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Endothelin-1 and H_2O_2-induced Signaling in Vascular Smooth Muscle Cells: Modulation by CaMKII and Nitric oxide

par

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Endothelin-1 and H_2O_2-induced Signaling in Vascular Smooth Muscle Cells: Modulation by CaMKII and Nitric oxide

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Résumé

L'endothéline-1 (ET-1) est un peptide vasoactif extrêmement puissant qui possède une forte activité mitogénique dans les cellules du muscle lisse vasculaire (VSMCs). Il a été démontré que l'ET-1 est impliquée dans plusieurs maladies cardio-vasculaires, comme l'athérosclérose, l'hypertension, la resténose après l'angioplastie, l'insuffisance cardiaque et l'arythmie. L'ET-1 exerce ses effets via plusieurs voies de signalisation qui incluent le Ca^{2+}, les protéines kinases activées par les mitogènes (MAPKs) y compris les kinases régulées par les signaux extracellulaires (ERK1/2) et la voie de la phosphatidylinositol 3-kinase (PI-3K)/protein kinase B (PKB). Plusieurs études ont démontré que les dérivés réactifs de l'oxygène (ROS) peuvent jouer un rôle important dans la signalisation d'ERK1/2 et de PKB induite par plusieurs facteurs de croissance et hormones.

Nous avons précédemment montré que l'ET-1 produit des ROS qui agissent comme médiateur de la signalisation cellulaire induite par l'ET-1. Le peroxyde d'hydrogène (H_2O_2), une molécule qui appartient à la famille des ROS, peut activer les voies de la MAPK et de la PKB dans les VSMCs. Par ailleurs, nos résultats suggèrent également que le Ca^{2+} et la calmoduline (CaM) sont essentiels pour la phosphorylation d'ERK1/2, de p38 et de PKB induite par le H_2O_2 dans les VSMCs. La Ca^{2+}/CaM-dependent protein kinases II (CaMKII) est une sérine/thréonine protéine kinase multifonctionnelle activée par le Ca^{2+}/CaM. Il a été montré que la CaMKII est impliquée dans les voies de signalisation induite par le H_2O_2 dans les cellules endothéliales. Cependant, le rôle de la CaMKII dans la phosphorylation d'ERK1/2, de PKB et de la proline-rich tyrosine kinase 2 (Pyk2) induite par l'ET-1 et le H_2O_2, de même que son rôle dans l'effet hypertrophique et prolifératif de l'ET-1 dans les VSMCs demeure inexploré.

Le monoxyde d'azote (NO) est une molécule vasoactive impliquée dans la régulation de plusieurs réponses hormonales. Le NO peut moduler la signalisation contrôlant la croissance cellulaire induite par plusieurs agonistes d'où son rôle protecteur dans le système vasculaire.

Des études ont montré que le NO peut inhiber la voie de Ras/Raf/ERK1/2 et la voie de PKB induite par le facteur de croissance endothélial (EGF) et l'angiotensine II (Ang II). Beaucoup d'autres travaux ont mis en évidence un *cross-talk* entre les voies de signalisation activées par l'ET-1 et le NO. La capacité du NO à inhiber la signalisation intracellulaire induite par l'ET-1 dans les VSMCs demeure inconnue. Le travail présenté dans cette thèse vise à déterminer le rôle du système Ca^{2+}-CaM-CaMKII dans la phosphorylation d'ERK1/2, de PKB et de Pyk2 induite par l'ET-1 et le H_2O_2 ainsi que son rôle dans la croissance et la prolifération cellulaire induites par l'ET-1 dans les VSMCs. Nous avons également testé le rôle du NO dans la phosphorylation d'ERK1/2, de PKB et de Pyk2 ainsi que la synthèse protéique induite par l'ET-1.

<u>*Dans la première partie*</u> de notre étude, nous avons examiné le rôle de la CaMKII dans la phosphorylation d'ERK1/2 et de PKB induite par l'ET-1 dans les VSMCs en utilisant trois approches différentes i.e. l'usage d'inhibiteurs pharmacologiques, un peptide auto-inhibiteur de la CaMKII (CaMKII AIP) et la technique de siRNA. Nous avons démontré que la CaMKII est impliquée dans la phosphorylation d'ERK1/2 et de PKB induite par l'ET-1 dans les VSMCs. Des études précédentes ont montré à l'aide d'inhibiteurs pharmacologiques comme le KN-93 que l'Ang II et les agents induisant une augmentation de la concentration en Ca^{2+} intracellulaire comme l'ionomycine,

provoquent la phosphorylation d'ERK1/2 via la CaM dans les VSMCs. Cependant, en utilisant différentes approches, nos études ont montré pour la première fois une implication de la CaMKII dans la phosphorylation d'ERK1/2 et de PKB induite par l'ET-1 dans les VSMCs. Nous avons également rapporté pour la première fois, un rôle crucial de la CaMKII dans la pathophysiologie vasculaire associée à l'ET-1 puisque l'activation de la CaMKII joue un rôle important dans l'hypertrophie et la croissance cellulaire.

Dans la deuxième partie, à la lumière des études précédentes qui montraient que les ROS agissent comme médiateurs de la signalisation induite par l'ET-1 dans les VSMCs, nous avons examiné si la CaMKII est également impliquée dans l'activation des voies d'ERK1/2 et de PKB induite par le H_2O_2. En utilisant des approches pharmacologiques et moléculaires, nous avons montré, comme pour l'ET-1, que la CaMKII joue un rôle critique en amont de la phosphorylation d'ERK1/2, de PKB et de Pyk2 induite par le H_2O_2.

Nous avons précédemment montré que la transactivation du récepteur de type I de l'*insulin-like growth factor* (IGF-1R) est nécessaire à l'activation de PKB induite par le H_2O_2. Pour cette raison, nous avons examiné l'effet de l'inhibition de la CaMKII par l'inhibiteur pharmacologique ou par le *knock-down* de la CaMKII sur la phosphorylation d'IGF-1R induite par le H_2O_2. Les résultats démontrent que la CaMKII joue un rôle critique en amont de la phosphorylation d'ERK1/2, de PKB et d'IGF-1R induite par le H_2O_2.

Dans la troisième partie de notre étude, nous avons également examiné le mécanisme moléculaire par lequel le NO exerce ses effets anti-mitogéniques et anti-hypertrophiques dans la signalisation induite par l'ET-1. En testant l'effet de deux différents donneurs de

NO (*S*-nitroso-*N*-acetylpenicillamine (SNAP), sodium nitroprusside (SNP)) et un inhibiteur de NO synthase, le N (G)-nitro-L-arginine methyl ester (L-NAME) dans la phosphorylation d'ERK1/2, de PKB et de Pyk2 induite par l'ET-1, nous avons observé que le NO a un effet inhibiteur sur la signalisation induite par l'ET-1 dans les VSMCs. Par ailleurs, le 8-Br-GMPc, un analogue du GMPc, a un effet similaire à celui des deux donneurs du NO, tandis que l'oxadiazole quinoxaline (ODQ), un inhibiteur de la guanylate cyclase soluble, inverse l'effet inhibiteur du NO. Nous concluons que le NO diminue la phosphorylation d'ERK1/2, de PKB et de Pyk2 induite par l'ET-1 d'une manière dépendante du GMPc. Le NO inhibe aussi les effets hypertrophiques de l'ET-1 puisque le traitement avec le SNAP diminue la synthèse des protéines induite par l'ET-1.

En résumé, les études présentées dans cette thèse démontrent que l'ET-1 et le H_2O_2 sont des activateurs de la phosphorylation d'ERK1/2, de PKB et de Pyk2 dans les VSMCs et que la CaMKII s'avère nécessaire pour ce processus, en agissant en amont de l'activation de IGF-1R induite par le H_2O_2 dans les VSMCs. Elles montrent également que le NO inhibe la phosphorylation d'ERK1/2, de PKB et de Pyk2 induite par l'ET-1. Enfin, nos travaux suggèrent aussi que l'activation de la CaMKII stimule la synthèse des protéines et de l'ADN induites par l'ET-1 alors que le NO inhibe la synthèse des protéines induite par ET-1.

Mots clés: Endothéline ; Peroxyde d'hydrogène ; CaMKII ; Monoxyde d'azote ; Système vasculaire ; PKB; ERK1/2; IGF-1R; Hypertrophie.

Abstract

Endothelin-1 has emerged as an extremely potent vasoactive peptide exhibiting potent mitogenic activity in vascular smooth muscle cells (VSMCs). A critical role of ET-1 in many cardiovascular diseases, such as atherosclerosis, hypertension, restenosis after angioplasty, heart failure and arrhythmia has been suggested. ET-1 exerts its effects through multiple signaling pathways which include Ca^{2+}, mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI-3K)/protein kinase B (PKB)/Akt pathways. Several studies have also demonstrated that reactive oxygen species (ROS) may play an important role in mediating the signals of several growth factors and peptides hormones linked to these pathways. We have previously reported that ET-1 generates ROS which mediates ET-1-induced signaling. H_2O_2, an important ROS molecule, activates both MAPKs and PKB signaling in VSMCs. In addition, we have also suggested that Ca^{2+} and CaM are essential to trigger H_2O_2-induced ERK1/2, p38 and PKB phosphorylation in A-10 VSMCs. Ca^{2+}/calmodulin (CaM)-dependent protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase which is believed to transduce the downstream effects of Ca^{2+}/CaM, and has been shown to be involved in H_2O_2-induced signaling in endothelial cells. However, a role of CaMKII in mediating ET-1 and H_2O_2-induced ERK1/2, PKB, Pyk2 phosphorylation, as well as its effect on hypertrophic and proliferative responses of ET-1 in VSMCs remains unexplored. Interestingly, a role of CaMKII in several cardiovascular diseases has been reported and studies showing that

pharmacological inhibition of CaMKII, by using KN-93, prevent arrhythmic activity improved vascular dysfunction in diabetes or in Ang II-induced hypertension.

Nitric oxide (NO) is also an important reactive species and vasoactive molecule involved in the regulation of several hormone-mediated responses. NO has been suggested to modify growth-promoting signaling events and thus may serve as a vascular protective agent. Studies have shown that NO can attenuate EGF and Ang II-induced Ras/Raf/ERK1/2 as well as increase in PKB phosphorylation signaling pathways. There is also evidence for a potential cross-talk between ET-1 and NO, however not much information on the ability of NO to modify ET-1-induced signaling in VSMCs is available. Therefore, the work presented in this thesis has investigated the role of CaMKII system in ET-1 and H_2O_2-induced ERK1/2, PKB and Pyk2 phosphorylation, as well as in cell growth and proliferation evoked by ET-1 in VSMCs. We also investigated the role of NO in ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation as well as protein synthesis.

In the first part of our studies, by using three different approaches, i.e. use of pharmacological inhibitors, a CaMKII AIP (autoinhibitor peptide) and siRNA techniques, we have investigated the involvement of CaMKII in ET-1-induced ERK1/2 and PKB phosphorylation in A-10 VSMC. We have demonstrated that CaMKII mediates the effect of ET-1 on ERK1/2 and PKB phosphorylation in A-10 VSMC.

By using pharmacological inhibitor alone such as, KN-93, earlier studies have reported that AngII and Ca^{2+} elevating agents, such as ionomycin, exert their effects on ERK1/2 phosphorylation via CaM-dependent pathways in VSMC. However, by using multiple approaches, our studies, have provided the first evidence to suggest an involvement of

CaMKII in mediating the effect of ET-1 on ERK1/2 and PKB phosphorylation in A-10 VSMC. We have also reported for the first time, a crucial role of CaMKII in vascular pathophysiology related to ET-1 by regulating the growth and hypertrophic events by using the technique of [^{3}H]leucine and [^{3}H]thymidine incorporation.

In the second part, in view of earlier studies showing that ROS mediates ET-1-induced signaling events in VSMC, we have also investigated if CaMKII is also implicated in H_2O_2-induced activation of ERK1/2 and PKB pathways. By using both pharmacological and molecular approaches, we show that similar to ET-1, CaMKII serves as a critical upstream component in triggering H_2O_2-induced ERK1/2, PKB and Pyk2 phosphorylation in VSMC. Furthermore, since we have previously reported that IGF-1R transactivation is needed for H_2O_2-induced PKB activation, we have investigated the effect of CaMKII inhibition and knocking-down on IGF-1R phosphorylation evoked by H_2O_2. Taken together, these results demonstrate that CaMKII plays a critical upstream role in mediating the effect of H_2O_2 on ERK1/2, PKB and IGF-1R phosphorylation.

In the third part of our studies, we have investigated the molecular mechanism by which NO exerts its anti-mitogenic and anti-hypertrophic effect on ET-1-induced signaling. By testing the effect of two different NO donors (SNAP and SNP) and L-NAME, an inhibitor of NO synthase, in ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation, we observed that NO has an inhibitory effect in ET-1-induced signaling in VSMC. In addition, 8-Br-cGMP, an analogue of cGMP, exerted similar effect to that of NO donors whereas, oxadiazole quinoxalin (ODQ), an inhibitor of soluble guanylyl cyclase (sGC), reversed the inhibitory effect of NO. We conclude that NO, in a cGMP-dependent manner, attenuated ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2 and also

antagonized the hypertrophic effects of ET-1, since SNAP treatment decreased the protein synthesis induced by ET-1.

In summary, the studies presented in this thesis demonstrate that both ET-1 and H_2O_2 induce ERK1/2, PKB and Pyk2 phosphorylation in VSMC and CaMKII activation is required for these events. We have also shown that CaMKII phosphorylation is upstream of H_2O_2-induced IGF-1R transactivation in VSMC. We have also provided evidence that NO attenuates ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. Finally, we have established that CaMKII activation stimulates ET-1-evoked protein and DNA synthesis, yet NO attenuates protein synthesis induced by ET-1.

Keywords : Endothelin; Hydrogen peroxide; CaMKII; Nitric oxide; Vascular; Protein Kinase B; Extracellular Signal-Regulated Kinase1/2; IGF-1R; Growth.

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List of abbreviations

a a	amino acid
AMP	adenosine monophosphate
Ang II	angiotensin II
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
ADH	anti-diuretic hormone
BAD	Bcl_2 associated death promoter
bEGF	basic fibroblast growth factor
CaM	calmodulin
CaMK	Ca^{2+}/CaM-dependent protein kinase
cAMP	cyclic adenosine monophosphate
DOCA	deoxycorticosterone acetate
EDRF	endothelium-derived relaxing factor
EGFR	epidermal growth factor receptor
ERK	extracellular signal-regulated kinase
ET	endothelin
ECE	endothelin converting enzyme
FKHR	forkhead transcription factor
GDP	guanosine diphosphate
GFR	glomerular filtration rate
GPCR	G-protein-coupled receptor
GTP	guanosine triphosphate
Grb-2	growth factor receptor binder-2
GSK-3	glycogen synthase kinase- 3
H_2O_2	hydrogen peroxide
HB-EGF	heparin binding EGF

IGF-1R	insulin-like growth factor type 1 receptor
IR	insulin receptor
JNK	Jun N-terminal kinase
kDa	kiloDalton
MAPK	mitogen activated protein kinase
MEK	mitogen extracellular regulated kinase
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NEP	neutral endopeptidase
NO	nitric oxide
$p70^{s6k}$	p70 ribosomal S6 kinase
$p90^{rsk}$	p90 ribosomal kinase
PDGFR	Platelet derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PH	pleckstrin homology
PI3-K	phosphatidylinositol 3-kinase
PI	phosphatidylinositol
$PI4,5P_2$	phosphatidylinositol 4, 5 triphosphate
$PI3, 4,5P_3$	phosphatidylinositol 3, 4, 5 triphosphate
PKB	protein kinase B
PKC	protein kinase C
PTK	protein tyrosine kinase
PTPase	protein tyrosine phosphatase
Pyk-2	proline-rich tyrosine kinase
R-PTK	receptor protein tyrosine kinases
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
sGC	soluble guanylate cyclase
SH2	src homology 2
SHC	src homology collagen
SHP2	SH2 domain-containing tyrosine phosphatase-2

SHR	spontaneously hypertensive rat
SOD	superoxide dismutase
SOS	son of seven less
TNF-α	tumour necrosis factor-α
TGF	transforming growth factor
VSMCs	vascular smooth muscle cells

Dedication

This thesis is dedicated to

Jacques de Champlain, OC, OQ, MD, PhD, FRCO, FAHA, FIACS, FCAHS
13 mars 1938 - 15 juillet 2009

Professeur émérite et chercheur titulaire
Départements de physiologie et de médecine
Faculté de médecine
Université de Montréal

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CHAPTER 1

INTRODUCTION

1 Endothelin

Endothelin (ET) was discovered by Yanagisawa and co-workers in 1988 (1) who also characterized and cloned it from porcine aortic endothelial cells (1). ET is one of the most potent vasoconstrictors that exhibits inotropic and mitogenic properties, influences salt and water homeostasis and stimulates the renin-angiotensin-aldosterone as well as sympathetic system (2-4). The overall effect of ET is usually to increase vascular tone and blood pressure. ET is believed to play an important role in vascular remodelling associated with experimental and human hypertension (4;5). Increased vascular smooth muscle cell (VSMC) hypertrophy, migration and proliferation are among the key events that contribute to remodeling of the vasculature associated with cardiovascular diseases. ET-1 exerts its physiological action by activating several signal transduction pathways linked to cellular hypertrophy, growth, migration and proliferation in several cell types including cardiomyocytes (6;7), kidney mesangial cells (8), and in the vascular system (9;10).

