsieh HJ. Activation of PKC-epsilon and ERK1/2 participates in shear-induced endothelial MCP-1 expression that is repressed by nitric oxide. *J Cell Physiol* 195: 428-434, 2003.

160. Taimor G, Rakow A and Piper HM. Transcription activator protein 1 (AP-1) mediates NO-induced apoptosis of adult cardiomyocytes. *FASEB J* 15: 2518-20, 2001.

161. Nishida E and Gotoh Y. The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Pharmacol Sci* 18: 128-131, 1993.

162. Zhang YM, Wang KQ, Zhou GM, Zuo J and Ge JB. Endothelin-1 promoted proliferation of vascular smooth muscle cell through pathway of extracellular signal-regulated kinase and cyclin D1. *Acta Pharmacol Sin* 24: 563-8, 2003.

163. Yoshizumi M, Kim S, Kagami S, Hamaguchi A, Tsuchiya K, Houchi H, Iwao H, Kido H and Tamaki T. Effect of endothelin-1 (1-31) on extracellular signal-regulated kinase and proliferation of human coronary artery smooth muscle cells. *Br J Pharmacol* 125: 1019-1027, 1998.

164. Cornwell TL, Arnold E, Boerth NJ and Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol Cell Physiol* 267: C1405-C1413, 1994.

165. Paolocci N, Ekelund UE, Isoda T, Ozaki M, Vandegaer K et al. cGMP-independent inotropic effects of nitric oxide and peroxynitrite donors: potential role for nitrosylation. *Am J Physiol Heart Circ Physiol* 279: H1982-88, 2000.

166. Lahteenmaki T, Sievi E and Vapaatalo H. Inhibitory effects of mesoionic 3-aryl substituted oxatriazole-5-imine derivatives on vascular smooth muscle

cell mitogenesis and proliferation in vitro. *Br J Pharmacol* 125: 402-408, 1998.

167. Zhan CD and Pan JY. Nitric oxide inhibits the expression of proto-oncogene c-fos induced by angiotensin II and endothelin-1 in cardiomyocytes. *Sheng Li Xue Bao* 52: 450, 2000.

168. Wang D, Yu X and Brecher P. Nitric oxide and N-acetylcysteine inhibit the activation of mitogen-activated protein kinases by angiotensin II in rat cardiac fibroblasts. *J Biol Chem* 273: 33027-33034, 1998.

169. Boerth NJ, Dey NB, Cornwell TL and Lincoln TM. Cyclic GMP-dependent protein kinase regulates vascular smooth muscle cell phenotype. *J Vasc Res* 34: 245-259, 1997.

170. Wang L and Proud CG. Ras/ERK signaling is essential for activation of protein synthesis by Gq protein-coupled receptor agonists in adult cardiomyocytes. *Circ Res* 91: 821-9, 2002.

171. Sugden PH, Fuller SJ, Mynett JR, Hatchett RJ, Bogoyevitch MA and Sugden MC. Stimulation of adult rat ventricular myocyte protein synthesis and phosphoinositide hydrolysis by the endothelins. *Biochim Biophys Acta* 1175: 327-32, 1993.

172. Bogoyevitch MA, Glennon PE, Andersson MB, Clerk A, Lazou A, Marshall CJ, Parker PJ and Sugden PH. Endothelin-1 and fibroblasts growth factors stimulate the mitogen-activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two

signaling pathways leading to myocyte hypertrophy. *J Biol Chem* 269: 1110-1119, 1994.

173. Yamboliev IA, Hruby A and Gerthoffer WT. Endothelin-1 activates MAP kinases and c-Jun in pulmonary artery smooth muscle. *Pulm Pharmacol Ther* 11: 205-208, 1998.

174. Clerk A and Sugden P. Regulation of phospholipase C and D in rat ventricular myocytes: stimulation by endothelin-1, bradykinin and phenylephrine. *J Mol Cell Cardiol* 29: 1593-1604, 1997.

175. Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB and Rhee SG. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phsophorylation. *J Biol Chem* 272: 217-221, 1997.

176. Griendling KK, Minieri CA, Ollerenshaw JD and Alexander RW. Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. *Circ Res* 74: 1141-1148, 1994.

177. Lo YY, Wong JM and Cruz TF. Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J Biol Chem* 271: 15703-15707, 1996.

178. Takizawa T, Gu M, Chobanian AV and Brecher P. Effect of nitric oxide on DNA replication induced by angiotensin II in rat cardiac fibroblasts. *Hypertension* 30: 1035-40, 1997.

179. Heller R, Polack T, Grabner R and Till U. Nitric oxide inhibits proliferation of human endothelial cells via a mechanism independent of cGMP. *Atherosclerosis* 144: 49-57, 1999.

180. Sandirasegarane L and Diamond J. The nitric oxide donors, SNAP and DEA/NO, exert a negative inotropic effect in rat cardiomyocytes which is independent of cyclic GMP elevation. *J Mol Cell Cardiol* 31: 799-808, 1999.

181. Lenormand P, Sardet C, Pages G, L'Allemain G, Brunet A and Pouyssegur J. Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. *J Cell Biol* 122: 1079-88, 1993.