1-1 Structure of ETs

ET is a 21 amino acid peptide which exists in at least three isoforms, ET-1, ET-2 and ET-3 (11). 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 amino acid residues 8-10 and a hydrophobic C-terminus (residues 16-21) containing the aromatic indole side chain at Trp^{21} (Fig. 1). ET-2 contains two amino acid substitutions (Trp^{6}-Leu^{7}) and shares 90% sequence homology with ET-1. ET-3 contains six amino acid

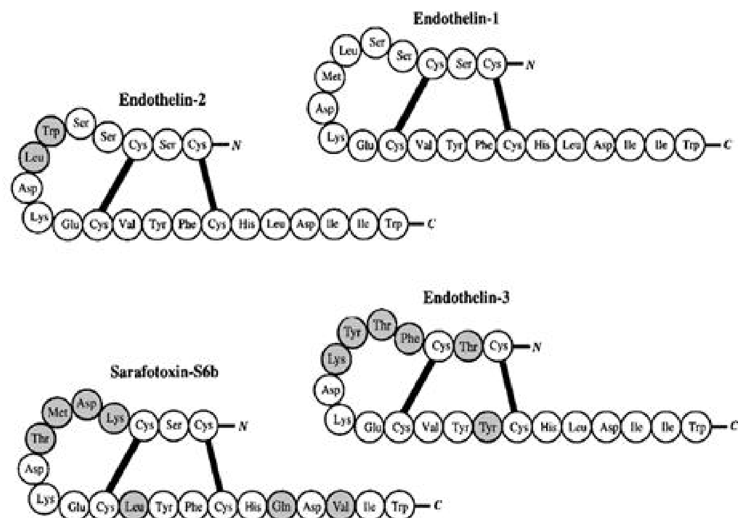

Fig. 1 : **Structures of endothelins.**
Amino acid sequences of the three members of the endothelin family and of the structurally related snake venom toxin sarafotoxin S6b. Each isoform contains two intra-chain disulphide bridges linking paired cysteine amino acid residues, thus producing an unusual semi-conical structure. Dark circles indicate where aminoacids differ from those of endothelin-1.
From (Haynes : J Hypertension 16:1081-1098, 1998)

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. The hydrophobic C-terminus of ET is essential for its bioactivity, as well as the loop configuration (12). Among the three ETs, ET-1 is the most important isopeptide in the vasculature (Fig. 1).

1-2 Regulation and sites of generation of ET-1

1-2-1 Regulation

Generation of ET-1 is increased by several factors, including vasoactive hormones, growth factors, hypoxia, shear stress, lipoproteins, free radicals, endotoxin and cyclosporin (13) (Fig 2) whereas factors that increase intracellular level of cyclic guanosine monophosphate (cGMP), including endothelium-derived nitric oxide, nitrovasodilators, natriuretic peptides, heparin and prostaglandins (13) are known to inhibit the production of ET-1 (Table1 shows a list of factors that influence ET-1 secretion). Each member of the ET family is represented by a separate gene that encodes a specific precursor for the mature isoform (11). In the 5' flanking region there are binding sites for activating protein 1 and nuclear factor 1, which mediate the induction of mRNA for ET-1 by angiotensin II (Ang II) and transforming growth factor-β, respectively (14). The 3' flanking region of the mRNA contains adenine-uracil-rich sequences that mediate selective destabilization of preproendothelin-1 mRNA, accounting for its relatively short biological half life of 15 min.

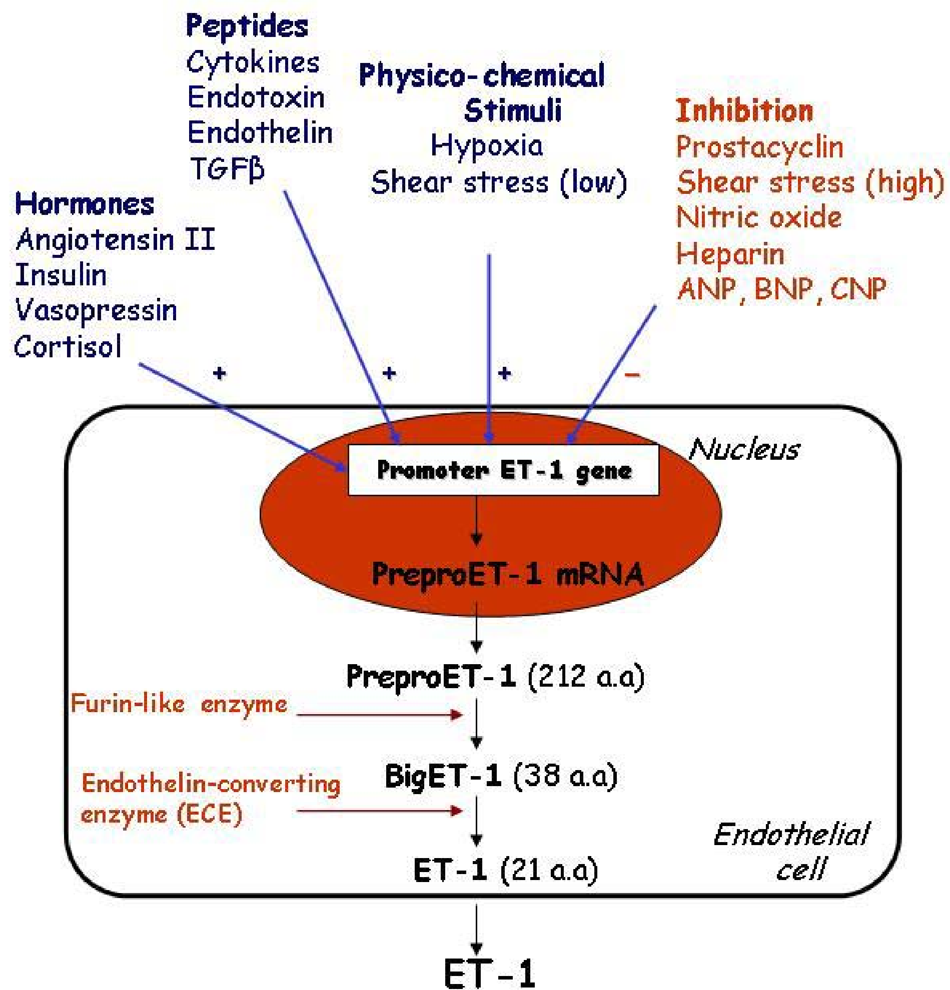

Fig. 2 : Regulation of ET-1 synthesis and its pathway of generation.
ET-1 synthesis is regulated by many factors; including hormones, growth factors and nitric oxide. The initial product of the human ET-1 gene is preproendothelin-1, a 212 amino acid peptide. Proendothelin-1 (BigET-1) is formed after cleavage of preproendothelin-1 by furin to generate a 38 amino acid peptide. The formation of mature ET-1 requires cleavage of bigET-1 by one of several unique endothelin converting enzymes (ECE). ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; CNP: C-type natriuretic peptides; a.a :aminoacids. Adapted from (Haynes WG and Webb DJ. *J Hypertens* 16: 1081-1098, 1998.)

TABLE 1. **Factors That Influence ET-1 Secretion***

Stimulating factors	Inhibiting factors
Vasoconstrictor	Vasodilators
Angiotensin II	Bradykinin
Vasopressin	Nitric oxide
Norepinephrine	Prostaglandins E_2 and I_2
Isoprostane 8-epi-prostaglandin $F_{2\alpha}$	Adrenomedullin
	Atrial and brain natriuretic peptides
Thrombogenic agents	Anticoagulants
Thrombin	Heparin
	Hirudin
Cytokines and growth factors	NA
Interleukin 1 and 3	
Tumor necrosis factor α	
GCSF	
Interferon-gamma	
Transforming growth factor β	
Endotoxin	
Physicochemical factors	Physicochemical factors
Mechanical strain	High levels of shear stress (>6 dyne/cm^2)
Pressure without cell distortion	NA
Hypoxia	NA
Low levels of shear stress (<2 dyne/cm^2)	NA
Hemodynamic pressure overload	NA
Aging	
Other factors	Other factors
Insulin	Nitrates
Serotonin	Progesterone
Corticosteroids	Estrogens
Erythropoietin	PPAR-α
Oxidized low-density lipoproteins	Calcium ionophores
Cyclosporine	Montelukast
Platelet aggregation	NA
Macrophage infiltration	NA
Formation of atherosclerotic lesions	NA

*ET-1 = endothelin 1; GCSF = granulocyte-macrophage colony-stimulating factor; NA = not applicable; PPAR-α = peroxisome proliferator-activated receptor-α.

From (Perez del Villar et al *Mayo Clin Proc*. 80: 84-96, 2005)

1-2-2 Site of generation

Endothelial cells express a high level of mRNA for preproendothelin-1 and intracellular converting enzyme. Compared to other cell types, endothelial cells are the major site of ET-1 generation (11). ET-1 is also produced by the heart, kidney, posterior pituitary and central nervous system (13). Human aortic vascular smooth muscle cells also express mRNA for ET-1, although its production is about 100-fold less than that in endothelial cells. Limited amounts of ET-2 are produced in endothelial cells, heart and kidney (15;16). ET-3 appears to be expressed in the endocrine, gastro-intestinal and central nervous systems, but not in endothelial cells (13)

1-3 Biosynthesis of ET

The initial product of the human ET-1 gene is preproendothelin-1, a 212 amino acid peptide (Fig.2). Proendothelin-1 is formed after removal of a short secretory sequence, and is then cleaved by furin to generate a 38 amino acid peptide, bigET-1 (1). BigET-1 does not appear to have any direct actions (17). The formation of mature ET-1 requires cleavage of bigET-1 by one of several unique ET converting enzymes (ECE). This family of metalloproteases is related to neutral endopeptidase-24.11 and Kell protein, but not to Ang II converting enzyme. ECE-1 is the physiologically active ECE (18). It possesses two splice variants, ECE-1a and ECE-1b, which have functionally distinct roles and tissue distributions (19). ECE-1a is expressed in the Golgi apparatus of 'producer' cells, such as endothelial cells, and appears to be responsible for intracellular processing of bigET-1 to ET-1 in such cells. ECE-1b is expressed in 'responder' cells, such as vascular smooth muscle cells, and is transported to the plasma

membrane where it acts to cleave extracellular bigET-1. Two more isoforms, ECE-1c and ECE-1d have also been identified (20). A second form of ECE (ECE-2) has been cloned and characterized (21). ECE-2 is similar to ECE-1 in that it is membrane bound, inhibited by phosphoramidon and exhibits selectivity for bigET-1. However, ECE-2 is active only at acidic pH (5.5) and is not expressed on the cell's surface (21). Thus, ECE-2 could act as an intracellular enzyme responsible for the conversion of endogenously synthesized big ET-1 in acidic environments. ECE-1 and ECE-2 are relatively selective for big ET-1, having much less activity in cleaving big ET-2 and big ET-3. It is probable that there is other, as yet unidentified, ECE that are responsible for cleavage of ET-2 and ET-3. Both ECE-1 and ECE-2 are inhibited by phosphoramidon, but not by selective neutral endopeptidase and angiotensin converting enzyme (ACE) inhibitors (13)

1-4 Plasma concentrations and clearance of ET-1

In healthy subjects, circulating concentrations of ET-1 in venous plasma are in the range 1–10 pmol/l (22;23). This concentration is lower than those which cause vascular contraction *in vitro* and *in vivo*, although concentrations at the interface between an endothelial cell and vascular smooth muscle are likely to be much higher. It has been reported that cultured endothelial cells secrete ET-1 into basolateral (abluminally) compartment and not in the apical (luminally) compartment (24). Thus, ET-1 appears to be primarily a locally acting paracrine substance rather than a circulating endocrine hormone. Venous plasma bigET-1 and the inactive C-terminal fragment of ET-1 concentrations have been used as a marker for endothelial synthesis of the peptide and

reflect better the exact amount of ET-1 generated as compared to ET-1 concentration (25), because circulating ET-1 is rapidly eliminated from the circulation. Although clearance of ET-1 from the circulation is very rapid and its biological half-life is about one min its pressor effects are maintained for about one hour (26). ET degradation happens mainly locally by endopeptidases (neutral endopeptidase, NEP) and cathepsins G generated from endothelial (in vascular) and epithelial cells (in lung) (27;28). ET-1 circulating is eliminated by kidney, liver and especially lungs which are responsible for 50% of elimination of ET-1 in human (29). A second metabolic pathway of ET appears to be mediated through receptor binding and then internalization (28). Pulmonary clearance of labelled ET-1 can be blocked by pretreatment with a large dose of unlabelled ET-1, suggesting that clearance of ET-1 is receptor-dependent (26). Blockade of ET receptors of the ET_B subtype, but not of the ET_A subtype, increases plasma concentrations of ET-1 and ET-3 (30) and prolongs the biological half life of exogenous [^{125}I]-endothelin-1(31). This blockade increases circulating level of ET-1 within 15 min (30;32) without affecting bigET-1 and C-terminal fragments concentrations (25), confirming that this increase is mediated by displacement of ET-1 from receptors rather than through peptide synthesis. These reports also suggest that ET_B receptors play an important role in ET-1 clearance.

1-5 ET-1 receptors

ET-1 exerts its biological actions through the activation of two receptor subtypes, ET_A and ET_B (33;34). Both ET_A and ET_B, receptors belong to a large family of transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs). They contain seven transmembrane domains of 22-26 hydrophobic amino acids in their ~400-

aminoacid sequences with an N-terminal extracellular region and C-terminal intracellular region (33-35). It is currently not clear whether receptors dimerization into homo-or heterodimers plays a role in ET-1 receptors activity and function in vivo (36). ET_A receptors are highly expressed in VSMC but are also found in cardiomyocytes, fibroblasts, hepatocytes, adipocytes, osteoblasts and brain neurons (33;37) and exhibit higher affinities for ET-1 and ET-2 than for ET-3 (33). Potent peptide and non-peptide ET_A antagonists have been synthesized, the prototype being the pentapeptide BQ-123 (38). ET_B receptors exist predominantly in endothelial cells and smooth muscle cells, but are also found in cardiomyocytes, hepatocytes, fibroblasts, osteoblasts, different types of epithelial cells and neurons and have equal subnanomolar affinities for all ET iso-peptides (34). ET-1 binding to ET_A and ET_B receptors on smooth muscles produces vasoconstriction, cell growth and cell adhesion (39) (Fig.3). The binding of ET-1 to endothelial ET_B receptors stimulates the release of nitric oxide and prostacyclin (Fig.3) which prevents apoptosis, inhibits ET converting enzyme-1 (ECE-1) expression in endothelial cells and plays an important role in ET-1 clearance (40;41). Several agonists selectively activate the ET_B over the ET_A receptors which include ET-3 (~2000-fold selectivity) and sarafotoxin S6c (~300 000-fold selectivity) (42). Up to date several selective and non-selective ET-1 receptors inhibitors have been discovered. For example, BQ-788 is a selective peptide antagonist of the ET_B receptor (43). The majority of currently used ET agonists are shown in Table 2.

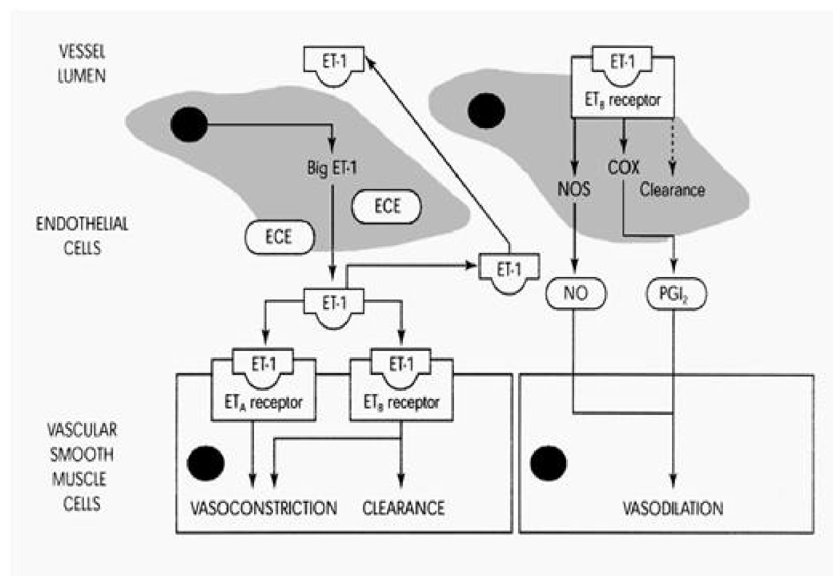

Fig.3. Localization and actions of ET_A and ET_B receptors.
Two ET receptors have been identified in the vasculature: ET type-A receptors (ET_A) reside in vascular smooth muscle cells and mediate vasoconstriction and cell proliferation, whereas ET_B receptors reside on endothelial cells and are mainly vasodilatory through NO and prostacyclin (which in turn can mediate the anti-apoptotic effects of ET-1), ET_B receptors on smooth muscle cells can elicit vessel contraction
From (Suresh et al. *J Card Fail.* 6:359-368, 2000)

TABLE 2. List of ET-1 receptors antagonist

ET_A-receptor antagonists	$ET_{A/B}$-receptor antagonists	ET_B-receptor antagonists	ECE inhibitors
A-127722 (non-peptide)	A-182086 (non-peptide)	A-192621 (non-peptide)	B-90063 (non-peptide)
ABT-627 (non-peptide)	CGS 27830 (non-peptide)	A-308165 (non-peptide)	CGS 26393 (non-peptide)
BMS 182874 (non-peptide)	CP 170687 (non-peptide)	BQ-788 (peptide)	CGS 26303 (non-peptide)
BQ-123 (peptide)	J-104132 (non-peptide)	BQ-017 (peptide)	CGS 35066 (non-peptide)
BQ-153 (peptide)	L-751281 (non-peptide)	IRL 1038 (peptide)	Phosphoramidon (peptide)
BQ-162 (peptide)	L-754142 (non-peptide)	IRL 2500 (peptide)	PP-36 (peptide)
BQ-485 (peptide)	LU 224332 (non-peptide)	PD-161721 (non-peptide)	SM-19712 (non-peptide)
BQ-518 (peptide)	LU 302872 (non-peptide)	RES 701-1 (peptide)	TMC-66 (non-peptide)
BQ-610 (peptide)	PD 142893 (peptide)	RO 468443 (non-peptide)	
EMD-122946 (non-peptide)	PD 145065 (peptide)		
FR 139317 (peptide)	PD 160672 (non-peptide)		
IPI-725 (peptide)	RO-470203 (bosentan) (non-peptide)		
L-744453 (non-peptide)	RO 462005 (non-peptide)		
LU 127043 (non-peptide)	RO 470203 (non-peptide)		
LU 135252 (non-peptide)	SB 209670 (non-peptide)		
PABSA (non-peptide)	SB 217242 (non-peptide)		
PD 147953 (peptide)	TAK-044 (peptide)		
PD 151242 (peptide)			
PD 155080 (non-peptide)			
PD 156707 (non-peptide)			
RO 611790 (non-peptide)			
SB-247083 (non-peptide)			
Sitaxsentan sodium (non-peptide)			
TA-0201 (non-peptide)			
TBC 11251 (non-peptide)			
TTA-386 (peptide)			
WS-7338B (peptide)			
ZD-1611 (non-peptide)			
Aspirin (non-peptide)			

From (Agapitov and Haynes *Renin Angiotensin Aldosterone Syst.* 3:1-15,2002)

1-6 Biological actions of ET

1-6-1 Action of ET in the vasculature

In VSMCs, activation of ET_A and ET_B by ETs is responsible for persistent vasoconstrictor response, whereas stimulation of ET_B, in the endothelium, causes a transient vasodilatation, which usually precedes vasoconstriction. The vasodilatation is attributed to the release of nitric oxide via activation of endothelial NO synthase. It has been reported that ET-1-induced PKB phosphorylation leads to phosphorylation and activation of NO synthase in endothelial cells (44). Prostacyclin production has been also involved in ET_B-mediated vasodilatation (41). It has been suggested that NO release and vasodilatation are mediated by ET_{B1}, whereas the subsequent vasoconstriction depends on ET_{B2} receptors (45). The ETs increase blood pressure *in vivo* in animals for at least one hour after a bolus dose (1) and in human, intravenous infusion of ET-1 increases blood pressure by 5–10% at doses of about one pmol/kg per min administered for over one hour (46). The coronary and renal vascular beds are most sensitive to the vasoconstrictor effects of systemic ET-1 (47). The mesenteric bed also constricts in response to systemic ET-1, whereas the hindquarter skeletal muscle bed exhibits only a little constriction (48). These differences among beds may be related to differences in constrictor (ET_A and ET_B) and dilator receptors (ET_B) in these beds. The pressor effect of bolus doses of ET is usually preceded by transient hypotension that is most marked for ET-3 (49). When concentrations of ET-1 rise more slowly, hypotension does not occur (50). The hypotensive response to bolus administration of ET is used for demonstrating the endothelial actions of the ETs.

As a long-term effect in VSMC, ETs have been proven to evoke mitogenic activity and this effect seems to be mediated through ET_A receptors activation (51;52). In addition to being a potent mitogen for VSMC, ET-1 also potentiates the mitogenic effects of growth factors such as PDGF, EGF and basic fibroblast growth factor (bFGF) and is considered as a co-mitogen with these growth factors (52).

1-6-2 Action of ET in the heart

The biological effect of ET-1 on cardiac tissue has been reported in several studies. ET has been found to exert a positive inotropic effect on human myocardium in vitro by activation of the sodium proton exchanger and increasing sensitization of cardiac myofilaments to Ca^{2+} (53). Recently, the activation of Na^+/H^+ exchange by ETs has been shown to be mediated by PKC (54). Na^+/H^+ exchange causing an increase in intracellular Na^+ concentration and alkalinisation (37). While the former effect activates the Na^+/Ca^{2+} exchange leading to an increase in intracellular Ca^{2+} concentration and myocardial contractility, the latter (alkalinisation) improves myofibrillar Ca^{2+} sensitivity (37).

At the same time ET-1 evokes prolongation of the duration of the action potential (55). Thus, this pharmacological action of ET-1 may contribute to the pro-arrythmogenic property of the peptide when it is endogenously released or exogenously applied (56). Interestingly, in postmyocardial infarction of heart failure, ET-1 induces more positive inotropic response compared to healthy heart because of a high responsiveness to ET-1 in this pathological state (57). ETs also influence heart function indirectly by inducing vasoconstriction in coronary artery. Furthermore, it has been suggested that, during

exercise, ET-1 level was significantly increased in heart and ET-1 participates in the modulation of cardiac function during exercise in rats (58).

1-6-3 Action of ET-1 in the nervous system

An intracerebroventricular administration of ET in conscious rats, dose-dependently elevated blood pressure and also increased heart rate (59). These effects depend on ET_A receptors activation and not ET_B receptors (60). Many other laboratories have confirmed these results (61;62) which suggested a central pressor action of ET. On the other hand, Makino et al have shown that intracerebroventricular administration of ET increases plasma catecholamine levels suggesting that the central pressor action of ET might be mediated by catecholamine release to the periphery (63). In contrast, however, Yamamoto et al have reported that intracerebroventricular ET-1 stimulates vasopressin secretion leading to increased blood pressure with a reduction in renal water and electrolyte excretion (62). It has been also reported that ET via both sympathetic nervous system and hypothalamo-pituitary-adrenal axis, and through its interaction with brain natriuretic peptide (BNP) in the central nervous system may regulate cardiovascular and hormonal functions. (63).

1-6-4 Action of ET in the Kidney

Many cells in the kidney produce ET-1 (64). Renal ET-1 and receptors localize predominantly in the medulla, and the production of ET-1 in this region exceeds that of anywhere else in the body (65). The contribution of ET-1 to regulation of renal function under normal physiological conditions has been studied by several groups. The effect of

ET-1 in the renal medulla is natriuretic, diuretic and hypotensive. Although, it is largely accepted that ET-1 action promotes sodium and water excretion thereby induce diuresis under physiological circumstances (66-68), other studies have suggested an attenuating role of ET-1 in urinary sodium excretion (69;70). Recently, by using transgenic mice exhibiting collecting duct-specific knockout of ET-1, data from Kohan et al have confirmed the fact that ET-1 promotes sodium and water excretion (71;72). Several potential mechanism by which ET-1 induces sodium and water excretion have been proposed, one of which involves ET-1-induced inhibition of tubular Na^+/K^+-ATPase activity in the proximal tubule and collecting duct (66). In addition, ET-1 has also shown to block reabsorption of water in the collecting duct by inhibiting the effects of anti-diuretic hormone (ADH) on tubular osmotic permeability (67). The natriuretic and diuretic effects of ET-1 appear to occur via ET_B receptor since the effects were specifically blocked only by ET_B receptor agonists and not by ET_A receptor antagonist (73). In addition, it has been reported that ET_B receptor knockout mice have hypertension secondary to renal retention of sodium (19).

Due to its potent vasoconstrictor action, ET-1 has been considered to be important in regulating renal vascular tone. ET-1 contracts afferent and efferent arterioles equally in vitro (74) and thus reduces both renal plasma flow and glomerular filtration rate (GFR) (75). Both ET_A and ET_B receptors participate in ET-1-mediated vasoconstriction of afferent arterioles (76).

1-6-5 Action of ET in the endocrine system

ET-1 has also been shown to stimulate secretion of several hormones throughout the body. ET-1 stimulates both cortical and medullo adrenal hormones, it enhances the release of aldosterone from isolated cortical zona glomerulosa cells (77) and of adrenaline from medullary chromaffin cells (78). ET-1 stimulates production and release of atrial natriuretic peptide (ANP) by cultured atrial myocytes in vitro and in vivo (79;80). An inhibitory role of ET-1 on renin release from isolated rat glomeruli (81) and from rat juxtaglomerular cells (82) has also been reported. On other hand ET-1 has been reported to stimulate endothelial ACE activity (83). Taken together these findings show that ET-1 has contrasting effects on the renin–angiotensin system. ET-1 and ET-3 have also been found to be implicated in the release of testosterone by stimulating basal and gonadotrophin-induced testosterone production although the effects of ET-3 were less marked in rat Leydig cells (84).

In adipose tissue, ET-1 inhibits adiponectin secretion through a phosphatidylinositol 4,5-bisphosphate/actin-dependent mechanism (85). It has been reported that a decrease in adiponectin (secreted by white adipose tissues) expression and secretion have been positively correlated with a decrease in insulin sensitivity (85). More recently, by using RT-PCR and real-time PCR analyses, Chai et al have proposed another mechanism by which ET-1 evokes insulin resistance, they have demonstrated that ET-1 is able to increase IL-6 secretion from adipocytes which is a critical step for insulin resistance.(86).

1-7 Role of ET-1 in cardiovascular diseases

Because of the ability of ET system to modulate a wide variety of cellular function it has been implicated in the pathophysiology of cardiovascular diseases such as, hypertension, atherosclerosis, coronary artery disease, heart failure as well as in diabetes, primary pulmonary hypertension, pulmonary fibrosis, scleroderma, renal failure, prostate cancer and its metastasis (87-90).

1-7-1 ET-1 in hypertension

1-7-1-1 ET-1 in experimental hypertension

Significant increases in plasma ET-1 levels are consistently seen in only certain models of hypertension. Deoxycorticosterone acetate (DOCA) salt-hypertensive rats, Dahl salt-sensitive rats, Ang II-induced hypertension, 1-kidney 1-clip Goldblatt hypertensive rats, and stroke-prone spontaneously hypertensive rats (SHRs) are among the models that exhibit an increase in systemic levels of ET-1 (91;92). As a consequence of the enhanced production of ET-1 in these models, ET-1 contributes to the remodeling of arteries in hypertension (93). Interestingly, the models of experimental hypertension that evoke increase in ET-1 systemic level also exhibit hypertrophic remodeling of resistance arteries with increased cross-sectional area, which is believed to be the results of the ET-1 action (94), rather than the eutrophic remodeling without true vascular hypertrophy often found in essential hypertension and in spontaneously hypertensive rats (93).

Flamant et al have suggested a critical role of EGF receptor transactivation in the vascular fibrotic response that is associated with ET-1-dependent vascular remodelling (95). It has also been reported that ET-1 stimulates DNA synthesis of VSMC which is an

index of proliferation and remodelling, via ET_A receptor (52) and through transactivation of EGFR (96). Studies using vessels from male Wistar rats have revealed the mechanism by which ET-1 induces remodelling. These studies have suggested that ET-1- induced inward eutrophic remodelling seems to response to sustained contraction, which involves collagen reorganization through β_3-integrins (97). It has been reported that ET-1 activates NADPH oxidase in VSMC and in blood vessels (98) and ET-1-induced proliferation may be mediated partly by increased ROS production (99). In aldosterone-induced hypertension systolic blood pressure, plasma ET-1, systemic oxidative stress, and vascular NADPH activity was increased. Enhancements of collagen, fibronectin and intercellular adhesion molecule (ICAM-1) have been shown to be associated with small artery hypertrophic remodeling in this hypertensive model (100). In the same study, BMS 182874, an ET_A receptor antagonism, attenuated vascular remodeling, fibrosis and oxidative stress as well as adhesion molecule expression in aldosterone-induced hypertension (100). Involvement of ET-1 in renal and cardiac target organ damage in hypertension has been documented. Studies have confirmed an implication of ET-1 in renal fibrosis through stimulation of growth factors and by inducing inflammation in hypertensive rats (101). In these studies an increase in renal ET-1 in hypertensive rats was shown to be associated with enhancements in transforming growth factor (TGF)-β1, basic fibroblast growth factor (bFGF), procollagen I expression and matrix metalloproteinase (MMP)-2 activity. In addition, it was also demonstrated that a selective ET_A antagonist was able to normalize these events. (101). Although, the role of ET-1 in promoting renal fibrosis has been confirmed, implication of ET-1 in renal damage associated with hypertension is still controversial. It has been demonstrated that in Ang

II-infused mice, the non-selective ET-1 receptor blocker, bosentan, prevented activation of the procollagen gene (102). By using the same blocker, Muller et al have shown a reduction in renal damage in rats overexpressing human angiotensinogen and human renin, that develop hypertension (103). On the other hand, Rothermund et al have suggested that ET-1 is not involved in renal damage and mortality in primary renin-dependent hypertension (104). Recently, it has been demonstrated that a selective ET_A receptor blockade not only reduces podocyte (cells of the visceral epithelium in the kidneys) injury and end-organ but also improves growth and survival independently of hypertension (105). In the heart, ET_A (106) or combined ET_A/ET_B (103) or ECE inhibitor (107) blockade prevented target organ damage in hypertension animal models. Recently, Vanourkova et al gave evidence showning that blockade of the ET-1 system prevented the rise in cardiac ET-1 concentration in transgenic rats with inducible malignant hypertension. They suggested that ET-1 receptor blockade may provide evidence for ET blockade as a tool to protect rats from hypertensive cardiac damage (108).

1-7-1-2 ET-1 in essential hypertension.

Systemic concentration of ET-1 does not reflect the real production of ET-1 because ET is generated and acts locally. Studies investigating the role of ET in essential hypertension have revealed no change in plasma ET-1 concentration in hypertensive patients as compared with normotensive (4), whereas, plasma ET-1 level rises in African Americans hypertensive subjects (109). It has been shown that the increases of plasma ET concentration seem to be related to aging, smoking and renal dysfunction rather than essential hypertension (110). ET_A receptor antagonists cause vasodilatation in forearm

vessels of both normotensive and essential hypertensive patients (111), and improve impaired vasodilation in hypertensive patients. On the other hand, the ET_B antagonist induces vasoconstriction on forearm resistance arteries in normotensive subjects (112;113) and had a vasodilator action on the forearm circulation of hypertensive subjects (113), indicating that a vasoconstrictor effect of ET_B receptors could be found in hypertensive but not normotensive individuals. African Americans patients appear to have increased numbers of smooth muscle vasoconstrictor ET_B receptors (109;114), which may explain the important role of the ET-1 system in these subjects. Both selective and dual-acting ET-1 receptor blockers can reduce systemic blood pressure in animal models and in hypertensive patients. Clinical studies recruiting 293 patients with mild-to-moderate essential hypertension revealed that bosentan, an antagonist of both ET_A and ET_B receptors, was able to significant lower diastolic blood pressure in these patients. This reduction was similar to that observed with the ACE inhibitor enalapril (115). Furthermore, in animal experimentation, combination of ET-1 receptor antagonist, bosentan and ACE inhibitor leads to additional hypotensive effect in hypertensive dogs (116). The selective ET_A antagonist darusentan reduced systolic blood pressure by 6.0 to 11.3 mm Hg (117). Elevation of liver enzymes, a side-effect found with bosentan, was not encountered with darusentan in this study. Recently, safety and efficacy of darusentan have been evaluated in the treatment of hypertension and heart failure (118). Although, the effect of ECE inhibitor has not been studied in essential hypertension in human, encouraging results have been reported in SHR where phosphoramidon, an ECE inhibitor, lowered mean arterial pressure when infused in conscious SHRs (119).

Data from recent study shows that moderate aerobic exercise reduces ET-1-mediated vasoconstrictor tone. These reductions in ET-1 system activation may be the mechanism by which exercising contribute to the known beneficial affects to prevent or treat hypertension. (120). Finally, as mention above, aging is cardiovascular risk factor which exhibits increased ET-1 system activation which may be one cause of aging-promoted hypertension (121).

1-7-2 ET-1 in atherosclerosis

Endothelial cell injury, inflammation, monocyte infiltration of the vessel wall, cytokines and growth factors releases, migration of VSMC to the intima, and lipid accumulation in foam cells are the main characteristics of atherosclerosis (87). Evidence from several groups has suggested the involvement of ET-1 in development and progression of atherosclerosis (87;122). It has been documented that ET-1 is a chemoattractant for monocytes and macrophages and acts as a comitogen for VSMC (123;124). Lerman et al have shown a significant increase in plasma ET-1 concentration and this increase is correlated with the number of atherosclerotic lesions in atherosclerotic patients (125). Together with the fact that ET-1 has a short half-life in the circulation (126), these raised plasma levels are likely to be due to increased tissue ET-1 production. Upregulation of ET-1 and ET receptors have been demonstrated in atherosclerotic lesions in human and experimental animal models (127). Recently, another parameter of ET system which is ECE-1, has been shown to be significantly increased in apolipoprotein E-deficient (apoE) atherosclerotic mice (128). By studying ET-1 mRNA expression in normal and atherosclerotic human coronary arteries, Winkles et al. have suggested that endothelial

cells, monocyte-derived macrophages and VSMCs within the atherosclerotic lesion are capable of synthesising ET-1 (129). In the same regard, it has been reported by using cultured human VSMC from atherosclerotic coronary arteries that ET-1 production is markedly increased in coronary atherosclerotic plaques which may contribute to the development or progression of coronary artery disease (130). Both ET_A and ET_B receptors have been shown to be linked to atherosclerosis pathology. It has been demonstrated that both receptors are localized in endothelial cells, smooth muscle cells and macrophages in atherosclerotic plaques in hyperlipidemic hamsters (131). Moreover, chronic ET_A receptor blockade normalized NO-mediated endothelial dysfunction and reduced atheroma formation in atherosclerotic apolipoprotein E-deficient mice (132). In human, infusion of the selective ET_A receptor antagonist BQ123, improves coronary vascular function in patients with atherosclerosis. These data suggest that ET-1 receptor blockade may be a new therapeutic strategy to improve coronary vascular function in patients with atherosclerosis (122). Additional mechanism by which ET-1 induces atherosclerosis has been revelled recently. Ballinger et al have demonstrated that ET-1, via ET_A receptors, induces changes in the structure of glycosaminoglycan (GAG) that increases its binding to low density lipoprotein (LDL) and modifies its lipid binding proprieties within the vascular wall (133).

Neointimal formation is the major cause of vessel occlusion observed following balloon angioplasty. Injury to the vascular endothelium and underlying tissue initiates intimal thickening with migration of medial VSMCs towards the intima where they change from the contractile to synthetic phenotype. The resultant thickened intimal layer forms a pronounced neointima, narrowing the vessel lumen and acting as pro-atherosclerosis

(134). ET_A receptors have been identified on proliferating cells in the neointima of porcine vein grafts and balloon-injured porcine coronary arteries (135). Infusion of exogenous ET-1 has been shown to potentiate the development of intimal hyperplasia following balloon catheter injury (136) whilst mixed ET_A/ET_B receptor antagonists or ECE inhibitor reduced angioplasty-induced neointima formation (135;137;138) which further support a role of ET-1 system in this process.

1-7-3 ET-1, Gi protein and hypertension

Guanine nucleotide regulatory proteins (G proteins) are a family of GTP-binding proteins that play an important role in the regulation of a variety of signal transduction systems, including the adenylyl cyclase/cAMP system. The activity of adenylyl cyclase is regulated by two G proteins, Gs (stimulatory) and Gi (inhibitory). Alterations in the levels of Gi proteins and cAMP levels that result in the impaired cellular functions lead to various pathological states such as hypertension. Studies have been shown an increased expression of G_i proteins and G_i protein mRNA in hearts and aortas from spontaneously hypertensive rats (SHR) and in hearts from experimental hypertensive rats including deoxycorticosterone acetate (DOCA)-salt hypertensive rats and 1 kidney 1 clip (1K1C) rats with established hypertension with established hypertension (139-142). VSMC from SHR exhibited enhanced levels of Giα protein as compared to WKY rats, which were restored to control levels by antioxidants, suggesting a role of oxidative stress in this process. (143). ET-1 treatment has been shown to increase both Giα-2 and Giα-3 expression without affecting Gsα expression level in A10 VSMC (144) this may explain the mechanism by which ET-1 contribute to established hypertension. Similar to ET-1,

Ang II and arginine vaspressin have been also reported to increase the levels of $G_i\alpha$ proteins in VSMC, whereas atrial natriuretric peptides (ANP) and nitric NO, which increase cGMP levels, decreased $G_i\alpha$ protein expression in the cells (144-146). In this context, it seems that ET-1 and NO have opposite action on Giα expression and adenylyl cyclise activity which explains le vascular protection effect of NO as well as the mechanism by which ET-1 contribute to establish hypertension.

1-7-4 ET-1 in heart failure

Circulating ET-1 levels have been shown to be increased not only in animal model but also in humans with heart failure (147;148). In addition, the degree of plasma levels of ET-1 correlates with the magnitude of alterations in cardiac hemodynamics and functional class (149). It seems that low cardiac output, observed in heart failure, serves as a stimulus for endot release because very high levels have been documented in animals with low cardiac output and low ventricular filling pressures produced by thoracic inferior vena cava constriction (150). Both selective (ET_A receptor) and non-selective (ET_A/ET_B receptors) ET receptor inhibitor have been shown to be beneficial in limiting heart failure complication. Indeed, oral ET-receptor antagonist, bosentan, improved systemic and pulmonary hemodynamics in heart failure patients. (151), same results have been seen with a selective ET_A receptor inhibitor (152). In fact a selective ET_A receptor blockade improved cardiac index in patients with congestive heart failure (152). Ohnishi et al compared the effects of selective ETA and mixed ET A/B receptor antagonism using FR139317 and TAK-044, respectively, in conscious dogs with heart failure. Both agents improved cardiac function in these studies (153). Recently, by using anesthetized rats,

Rufanova et al have shown that myocardial contractility was restored and cardiac relaxation significantly improved after the application of PP36 an inhibitor of ECE application suggesting a crucial role of ET production in this pathophysiology state (154).

1-8 ET-1induced signaling in vascular smooth muscle cells

ET-1 exerts its physiological actions through the activation of multiple signaling pathways which include the PLC/DAG/IP3, MAPKs, and PI3-K/PKB pathways. As shown in Fig. 4 and 8, many receptor and non-receptor tyrosine kinases also play a role in initiating the ET-1-induced signaling events. The cellular events triggered by the activation of these signalling pathways play important role in regulating the cellular growth, proliferation, contraction and survival of VSMC, and aberration in this pathways results in the pathological states.

1-8-1 ET-1-induced activation of phoshoinositide cascade

The binding of ET-1 to its receptor activates heterotrimeric guanine nucleotide binding (G) proteins such as Gq which is the best characterized signal transducer for ET_A receptor. 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 with the membrane (35;155). In the inactive Gq heterotrimer, αq is ligated to guanosine diphosphate (GDP) and exchange of GDP for guanosine triphosphate (GTP) on αq leads to the dissociation of αq(GTP) and βγ and both remain associated with the membrane (35). Their dissociation leads to the activation of phosphoinositide-specific phospholipase C β (PLC β) (156;157), which

then hydrolyzes the membrane phospholipid, phosphatidylinositol-4',5'-bisphosphate [PtdIns(4,5)P_2] to generate two second messengers: hydrophobic diacylglycerol (DAG), which remains in the membrane, and soluble inositol-1',4',5'-trisphosphate [Ins(1,4,5)P_3] (156;157). Ins(1,4,5)P_3 diffuses into the cytoplasm and activates calcium channels of the sarcoplasmic reticulum, which leads to an increase in Ca^{2+} levels in the sarcoplasm and cell contraction. DAG together with Ca^{2+} activates the phosphatidylserine-dependent protein kinase, protein kinase C (PKC) (Fig.4). The PKC family of serine/threonine kinases have been subdivided into three groups: the classical or conventional PKCs, which require DAG and Ca^{2+}, include the isoenzymes α, β and γ (158), the novel PKC (nPKC) which are DAG-dependent, but Ca^{2+}-independent, include the isoenzymes δ, ε, η, θ, μ and ν (158;159) and the atypical PKC (aPKC), which are independent of DAG and Ca^{2+} and include ξ and λ isoenzymes (158;160). ET-1 has been shown to activate PKC in cardiomyocytes (161) and in other cells (162;163). Many studies have implicated PKC in the deleterious vascular effects of a variety of pathologies including diabetes and hypertension (164). PKC has been shown to be involved in ET-1-induced signalling in VSMCs (165;166). Moreover, growing evidence suggests that PKC activity modulates proliferation (166;167) and contraction of VSMCs evoked by ET-1 (168). Recntly, by using human VSMC, Chen et al have reported an implication of PKC in ET-1-induced ERK activation through ET_A receptors (169). Thus, ET-1-induced activation of PKC and its downstream effects appear to be important in regulating vascular functions.

In addition to PLC via Gq, ET_A and ET_B receptor subtypes are functionally coupled to adenylate cyclase, via Gs in VSMC and Gi in endothelial cells respectively. (170;171). It

has been also reported in other cell types that the calcium signal induced by ET-1 is consequent to both an activation of PLC and inhibition of the calcium pump, both effectors being coupled to the ET_B receptor by different G proteins, Gq and Gs, respectively (172).

1-8-2 Calcium calmodulin system in ET-1 signaling

It has been shown that, in VSMC, ET-1 evokes increase in intracellular calcium concentration via a stimulated plasma membrane Ca^{2+} entry and/or sarcoplasmic reticulum Ca^{2+} release (173-175). Cytoplasmic Ca^{2+} is tightly controlled and in the unstimulated state it is believed to be around 100 nM. In response to various signalings this concentration can rise up to 300-500 nM this increased Ca2+ signals participates in many cellular functions including short-term contractile, secretory, or metabolic responses to longer term regulation of transcription, growth, and cell division (176).

Through the activation of PLCβ, ET-1 generates the second messengers IP3 and DAG. IP3 diffuses rapidly within the cytosol and interacts with IP_3 receptors (IP_3R) located on the endoplasmic reticulum, which function as calcium channels to release calcium stored in the endoplasmic reticulum lumen and generate the initial calcium signal phase as mention in the previously section. The resulting depletion of calcium stored within the endoplasmic reticulum lumen functions as the primary trigger for a message that is returned to the plasma membrane. The lowered luminal Ca^{2+} causes dissociation of Ca^{2+} bound to the low-affinity EF-hand Ca^{2+} -binding site on the N-terminus of *st*romal-*i*nteracting *m*olecule (STIM)1, which is considered as a ‘‘sensor’’ of Ca^{2+} within ER Ca^{2+} store (177). This Ca^{2+} dissociation causes STIM1 molecules to aggregate and be translocated close to the plasma membrane (177). At this level STIM1 interacts directly

with Orai1 protein, (178) which serve as highly Ca^{2+}-selective store-operated channel (179) and allow entry of external calcium. This sustained calcium entry phase mediates longer term cytosolic calcium signals and provides a means to replenish intracellular stores (180).

The other product of PLC activation, DAG, also has important effects on calcium entry channels. Transient receptor potential channels (TRPC) have been reported to play a major role in GPCR-induced Ca^{2+} entry in VSMC and other cell types (180;181). DAG has been shown to triggers Na^{+} entry via TRPC6 activation (178;182) which in turn induces membrane depolarization resulting in activation of Ca^{2+} entry through L-type voltage-activated Ca^{2+} channels (178).The TRPC6 channels also appear to be activated by mechanical stretch, resulting again in depolarization and L-type Ca^{2+} channel activation (183).

An important effector of Ca^{2+} is calmodulin (CaM) and many diverse actions of Ca^{2+} and CaM signaling are known to be mediated through Ca^{2+}/CaM-dependent protein kinases (CaMK). CaMKs are serine/threonine kinases that are regulated by Ca^{2+} liganded CaM. Myosin light chain kinase (MLCK) and phosphorylase kinase are Ca^{2+}/CaM-dependent protein kinases which are dedicated to a particular substrate. Elongation factor-2 (EF-2) kinase (originally termed CaMKIII) is also a kinase dedicated to the phosphorylation of a single substrate (184). In contrast, CaMKs are multifunctional CaMKs (185) some member of this family e.g. CaMKI, II and IV exhibit a board substrate specificity whereas others members are more selective. In the context of cardiovascular system, CaMKII has generated a lot of interest because of its potential involvement in myocardial physiology and diseases (186)

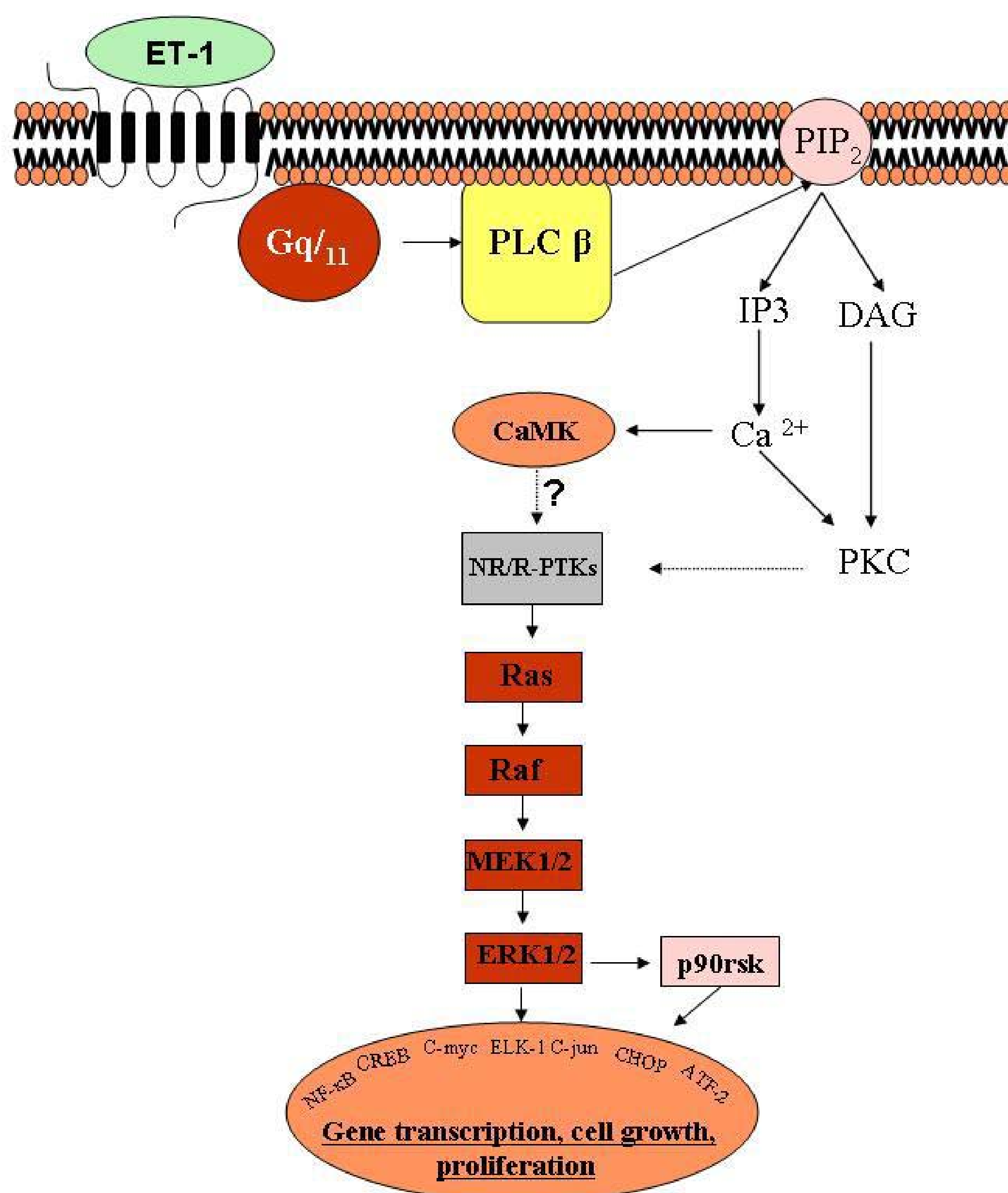

Fig.4: Activation of MAPK pathway by ET-1 in VSMC. ET-1 receptor stimulation leads to G-protein bγ activation which activates PLC β. Activated PLC β converts PIP2 to IP3 and diacylglycerol (DAG). IP3 elevates the concentration of intracellular calcium and participates in muscle contraction. DAG activates PKC. PKC and/or Ca^{2+}/Calmodulin (CaM)-dependant protein kinase (CaMK) activate receptor and non-receptor tyrosine kinases. Activation of these components signals the stimulation of Ras/Raf/ MEK /ERK1/2, p38 mapk and JNK. The MAPK family members are translocated to nucleus and regulate nuclear events by activating transcription factors through phosphorylation.

1-8-2-1 CaMKII

There are four known isoforms of CaMKII (α, β, γ, δ) which are encoded by separate genes. α and β-CaMKII are the predominant neuronal isoforms and the δ and γ isoforms are expressed in diverse tissues including the heart (187). In heart, the predominant isoform is δ (188) whereas both α and δ isoforms have been shown to be express in VSMC (189;190).

1-8-2-1-1 Structure of CaMKII

Monomeric structure

The CaMKII monomer consists of an NH_2-terminal catalytic domain, a centrally located regulatory domain, and a COOH-terminal association domain (Fig.5). The catalytic domain is needed to catalyze the transfer of the phosphate from ATP to serine or threonines embedded within a CaMKII consensus motif (191). The regulatory domain contains a pseudosubstrate sequence that, under basal conditions, binds and constrains the catalytic domain. A CaM binding domain is located in the regulatory domain. The pseudosubstrate sequence is found around an activating "autophosphorylation" site at Thr^{286} for α isoform (Thr^{287} for δ) (192) and a recently identified activating oxidation site (Met^{281}/Met^{282}) (193). Phosphorylation of Thr^{286} or oxidation of Met^{281} and Met^{282} prevents the re-association of the catalytic and regulatory domains even after dissociation of Ca^{2+}/CaM complex, thereby enhancing the CaMKII signal. An inhibitory autophosphorylation site, Thr 306/307, reacts with the Ca^{2+}-CaM binding sequence. Basal phosphorylation occurs preferentially at Thr^{306}, preventing Ca^{2+}/CaM binding, resulting in decreased CaMKII activity (194). The association domain is responsible for the assembly of CaMKII monomers into the holoenzyme.

Holoenzyme structure

The partial structure of the CaMKII holoenzyme has been determined by crystallographical studies. These studies have revealed that the ~600 kDa holoenzyme consists of two central rings stacked on top of each other, with a diameter of ~145 Å, formed by interaction of multiple association domains. This central ring serves as the scaffold from which an outer ring, consisting of the regulatory and catalytic domains,

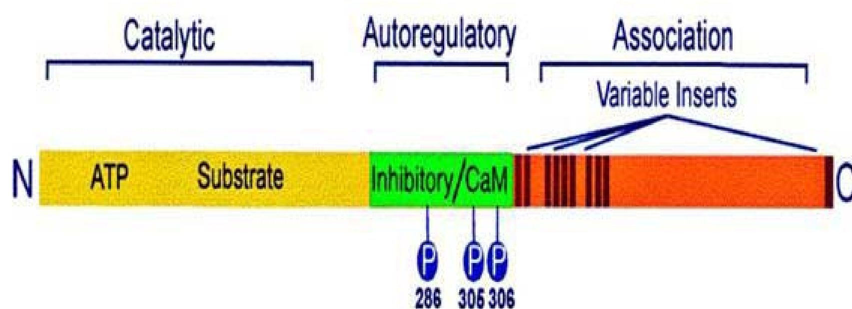

Fig.5 Linear diagram of a prototypical CaMKII subunit. The catalytic domain is autoinhibeted by pseudosubstrate autoregultory sequence that is disinhibited following Ca2+/CaM binding. The association domain produces the native form of the enzyme, a multimeric holoenzyme composed of 12 subunits. Isoform differences present in the α, β, γ and δ isoforms of CaMKII are contributed primarily by a region of multiple alternatively spliced sequences, termed variable insert, which reside in the association domain. Conserved site of autophosphorylation are indicated in autoregulatory domain.
From (Hudmon and Schulman *Biochem. J.* 364: 593-611, 2002)

arises (195). The holoenzyme is either a dodecameric (196;197) or a tetradecameric (195) structure. This arrangement allows for a high concentration of catalytic domains that can

interact with target proteins, including adjacent CaMKII monomers within the holoenzyme. CaMKII autophosphorylation (at Thr^{286} for α or Thr^{287} for δ) critically depends on the arrangement of monomers within the holoenzyme. In vitro studies revealed that the holoenzyme structure is critical for autophosphorylation graded activity responses related to the frequency and duration of intracellular Ca^{2+} transients (198).

1-8-2-1-2 Activation of CaMKII

CaMKII requires Ca^{2+}/CaM for activation (Fig.6). CaM is a bi-lobed intracellular protein that contains four Ca^{2+}-binding EF hands (2 EF hands in each lobe). Under basal conditions, CaMKII is inactive because of intramolecular binding of the catalytic domain to the regulatory domain. However, a fraction of CaMKII is active even under this condition, because autophosphorylated $Thr^{286/287}$ is detectable even in quiescent cells (199). This inhibitory interaction between the catalytic and regulatory domains prevents substrate and ATP binding (200-202). Calcified CaM (CaM and Ca^{2+} complex) binds to the regulatory domain, inducing a conformational change that frees the catalytic domain from its pseudosubtrate (203). The allosteric rearrangement of CaMKII upon binding with Ca^{2+}/CaM allows access to the ATP binding pocket, which in turn allows CaMKII to catalyze the transfer of a phosphate donor group to downstream targets, including its autophosphorylation. Autophosphorylation, which occurs by an intra-holoenzyme reaction (204), has several important implications for CaMKII activity. First, $Thr^{286/287}$ autophosphorylation results in a 1,000-fold increase in the affinity for binding with CaM, a property known as "CaM

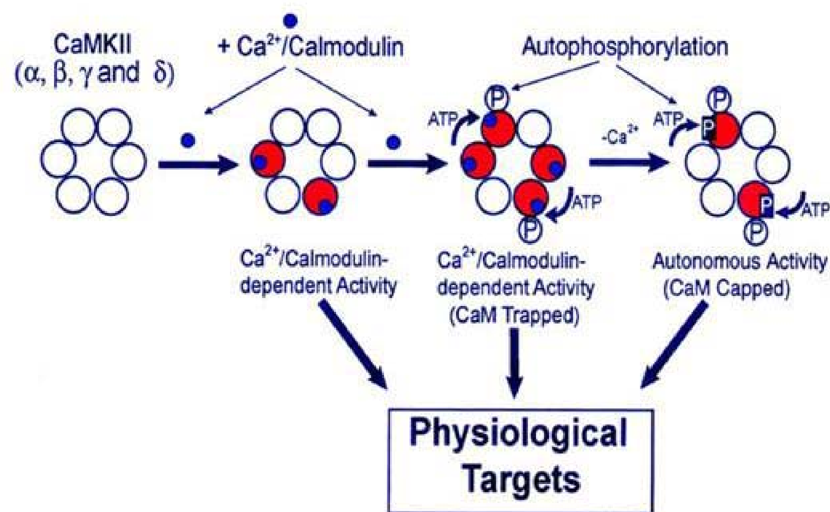

Fig.6 CaMKII undergoes multiple autoregulatory states that may have an impact on its function following Ca^{2+}/CaM activation From (Houdmon and Schulman *Biochem. J.* 364, 593-611, 2002)

trapping" (205). Second, autophosphorylation results in the ability of the kinase to maintain catalytic activity even in the absence of CaM binding (192). Under resting conditions, phosphorylation occurs preferentially at Thr^{306}, preventing Ca^{2+}/CaM binding, which in turn results in decreased CaMKII activity.

1-8-2-1-3 Role of CaMKII in heart diseases

There is a growing evidence for a pathophysiological role of CaMKII in cardiac hypertrophy and heart failure for review, see reference (206). ET-1 has been shown to increase the activity of CaMKII and induces hypertrophy in cardiomyocyte. Pretreatment with KN-62, a CaMK inhibitor, was able to suppress ET-1-induced cardiomyocyte hypertrophy measured by β-myosin heavy chain promoter activation and [^{3}H] phenylalanine uptake (207). Hypertrophic responses induces by Leukemia inhibitory factor (LIF), a glycoprotein cytokine, was also attenuated by CaMKII inhibitor in cardiomyocytes (208). Furthermore, an increase in CaMKII expression (209) and activity (210) has been reported in hypertrophied myocardium from spontaneously hypertensive rat (SHR). More recently, by using hearts extracted from CaMKIIδ–null mice, a critical role of CaMKII in pathological cardiac hypertrophy and remodeling has been reported (211). CaMKII upregulation and activation has been found to play a role in development and maintenance of heart failure (208). Further evidence linking CaMKII and heart failure comes from studies suggesting that mice with genetic CaMKII inhibition reverse heart failure-associated changes (212). The mechanism by which CaMKII induces its cardiac alteration seems to be modulation of ryanodine receptor thereby regulating the Ca^{2+} leak from sarcoplasmic reticulum (213). CaMKII activation plays a role not only in

heart failure and cardiac hypertrophy but also in cardiac arrtythmias. It has been reported that CaMKII is a proarrhythmic signaling molecule in cardiac hypertrophy in vivo. (214) and calmodulin kinase inhibition prevents development of the arrhythmogenic transient inward current. (215).

1-8-2-1-4 Role of CaMKII in vascular diseases

Similar to the cardiac system, an involvement of CaMKII in regulating vascular function has also been suggested. It has been shown that CaMKII regulates proliferation of VSMC in thoracic aorta, (216) and in rat carotid arteries (217). Others studies indicated involvement of CaMKII in cell migration. siRNA-mediated suppression of CaMKIIδ_2 resulted in the inhibition of wound-induced Golgi reorganization and ERK1/2 activation leading to cell migration (218) and insulin-stimulated cGMP inhibits VSMC migration by inhibiting CaMKII (219). In addition, by using an antisense knockdown of CaMKII gamma, it has been reported that CaMKII is essential for ERK-mediated signaling in differentiated smooth muscle cells (220). This notion is further supported by studies showing that pharmacological blockade of CaMKII by KN-93 improved vascular hyperplasia and hypertension in AngII-induced hypertensive rats (221), and normalized aberrant vascular reactivity in diabetes-induced vascular dysfunction (222;223). Furthermore, norepinephrine and Ang II-induced arachidonic acid release, which is considered as the first step of inflammation, have been shown to be mediated by CaMKIIα activation in rabbit aortic smooth muscle cells (189;224). More recently, CaMKII has been also found to be induced in smooth muscle cells during the response of an artery to injury and is a positive regulator of proliferation and migration in the vessel

wall contributing to neointima formation and vascular remodeling (217). Since a hightened proliferation and migration of VSMC are hallmarks of vascular disease, CaMKII has been suggested to play an important role in the pathogenesis of vascular diseases (221;222).

1-8-3 ET-1-induced activation of MAPK cascade in VSMC

ET-1 receptor activation also leads to the stimulation of MAP Kinase cascade (Fig.4). The first component of this cascade is Ras, a member of the small GTP-binding protein family. Ras cycles between an active GTP-bound conformation and an inactive GDP bound form (225). Once activated, Ras, bound to membrane, recruits Raf, also known as mitogen activated protein kinase kinase kinase MAPKKK (226). Raf phosphorylates MEK or MAPKK at specific serine/threonine residues, which in turn, phosphorylates MAPKs, such as extracellular signal regulated kinases 1 and 2 (ERK1/2) on threonine and tyrosine residues (227). MAPKs are serine/threonine protein kinases, which are activated in response to a variety of external stimuli such as growth factors, hormones and stress (228-233). Several reports have demonstrated that ET-1 activates ERK1/2 signaling pathway in many cell types including cardiomyocytes (6), fibroblasts (234), glomerular mesangial cells (235) and VSMCs (10). ERK1/2, $p38^{mapk}$ and c-Jun N-terminal kinases (JNK) are the principal MAPKs (236;237). MEK1/2 phosphorylate ERK1/2, which promote growth signaling, whereas, MEK 4/7 and MEK 3/6 phosphorylate JNK and $p38^{mapk}$ respectively, which control survival, differentiation and inflammation (41;230;237;238). Several studies have suggested that the activation of MAPKs in response to ET-1 was responsible to promote proliferation in VSMC

(10;239). It has been demonstrated that ERK5, a new member of the MAPK family, which is involved in cell differentiation and cell cycle, is activated by ET-1 and angiotensin II , another vasoactive peptide with similar effects as ET-1, in VSMCs (240). A role of MAPKs activation in regulating the contraction of VSMCs in response to ET-1 and angiotensin II has also been reported (241-243).

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 (244). Also, ERK1/2 and other MAPK family members are translocated from the cytosol to the nucleus (237;245) and phosphorylate many transcription factors which lead to activation of genes involved in growth and differentiation (237;246)(Fig.4). ET-1 also activates (but to a lesser degree than ERKs) JNK and $p38^{mapk}$ cascades in cardiomyocytes (6;247), in VSMC (248) as well as in mesangial cells (8). In addition to Ras, several other small G-protein families such as Rho, Rab and Ran have been shown to be stimulated by ET-1 (225). ET-1 activates members of the Rho family in cardiomyocytes (249) and fibroblasts (250), which are positive regulators of $p38^{mapk}$ pathway (251). The precise events that mediate ET-1 induced activation of Ras/Raf/MEK and ERK1/2 remain poorly characterized, however, there is some evidence supporting the involvement of PKC in Ras activation in many cell types including cardiomyocytes (161) and rat myometrial cells (162). 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

(252;253) and rat kidney mesangial cells (254). Pyk2 is also activated by ET-1 in VSMCs and may serve as an upstream regulator of MAPK cascade in VSMCs (10). In several other cell types, Ca^{2+}- and PKC-dependent Pyk2 activation (255) has been shown to link GPCRs to upstream regulators of MAPK-signaling, such as Src, Shc, Grb2, son of the sevenless (SOS) and the Ras guanosine nucleotide exchange factor (254;256;257). Previous data has 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 leads to c-Src activation in many cell types including mesangial cells (254;256) and cardiomyocytes (258). Activated c-Src bound to Pyk2 might directly phosphorylate adjacent cellular proteins, such as p130Cas, which then act as docking protein to recruit its effectors able to activate JNK (259;260) in cardiomyocytes (247). Other studies have suggested that CaMKII may serve as an upstream regulator of MAPK pathway in VSMCs through the activation of Pyk2 (261), however a role of CaMKII in ET-1 evoked ERK1/2 activation is not Known.

1-8-4 ET-1-induced PI3-K activation in VSMC.

Phosphatidylinositol-3 kinases (PI3-Ks), which are a family of both protein and lipid kinases have also emerged as an important effector of ET-1 action. PI3-K catalyzes the transfer of phosphate from ATP to the 3'position of the inositol ring of the membrane-localized phosphoinositides such as phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns-4-P) and phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P_2) and generates PtdIns-3-P, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 respectively (Fig.7). These phospholipids act as second messengers to activate several proteins kinase such as

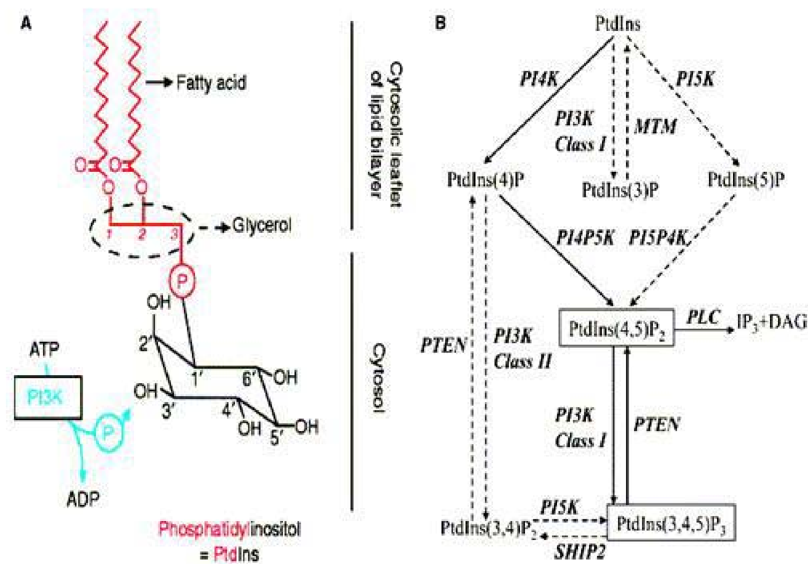

Fig. 7 Major pathways of phosphoinositol metabolism. (A) Structure of phosphoinositol (PtdIns) which is phosphorylated on the myoinositol head group to produce different phosphoinositides (B) Metabolic pathways involved in PtdIns metabolism and the key role of lipid kinases and phosphatases. IP3, inositol 1,4,5-triphosphate; DAG, diacylglycerol. Solid lines refer to well-established pathways.
From (Oudit GY et al *PJ Mol Cell Cardiol*. 37:449-71, 2004).

PtdIns-3,4,5-P_3 dependent protein kinase (PDK), protein kinase B (PKB)/Akt and 70 KDa ribosomal protein S6 kinase ($p70^{S6K}$)(262).

PI3-Ks are divided into three classes based on their structure and mechanism of regulation (262). Class I PI3-Ks 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 (263). Class II PI3-Ks generate PtdIns-3-P and PtdIns-3,4-P_2 and possess a lipid binding domain, whereas, Class III PI3-Ks generate PtdIns-3-P only (263). Class I PI3-Ks are ubiquitously expressed and represent the dominant form in cardiovascular tissues (264)(Fig.7). Class I PI3-Ks are subdivided further into class IA and IB, and are heterodimeric proteins consisting of a catalytic and a regulatory (accessory) subunit. The catalytic subunits of class IA- PI3-K exist in three isoforms: p110α p110β and p110δ, among these, p110α has more ubiquitous distribution than p110β and p110δ. Similarly, the regulatory adaptor subunit for classIA-PI3-K also has several isoforms: p85α ,p85β and p55γ which are products of three genes and their splice variants (237). The class IA PI3-Ks are believed to be typically activated in response to tyrosine kinase coupled stimuli. In contrast to class IA PI3-K, the class IB enzyme (PI3-Kγ) consist of a catalytic subunit PI3-K (p110γ) and a regulatory subunit (p101) and are usually activated by G βγ subunits of G proteins liberated by GPCR activation (264).

Except for rat glomerular mesangial cells where ET-1 was shown to directly increase the catalytic activity of PI3-K (161), a direct activation of PI3-K in VSMC has not been documented. However, an involvement of PI3-K as an upstream mediator of several ET-1 induced responses has been reported. For example, a role of PI3-K in ET-1-induced Ca^{2+} influx in carotid artery and in basilar artery contraction was recently shown by using

wortmannin and LY 294002 specific inhibitor of PI3-K (265;266). In mesangial cells, ET-1 receptor activation has been shown to stimulate PI3-K phosphorylation through Ras (257). Also, in rabbit internal carotid artery VSMCs, PI3-K appeared to be involved in ET-1-induced Pyk2 tyrosine phosphorylation (265). Conversely, studies using angiotensin (Ang II), a vasoactive peptide with similar effects to ET-1, have suggested that Pyk2 regulates PI3-K cascade specifically via interaction of Pyk2 with p130Cas which lead to their association with PI3-K in VSMC (49). Moreover, the recent work demonstrating that mice lacking PI3-Kγ are protected from angiotensin-evoked smooth muscle contraction and hypertension provides further support for an important role of PI3-K signalling in vascular pathobiology (267).

1-8-5 ET-1-induced PKB activation in VSMC.

Several downstream targets of PI3-K have been identified. However, the most widely studied target is PKB, also known as Akt (a product of akt proto-oncogene). PKB is a serine/threonine kinase with three identified isoforms in mammalian system: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (268-270). They are activated by dual phosphorylation on threonine and serine residues (Thr^{308} and Ser^{473} for PKBα, Thr^{309} and Ser^{474} for PKBβ and finely Thr^{302} and Ser^{472}) for PKB γ (271) and contain a central catalytic kinase domain with specificity for serine or threonine residues in substrate proteins (271). N-terminal of PKB possesses a pleckstrin homology (PH) domain that binds phospholipids generated by PI3-K activation. 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 (272).

The lipid products of PI3-K 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 (273). This binding induces translocation of PKB to the plasma membrane where its phosphorylation on Thr308 by PDK-1 and Ser473 by a hypothetical kinase termed as PDK-2 is required for its complete activation (273) (Fig. 8).

ET-1 has been shown to increase PKB activation in cardiomyocytes (274), in myofibroblast (275), in human umbilical vein endothelial cells (276) and in human osteoarthritis chondrocytes as well as in A-10 VSMCs (10). An involvement of PKB in vascular disease was suggested from studies in which an enhanced PKB activity was associated with angiotensin-induced hypertension in New Zealand White rats (277). Furthermore, a role of PKB in regulating the ploidy levels and hypertrophy of VSMCs has also been suggested in a model of hypertension (278). Several different substrates of PKB have been identified which include members of the cell survival/apoptosis cascade such as Bcl_2 associated death promoter (BAD) (272;279), caspase (280) and glycogen synthase kinase 3 (GSK-3) (281), as well as key regulators of protein synthesis and cell growth such as mammalian target of rapamycin mTOR (282) (Fig.8). Thus, the stimulation of PI3-K/PKB signalling in response to ET-1 receptor activation has implications in regulating not only survival and death but also hypertrophic responses in VSMCs.

1-8-6 ET-1-induced growth factor receptor transactivation

There is an increasing body of evidence to suggest that vasoactive peptides such as ET-1 whose receptor belong to GPCR family stimulate intracellular signalling pathway through

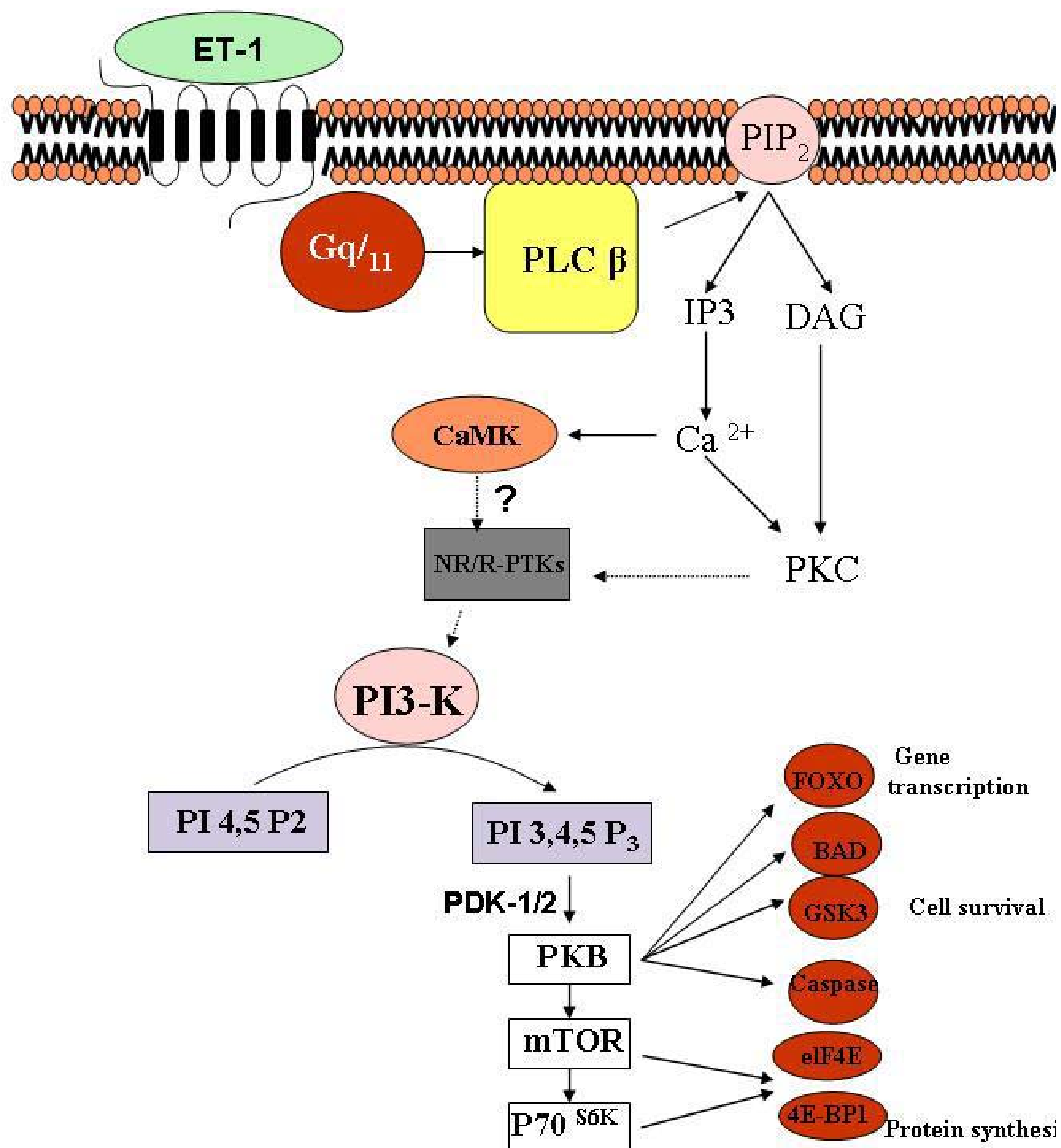

Fig.8: Activatin of PI-3K/Akt pathway by ET-1 in VSMC. ET-1 receptor stimulation leads to G-protein bγ activation which activates PLCβ. Activated PLCβ converts PIP2 to IP3 and diacylglycerol (DAG). IP3 elevates the concentration of intracellular calcium and participates in muscle contraction. DAG activates PKC. PKC and/or Ca2+/Calmodulin (CaM)-dependant protein kinase (CaMK) activate receptor and non-receptor tyrosine kinases such as Src and Pyk2. Activation of these components can eventually contribute to the activation of PI3-K. PI3K activation leads to the production of PI(3,4,5)P3 from PI (4,5) which results in recruitment and activation of PKB. PKB has several effectors.

transactivation of receptor protein tyrosine kinases (R-PTK) (283). Among the various R-PTK, the role of EGF-R, IGF-R and PDGF-R in this process has been investigated in some detail(284;285).

1-8-6-1 Role of EGF-R

The EGF-R is a R-PTK that is ubiquitously expressed in a variety of cell types, with the most abundant expression in epithelial cells and many cancer cells (286-288). It contains an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase autophosphorylation and regulatory domain (reviewed in (289)). EGF-R undergoes dimerization to induce autophosphorylation of tyrosine residues in its tyrosine kinase domain in response to ligand binding (287;290). Dimerization activates the intrinsic PTK activity of the intracellular domain leading to the phosphorylation of several key tyrosine residues. Phosphorylated tyrosines serve as docking sites for binding with Src homology 2 (SH-2) domain containing signaling proteins which trigger downstream events. The phosphorylation of EGF-R on Tyr^{1068} recruits of the adaptor protein Grb2, leading to the activation of Ras/ERK1/2 pathway.

ET-1 has been shown to transactivate EGF-R by tyrosine phosphorylation in many cell types (96;258;291), and a critical role of this transactivation in mediating ET-1-induced ERK1/2 activation has been suggested (96). An increase in EGF-R phosphorylation and ERK1/2 activation was also reported in freshly isolated rat aorta in response to ET-1 stimulation, and PD153035, an EGF-R PTK inhibitor, blocked ERK1/2 phosphorylation in these studies (258). An involvement of ET-1-induced EGF-R transactivation has been implicated in protein and DNA synthesis and c-Fox gene transcription in VSMCs

(96;292). In contrast to the results of Iwasaki et al showing that ET-1-induced protein synthesis could be blocked by AG1478, an other blocker of EGF-R , in vivo studies have failed to show any inhibitory effect of this inhibitor on total protein synthesis is small mesenteric arteries (293). However, these investigators did not examine the effect of AG1478 on EGF-R phosphorylation. A requirement of EGF-R transactivation in ET-1-induced vascular contraction has also been shown in rabbit basilary artery rings (294) and in mouse aortic ring segments (95). Chansel et al reported that the ET-1-induced vasoconstriction in carotid artery segments in mice dependent also on EGF-R transactivation (291). This contractile response was dependent on ERK1/2 activation because pharmacological blockade of this pathway inhibited ET-1-induced contraction in basilary artery rings (294). Although, several studies in VSMCs have suggested an involvement of ET_A receptor in ET-1-induced EGF-R transactivation, recently , by using either the full-length ET_B receptor or a N-terminally truncated, a role of ET_B receptor in this process has also been suggested (295). It thus appears that EGF-R transactivation plays a role not only in growth promoting /hypertrophic responses of ET-1, but also in mediating the contractile events induced by these two vasoactive peptides.

1-8-6-2 Role of IGF-1R

The IGF-1R is also a R-PTK that shares structural and functional homology with the insulin receptor. The mature receptor is a tetramer consisting of 2 extracellular α-chains and 2 intracellular β-chains (296). The β-chains include an intracellular tyrosine kinase domain that is believed to be essential for most of the receptor's biologic effects (297). Binding of IGF-1 or Insulin (at very high concentrations) induces the activation of PTK

domain of IGF-1Rβ subunit which in turn activates the autophosphorylation of the receptor (reviewed in (298)). One of the earliest steps in signal transduction initiated by the IGF-1R is the phosphorylation of adaptor/docking proteins such as insulin receptor substrate (IRS-1 or IRS-2) and Shc (299;300). IRS-1, an important substrate for both the insulin and the IGF-1 receptor, contains multiple tyrosine phosphorylation sites that recognize and bind to SH2-domain containing signaling molecules, such as Grb2, Nck, the p85 subunit of PI3-K and the SH2 domain-containing tyrosine phosphatase-2 (SHP-2) (299). Of these, the binding of Grb2/Sos to tyrosine-phosphorylated IRS-1 activates Ras, which then stimulates the Raf-1/MAPK cascade (301). Shc can also interact directly with IGF-1R (302). After tyrosine phosphorylation of Shc, it recruits the Grb2/Sos complex and activates the Ras/Raf-1/MEK/ERK pathway (301). The activated IGF-1R also triggers the activation of PI3-K and its downstream targets PKB and p70s6k (303;304).

It has been shown that ET-1 transactivates IGF-1R via Src kinase, and that activation of IGF-1R results in PI3-K mediated Akt phosphorylation therby ET-1 triggers the development and progression of prostate cancer cell (305). In VSMC, we have shown recently a requirement of IGF-1R in ET-1-induced PKB phosphorylation. These studies have shown that ET-1 was able to phosphorylate IGF-1R and inhibition of IGF-1R using AG1024, a selective pharmacological inhibitor of IGF-1R-PTK activity, attenuated both PKB phosphorylation and cell growth evoked by ET-1 (306).

There is also some evidence indicating that transactivation of EGFR mediates the responses of IGF-1R in some cell types suggesting the existence of a cross-talk between IGF-R and EGF-R transactivation (307). Moreover, the studies showing that dominant negative or antisense oligonucleotide of IGF-1R are able to attenuate neointima

formation in an injured carotid artery rat model (308) and reduce AT_1 receptors expression and function in spontaneously hypertensive rats (309) supports a potential pathogenic role of upregulated IGF-1R signalling in vascular disease.

Although several studies have shown that PDGFR undergoes tyrosine phosphorylation in response to Ang II *in vitro* and *in vivo* (310;311) an effect of ET-1 on PDGFR transactivation has not been established in VSMC (292)

1-8-6-3 Mechanism of growth factor receptors transactivation

The mechanism by which vasoactive peptides transactivate growth factor receptors is not fully understood, yet several possible mechanisms have been suggested. The first mechanism involves metalloproteinase-induced cleavage of pro-heparin binding EGF (proHB-EGF) to HB-EGF which binds to the ectodomain of EGFR, and activates downstream signalling events (312). Both ET-1- and AngII-induced EGFR transactivation have been found to be sensitive to inhibition by a series of metalloproteinase inhibitors such as GM6001, doxycyclin and batimastat (291;312). These inhibitors were also shown to block downstream signaling induced by ET-1 and AngII in VSMC (291;313). Several matrix metalloproteinases have been identified and some of these have been implicated in cardiovascular pathophysiology. For example, an involvement of MMP2/9, a member of MMP family, in the development of pressure-induced enhanced myogenic tone in mouse resistance artery has been reported (314). A role of ADAM 17, also known as tumor necrosis factor (TNF) alpha-converting enzyme (TACE), in mediating AngII-induced VSMC hypertrophy and EGFR transactivation was recently demonstrated (315). Interestingly, another ADAM family member, ADAM10,

was shown to have no role in AngII-induced EGFR transactivation in this system (315), whereas a requirement of ADAM 12 in AngII-induced cardiac hypertrophy and HB-EGF release was demonstrated (316). It seems that requirement of specific MMPs/ADAMs to release HB-EGF from pro-HB-EGF is dependent on cell type and physiological context. Although, MMPs are crucial for EGFR transactivation, a similar role of MMPs in PDGFR or IGF-1R transactivation remains to be established.

In VSMCs, ET-1 and other vasoactive peptides mediate their responses through the generation of reactive oxygen species (ROS) (10;240). ROS have been suggested to serve as critical signaling molecules (317), and it may be possible that ROS generation could be among the mechanisms by which vasoactive peptides transactivate EGFR. In fact, by using different antioxidants, a requirement of ROS generation in ET-1 and Ang II-induced phosphorylation of EGFR has been demonstrated (318). There is also some suggestion for a role of ROS generation in MMP activation (319)

The precise mechanism by which ROS contributes to the transactivation of growth factor receptors-PTKs is still unclear. However, in view of the ability of ROS to inhibit PTPases, such as PTP-1B(320)and SH-2 domain-containing tyrosine phosphatase -2 (SHP-2) (321;322), it is possible that ROS can shift the equilibrium of the phosphorylation-dephosphorylation cycle, culminating in a net increase of tyrosine phosphorylation of R-PTK and /or other PTKs (284;323;324). The activated PTKs can thus promote the assembly of signaling components essential to trigger the ERK1/2 and PKB signaling pathways. It should be noted that a potential role of several non-receptors PTKs, such a Src and Pyk-2, in inducing AngII-induced EGFR transactivation has also been demonstrated (318;325).

2 Reactive oxygen species

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 (326;327). More recent reports have confirmed this concept (328). Under oxidative stress conditions, excessive endogenous formation of ROS overcomes cellular antioxidant defense mechanisms, which results in ROS-initiated modification of lipids, proteins, carbohydrates and DNA (327). 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.9). Mitochondria is among the sources of endogenous ROS, which convert 1-2 % of consumed molecular oxygen into superoxide anion (329). In VSMCs and endothelial cells, NADH/NADPH oxidases represent the most important source of $O_2^{\cdot-}$ (330). 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 (233) (Fig.9).

2-1 ROS mediates ET-1 signaling

ET-1 has been shown to activate NADPH oxidase, thereby increasing $O_2^{\cdot-}$ levels in endothelial cells (331) and cardiomyocytes (332) and stimulates $O_2^{\cdot-}$ production in pulmonary smooth muscle cells (333). Studies, from Fei lab, have shown that ET-1 can activate NADPH oxidase in VSMC (334) and that, in vivo, free radicals generated in this

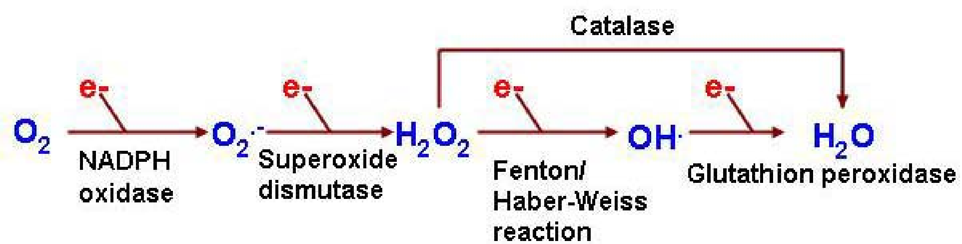

Fig 9. Key steps in the production of reactive oxygen species

manner could play important roles in mineralocorticoid-induced hypertension, (98;335;336). Findings also suggest that ET-1 can increase $O_2^{\cdot-}$ levels via activation of NADPH oxidase in DOCA-salt rats (98). More recently, it has been documented that ET-1 induces Ca^{2+} sensitization through activation of RhoA/ROK signaling induced by ROS in pulmonary VSM (337) and induces the activation of L-type Ca^{2+} channels via stimulation of NAD(P)H-derived superoxide production in cardiac myocytes (332). Increased ROS generation has been associated with a variety of cardiovascular pathologies (326) including hypertension (338) and atherosclerosis (339). Pathogenesis of cardiovascular diseases by activating ROS are thought to participate in the cellular signaling pathways responsible for promoting cell growth (340) and proliferation (234). It has been demonstrated that ET-1 induces JNK and p38mapk activation through ROS generation but not ERK1/2 (341). These findings are consistent with those of Fei et al. (334), 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 study demonstrated the involvement of ROS in ET-1-induced activation of ERK1/2 pathway as well as JNK and p38mapk in cardiac fibroblasts (234). Moreover, our laboratory has demonstrated a role of ROS in ET-1-induced activation of ERK1/2, PKB, and Pyk2 signaling in VSMCs (10;284;324).

ROS, such as H_2O_2 has been shown to induce increases in cytoplamic Ca^{2+} in a number of cell types including cells from vascular system (342). This finding was first reported by Hyslop et al. (343). The mechanism by which H_2O_2 induces intracellular Ca^{2+} is not fully clarified, however several hypotheses have been proposed. A potential role of extracellular Ca^{2+} entry into cells through voltage dependent Ca^{2+} channels and

intracellular Ca^{2+} from caffeine- and noradrenaline-sensitive stores in mediating H_2O_2-induced contractile responses of aortic ring has been suggested (344). It has also been reported that the activation of the tyrosine kinase c-Src contributes to the activation of store-mediated Ca^{2+} entry in platelets by H_2O_2 (345). In addition the increase in cytoplasmic Ca^{2+}evoked by H_2O_2 in endothelial cells, appears to be derived totally from intracellular stores since BABTA-AM could attenuate this increase (346). Recently, Hecquet et al. have demonstrated a novel mechanism of H_2O_2-mediated disruption of endothelial barrier function that is attributable to a rise in intracellular Ca^{2+} mediated by Ca^{2+} entry through oxidant-sensitive channels named transient receptor potential melastatin (TRPM2) (347).

2-2 ROS in cardiovascular pathophysiology

Oxidative stress plays an important role in the pathophysiology of cardiovascular diseases such as hypertension, atherosclerosis, diabetes, cardiac hypertrophy and heart failure. Although several sources of ROS may be involved, a family of NADPH oxidases appears to be important for redox signaling. It has been reported that both NADPH oxidase subunit expression and activity are increased in Ang II-induced hypertension in rats (338;348) and the fact that ROS concentration is increased in hypertensive mice (349) indicate strongly the critical role of oxidative stress in hypertension. Treatment with an NADPH oxidase inhibitor decreases vascular O_2^- production and attenuates Ang II-induced elevation in blood pressure (350). Some subunits of NADPH oxidase have been reported to be implicated in hypertension. Nox1-deficient mice and p47phox-deficient mice have reduced vascular O_2^- production and blunted pressor responses to

Ang II (351;352) whilst transgenic mice overexpressing Nox1 in smooth muscle show enhanced O_2^- levels and blood pressure in response to Ang II (353). In SHR, the mechanism by which oxidative stress induced hypertension seems to be the enhanced expression of Giα proteins and adenylyl cyclase singaling in VSMCs (143). Furthermore, many other studies reported that antioxidants such as N-acetyl-cysteine, tempol and vitamins E and C prevent the development of hypertension in several animal models (354-356).

Evidence supports important pathophysiological roles for redox-sensitive signaling pathways in the processes underlying left ventricular hypertrophy, adverse left ventricular remodelling and congestive heart failure. Experimental (357) and clinical (358) studies have suggested that oxidative stress is increased in heart failure, and also have indicated that the degree of free radical production is linked to the severity of the disease. Hypertrophy of isolated cardiomyocytes induced by ET-1 or Ang II has been shown to involve increased ROS production (359). Similarly, the inhibition of cellular SOD activity, which leads to increased intracellular ROS levels, induces hypertrophy of isolated cardiomyocytes (360). Increased ROS production also promotes the development of interstitial and perivascular fibrosis as well as promoting increased extracellular matrix turnover, at least in part through the activation of MMP(361). Recently, evidence suggested that ROS may also induce specific changes in the function of proteins involved in myocardial excitation–contraction coupling, which include the sarcoplasmic reticulum Ca^{2+} ATPase pump, ryanodine receptor and contractile proteins (362). Thus it appears that ROS, via its effects on signaling pathways linked to hypertrophy, proliferation, and growth may contribute to various cardiovascular pathologies.

3 Nitric oxide

NO is a free radical that was previously described as a non-prostaglandin, endothelium-derived relaxing factor (EDRF) (40;363) and is involved in the regulation of a large number of biological processes (40;363). Efforts to understand the role of NO in the cardiovascular nervous and immune systems have revealed that it can modulate a variety of cellular and pathologies events including cell proliferation, growth, apoptosis, inflammation, kidney function, diabetes, oxidative stress and aging (364;365). Most notably, NO has emerged as an important cardiovascular protective agent by its ability to exert anti-hypertrophic, anti-proliferative and anti apoptotic effect in cardiovascular system (366;367).

3-1 Relationship between ET-1 and Nitric oxide

Both ET-1 and NO are two endothelium- derived mediators that act as mutual antagonists in maintaining vascular tone as well as other physiological and pathological processes (368;369). In endothelial cells, the binding of ET-1 to ET_B receptors, stimulates the release of NO which diffuses in to the smooth muscle cells and induces physiological effects. It has been suggested that long term exposure of endothelial cells to ET-1 decreased NO levels by both degradation and attenuation of NO production resulting in endothelial dysfunction (370). It was also shown that endothelium-restricted overexpression of ET-1 caused endothelial dysfunction and a decrease in the level of NO (371). And the results from pre-clinical and clinical data further support this notion that the blockade of ET receptor function improves NO bioavailability and endothelial

function in pathological situations (370). However the precise molecular details by which NO antagonize the physiological effects of ET-1 in VSMCs remains to clarify.

3-2 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 (364;365;372) (Fig. 10). Reaction products are NO, $NADP^+$ and citrulline. 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 (372). 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 (363). 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 (364).

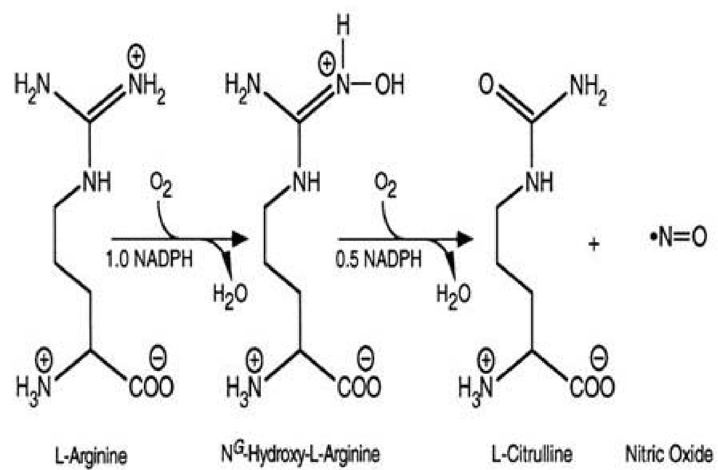

Fig.10. The NOS-catalysed reaction
From (Andrew, P. J et al. *Cardiovasc Res* 43:521-531;1999)

3-3 NOS isoforms

Three distinct NOS enzymes, each a product of a unique gene, have been identified and characterized (363;364;373). 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. Although, it is largely believed that in normal condition VSMCs do not express iNOS, regulation of iNOS gene expression in VSMCs has been reviewed (374). 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 it to the plasmalemmal caveolae of endothelial cells. Human enzymes exhibit approximately 51-57 % homology at the aminoacid level (363;373). 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 (373).

3-4 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) (375). When NO binds to the pentacoordinate

ferrous heme of the sGC that appears to be uniquely tuned to interact with NO, a conformational change occur in the enzyme, stimulating its catalytic activity (376). 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) (375;377), which in turn, facilitates the phosphorylation of various proteins as well as the reduction of intracellular calcium concentrations by different mechanisms (378). Moreover, NO also targets many proteins either by nitrosylation of thiol residues, nitration of tyrosine or oxidizing DNA and proteins (378). Increasing evidence indicates also that NO may inactivate NADPH oxidase by inhibiting its assembling process, thus reducing the ROS levels (379). 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 (380). 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 among the important pathway in mediating NO action (375).

3-5 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 (378). Two major forms of guanylate cyclases are known, the particulate guanylate cyclase and the soluble guanylate cyclase. It is generally believed that activation of soluble guanylate cyclase (sGC) is the principal intracellular event that initiates relaxation (381;382). The activity of the sGC is regulated by

nitrovasodilators, oxidation products of fatty acid and free radicals (383). 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 (383)(Fig.12). N-terminal of each subunit contains heme as a prosthetic group which serves as a site for NO binding (383). sGC lacking the heme moiety, is not able to be activated by NO (383). 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 (384;385). C-terminal of each subunit possesses a catalytic domain with a high homology sequence between the monomers (384;385). 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 (384;385). 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 (385). 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 (375) (Fig. 11). Recently, the vital importance of sGC for mammalian physiology was directly confirmed by generation of sGC knockout mice. The absence of sGC protein resulted in a significant increase in blood pressure (386)

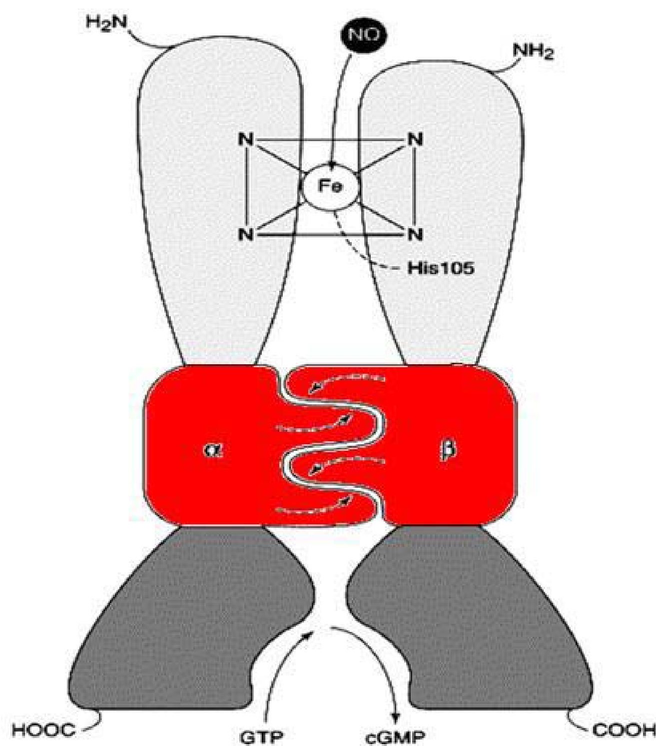

Fig.11. Schematic representation of a soluble guanylate cyclase α/β heterodimer. The N-terminal region constitutes a haem-binding domain with His105 providing the axial ligand to the fifth coordinate of the haem-iron. The central portion of each subunit contains sequences which mediate dimerization of the monomers, a prerequisite for catalytic activity. The C-terminal region forms the catalytic domain, responsible for substrate binding (GTP) and conversion to cGMP.
Adapted from (Hobbs et al.*Trends Pharmacol Sci*. 18:484-491, 1997)

3-6 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) (387)(Fig.12). There are at least seven known distinct mammalian PDE families. Each one differs from the other in biochemical and physical properties, responses to specific effectors, inhibitors and regulatory control mechanisms (387). Type V PDE has been isolated from a number of tissues including human platelets (388), trachea (389) and VSMC (390) 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 (388).

3-7 NO in signal transduction

The endothelium serves as the principal physiological source of NO in blood vessels (391). As evidenced, NO contributes to the regulation of several hormone-mediated responses (392;393). In addition to its vasodilating effect, NO can also inhibit atherogenesis (363), thrombocyte aggregation (394) and VSMC proliferation (387;391) and migration (395). There is also increasing body of evidence suggesting that NO is able to antagonize the physiological and pathophysiological effects (162;375) of growth factors and vasoactive peptides such as EGF (387), PDGF (396) and bFGF (395). This is probably achieved by inhibiting one or several of the signaling events induced by these factors (363;391;395;397). According to several studies, mitogens such as ET-1 stimulate

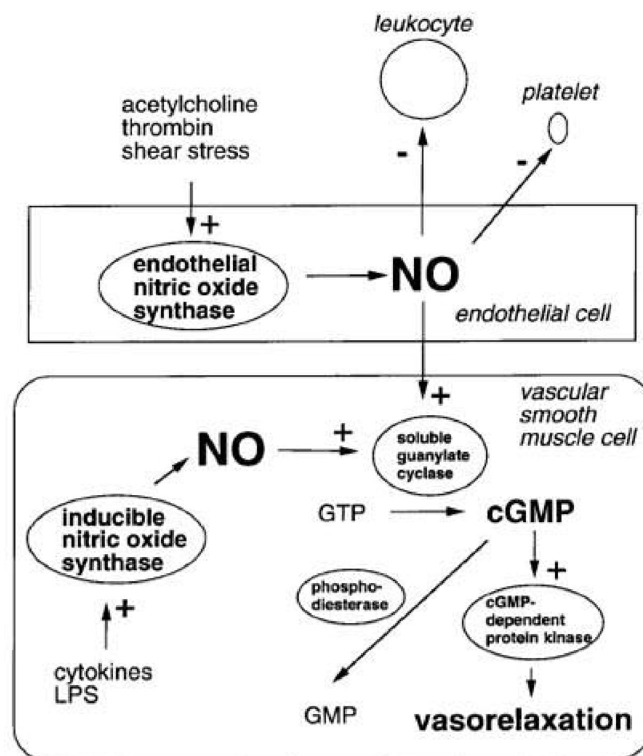

Fig.12. The nitric oxide/cGMP signal transduction

From (Lloyd-Jones and Bloch . *Annu Rev Med* 47: 365-375, 1996)

the synthesis of DNA and cell proliferation by activating the phosphorylation cascade of MAPK (234;239;398). The potential mechanism that could modulate VSMC proliferation is the release of NO by the endothelium either via a cGMP-dependent (375;387) or a cGMP-independent mechanisms (399;400). In cardiomyocytes, ET-1-induced protein synthesis (401;402) has also been shown to be inhibited by NO (403). Furthermore, NO was found to suppress the Ang II-induced activation of three major MAPKs, ERK1/2, p38mapk and JNK (404) as well as Pyk2 (393) in cardiac fibroblasts. However, it is not known whether NO, similar to its effect on growth factor and Ang II-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 (392). These data implicate PKB signaling cascade as an important mitogenic pathway that is subjected to modulation by NO in VSMC (392). 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.

4 Hypothesis and objectives

As discussed above, ET-1 and NO are very potent active molecules, by virtue of their ability to regulate various cardiovascular function they play an important role in health and diseases. ET-1 activates multiple signaling pathways including MAPK and PI3-K/PKB which mediate the hypertrophic and proliferative responses in VSMC. An important role of ROS generation in mediating the effect of ET-1 has been demonstrated and both ROS and ET-1 have been reported to activate similar signaling pathways. Moreover, Ca^{2+} has emerged as a key second messenger to transduce the effect of ET-1 as well as ROS. Furthermore, NO has been shown to serve as an anti-hypertrophic and serve as and anti-proliferative agent in many cell types. However, the precise mechanism by which Ca^{2+} contribute to ET-1 and ROS-induced signaling and physiological responses, and how NO can modulate these effects in VSMCs remain unexplored therefore the following studies have been undertaken to better understand this process.

Thus, the purposes of the present studies were to elucidate:

1. The implication of CaMKII which is downstream effector Ca^{2+} action in ET-1 induced ERK1/2 and PKB signaling in VSMCs. Therefore, the first objective of the present studies was to examine the role of CaMKII in ET-induced signaling as well as growth and proliferation in VSMCs.

2. Since ET-1 mediates its effects through the generation of ROS, we investigated the role of CaMKII in H_2O_2 induced ERK and PKB phosphorylation as well as IGF-1R phosphorylation.

3. To better understand the molecular mechanism by which NO antagonizes the anti proliferative and hypertrophic effect of ET-1, we have also investigated the effect of NO system on ET-1-sensitive signaling. In these studies, 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 VSMCs. We then used 8-Bromo-cGMP, a cyclic GMP analogue and ODQ, an inhibitor of sGC, to examine whether NO acting through a cGMP-dependent mechanism.

Will be submitted soon to Cardiovascular Research

CHAPTER-2

Involvement of Calmodulin and Calmodulin-dependent protein kinase II in ET-1-Induced Activation of ERK1/2 and Protein Kinase B as well as Growth and Proliferation in Vascular Smooth Muscle cells.

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Running title: CaMKII in ET-1-induced signaling in VSMC

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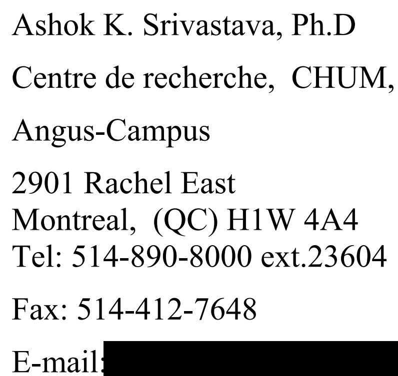

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Abstract

We have shown earlier that in vascular smooth muscle cells (VSMC), H_2O_2-induced activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and protein kinase B (PKB), two key mediators of growth-promoting and proliferative responses, are mediated through Ca^{2+} and Calmodulin (CaM)-dependent downstream signals. Moreover, endothelin-1 (ET-1), a powerful vasoactive peptide with a pathogenic role in vascular disease, requires H_2O_2 generation to elicit its responses. Therefore, in the present studies, we have investigated a possible role of CaM and its effector, Ca^{2+}/CaM-dependent protein kinaseII (CaMKII) in mediating the ET-1-induced ERK1/2 and PKB phosphorylation by using pharmacological inhibitors, CaMKII inhibitor peptide and small interfering RNA (siRNA) technique. W-7 and calmidazolium, antagonists of CaM, as well as KN-93, a specific inhibitor of CaMKII, attenuated ET-1-induced ERK1/2 and PKB phosphorylation in a dose-dependent fashion. However, KN-92, an inactive analogue of KN-93 was without effect. Transfection of VSMC with CaMKII inhibitory peptide (AA 281-309) corresponding to auto-inhibitory domain (AID) of CaMKII, attenuated ET-1-induced phosphorylation of ERK1/2 and PKB. Furthermore, significant knock-down of CaMKII expression by using CaMKIIα siRNA and reduced ET-1-induced ERK1/2 and PKB phosphorylation, whereas, control siRNA was without any effect on these events. Blockaded of CaM and CaMK-II by W-7 and KN-93, respectively, significantly reduced ET-1-induced increase in protein and DNA synthesis. In addition, ET-1 also induced Thr^{286} phosphorylation of CaMKII which is associated with its enhanced catalytic activity. Taken together, these data demonstrate that CaM/CaMKIIα

plays an important role in mediating the stimulatory action of ET-1 on ERK1/2 and PKB phosphorylation as well as on hypertrophic and proliferative responses in VSMC.

Key words: Cell signaling, endothelin-1, calmodulin, CaMKII, VSMC. Hypertrophy, proliferation: ERK-1/2, PKB/Akt

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide (1) which also exhibits mitogenic activity in vascular smooth muscle cells (VSMC) (2-4). A possible role for ET-1 in the pathogenesis of many diseases, such as atherosclerosis (5), hypertension (6) and restenosis after angioplasty has been suggested (7). ET-1 exerts its biological actions through the activation of two receptor subtypes, ET_A and ET_B (8, 9). Both receptors belong to a large family of transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs). ET_A receptors are highly expressed in VSMC but are also found in cardiomyocytes, fibroblasts, hepatocytes, adipocytes, osteoblasts and brain neurons (8, 10) and exhibit higher affinities for ET-1 and ET-2 than for ET-3 (8). ET_B receptors exist predominantly in endothelial cells and smooth muscle cells, but are also found in cardiomyocytes, hepatocytes, fibroblasts, osteoblasts, different epithelial cells and neurons and have equal subnanomolar affinities for all ET iso-peptides (9). ET-1 binding to ET_A receptors on smooth muscle produces vasoconstriction, cell growth and cell adhesion (11) whereas the binding of ET-1 to endothelial ET_B receptors stimulates the release of nitric oxide and prostacyclin (12, 13).

ET-1 exerts its effects through multiple signaling pathways which include Ca^{2+} (14), mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun-NH2-terminal kinase (JNK) and p38mapk (15-19) and Akt/phosphatidylinositol 3-kinase (PI-3K)/protein kinase B (PKB) (19, 20). Activation of receptor and non-receptor protein tyrosine kinases (PTKs) in transducing ET-1-induced signaling responses have also been suggested (18, 21-24). PTKs activated by

ET-1 include epidermal growth factor (EGF) (24), c-Src (21, 23, 25) and a Ca^{2+}-dependent PTK, Pyk2 (26).

Several studies have demonstrated that reactive oxygen species (ROS) play an important role in mediating the signals of several growth factors, peptides hormones, and cytokines, such as platelet-derived growth factor, EGF, angiotensin II, insulin and interleukin-1. ET-1 has also been shown to augment ROS production in VSMC (27, 28). H_2O_2, an important ROS molecule, has been reported to activate both MAPKs and PKB signaling in many cell types including VSMC (29-31). We have shown earlier that Ca^{2+} and CaM are essential to trigger H_2O_2- induced ERK1/2, p38 and PKB phosphorylation in A-10 VSMC (32). CaM exerts its effects through Ca^{2+}/ CaM dependent protein (CaMKII) which is a multifunctional serine/threonine protein kinase and is believed to transduce the downstream effects of Ca^{2+}/CaM (32). CaMKII is maintained in an inactive state by an autoinhibitory domain (AID), the binding of Ca^{2+}/CaM complex to the CaM binding domain reverses this auto inhibition by changing the confirmation of CaMKII inducing its autophosphorylation at Thr^{286} (33).

Angiotensin II (Ang II) has been shown to increase CaMKII phosphorylation and activity, and a role of CaMKII in AngII-induced activation of ERK1/2 has been demonstrated in VSMC (34). However, a role of CaMKII in mediating ET-1-induced ERK1/2 and PKB phosphorylation and its effect on hypertrophic and proliferative responses in VSMC remains unexplored. Therefore, in the present studies, by using a series of pharmacological inhibitors and genetic approaches, we investigated the

involvement of CaM and CaMKIIα in ET-1-induced ERK1/2 and PKB phosphorylation, as well as protein and DNA synthesis, in A-10 VSMC.

Materials and Methods

Materials

ET-1 was purchased from American Peptide Inc (USA). BQ-123, BQ-788, calmidazolium, W-7, KN-93, KN-92 and CaMKII autoinhibitory domain (AID) specific peptide inhibitor (AA 281-309, autoinhibitory peptide (AIP)) were obtained from Calbiochem and Lipofectamine was form Invitrogen, Canada. Monoclonal phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 antibody, polyclonal ERK1/2 antibody, CaMKIIα siRNA (catalogue number sc-29901), control siRNA (catalogue number sc-37007), transfection reagent for siRNA (catalogue number sc-29528), phospho-specific-Thr^{286}-CaMKII, anti-CaMKIIα, and β-actin antibodies were from Santa Cruz Biotech (Santa Cruz, CA). CaMKIIα siRNA is a pool of 3 target-specific, 20-25 nucleotide siRNAs designed to knock down gene expression of CaMKIIα. The non-specific siRNA (scrambled) consist of non-targeting 21 nucleotides with no homology to rat genes. The phospho-specific-Ser^{473}-PKB and total PKB antibodies as well as horseradish peroxidase-conjugated anti-rabbit antibodies were procured from New England Biolabs (Beverly, MA). The enhanced chemiluminescence (ECL) detection system kit, (^3H) Thymidine and L-(4,5-^3H) leucine were from was 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 Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum at 37^0C in a humidified atmosphere of 5% CO_2 as described earlier (26). The cells were grown to 80-90% confluence in 60-mm plates and incubated in serum-free DMEM 5h prior to the treatments.

Transfection with CaMKII inhibitory peptide

A-10 VSMCs at 80-90 % confluence were transfected with a CaMKII-specific AID domain inhibitory peptide (2ng/ml) by using lipofectamine (4µg/ml) (35). Transfections were performed in serum and antibiotics-free media DMEM for 2h then 10% of serum was added. Protein phosphorylation by ET-1 in peptide-transfected cells was examined 48h later.

siRNA transfection protocol

A-10 VSMCs at 80-90 % confluence were transfected with CaMKIIα siRNA or control scrambled siRNA (final concentration of CaMKIIα or scrambled, non-specific siRNA was 70 nM) according to the manufacturer's protocol. Transfections were performed in serum and antibiotics-free DMEM. A mixture of CaMKIIα siRNA or control scrambled siRNA and transfection reagent was added to cells and incubated for 6h at 37^oC then serum was added to reach 10% of FBS. Cells were incubated for an additional 48h before stimulation with ET-1.

Cell lysis and Immunoblotting

Cells incubated in the absence or presence of various agents were washed twice with ice-cold PBS and lysed in 200 µl of buffer (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 2 mM benzamidine, 2 mM ethylenebis(oxyethylenenitrolo)-tetraacetic acid, 2 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5 µg/ml leupeptin) on ice. 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 7.5% SDS-polyacrylamide gel (SDS-PAGE), transferred to PVDF membranes (Millipore, MA, USA) and incubated with respective primary antibodies, (monoclonal phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 antibody (1:4,000), polyclonal phospho-specific-Ser^{473}-PKB antibody (1:1,000). phospho-specific-Thr^{286}-CaMKII antibody (1:2,000)). The antigen-antibody complex was detected by a horseradish peroxidase-conjugated second antibody (1:2000), and protein bands were visualized by ECL. The intensity of specific bands was quantified by NIH Image software as described previously (36).

Measurement of [Methyl ^{3}H-4] thymidine incorporation: DNA Synthesis in the cells was evaluated by incorporation of [Methyl ^{3}H-4] thymidine into cells. A10 VSMCs at 80-90% confluence were serum-starved for 16hrs to induce cell quiescence. The cells were then incubated with ET-1 (100nM) alone or after pretreatment with calmidazolium (10µM) or KN-93, (10µM) or KN-92 (10µM) for 30 min, left to incubate for another 24hrs, in [^{3}H] thymidine (1µci/dish). 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 ice cold water and resuspended in 500ml of 0.4 M NaOH and radioactivity incorporated into DNA was determined by a liquid scintillation counter (28).

[^{3}H] Leucine incorporation: Protein Synthesis was assessed by incorporation of [^{3}H] leucine into protein. A10 VSMCs at 80-90% confluence were serum-starved for 16hrs to induce cell quiescence. The cells were treated with ET-1 (100nM) alone or after pretreatment with calmidazolium, or KN-93 or KN-92 for 30 min, and left to incubate for another 24 hrs. [3H] leucine, 1µci/dish, was added at the same time as ET-1. 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 ice cold water and resuspended in 500 µl of 0.4 M NaOH and radioactivity incorported into protein was determined by liquid a scintillation counter (28).

Statistics

The data are means ± SE of at least three individual experiments. Statistical significance was determined with paired or unpaired Student's *t* test and $p<0.05$ was considered significant.

Results

ET-1 induces ERK1/2, PKB and CaMKII phosphorylation via ETA and not ETB receptors in A-10 VSMCs

Several studies have reported the involvement of ET_A receptors in ET-1-induced signalling in VSMC. However, specific involvement of ET_A receptors in ET-1-induced PKB activation has not been investigated. Therefore, by using BQ-123 and BQ-788, blockers of ET_A and ET_B receptors, respectively, we assessed the specific contributions of these receptors in mediating the effect of ET-1 on PKB phosphorylation. As shown in Fig.1, pretreatment of A-10 VSMC with BQ-123 for 30 min caused a significant reduction in ET-1-induced ERK1/2 (Fig.1A) and PKB (Fig. 1B) phosphorylation whereas; BQ-788 was without effect. These data established the implication of ET_A and not ET_B receptor subtype in ET-1-induced PKB and ERK1/2 phosphorylation. The two inhibitors alone did not affect the basal phosphorylation of ERK1/2 and PKB.

Dose-dependent attenuation of ET-1-induced ERK1/2 and PKB phosphorylation by CaM and CaMKII inhibitor in A-10 VSMCs.

We have shown earlier that CaM is required for H_2O_2-induced PKB phosphorylation in A-10 VSMC (32). Since ET-1 induced ROS production we examined the involvement of both calmodulin and its downstream effecter, CaMKII, in ET-1-induced ERK1/2 and PKB phosphorylation. As shown in Fig.2, pretreatment of A-10 VSMCs with W-7 and calmidazolium, specific inhibitors of CaM, for 30 min, dose-dependently attenuated ET-1-induced phosphorylation of these two signaling component. However, PKB appeared to be more sensitive to the inhibitory effect of both W-7 and calmidazolium than ERK1/2. Almost complete attenuation of ET-1-stimulated phosphorylation of PKB was observed

at 10 μM of W-7 or calmidazolium (Fig.2 A and C). In contrast, a higher concentration of these inhibitors was required to inhibit ET-1-enhanced phosphorylation of ERK1/2 (Fig.2 B and D). We next investigated the effect of the blockade of CaMKII on ET-1-induced responses using KN-93. KN-93 inhibits CaMKII activity by competitively binding to CaM binding domain of CaMKII. As shown in Fig.3, KN-93 treatment dose-dependently inhibited ET-1-induced ERK-1/2 (panel B) and PKB (panel A) phosphorylation. However, KN-93 inhibited the PKB phosphorylation more potently than the phosphorylation of ERK1/2. (Fig.3 A and B).

CaMKII inhibitory peptide (AIP) and CaMKII siRNA attenuates ET-1 -induced ERK1/2 and PKB phosphorylation.

To provide additional proof for the involvement of CaMKII in ET-1-induced responses, we have used a peptide corresponding to the autoinhibitory domain (AID) of CaMKII (AA 281-309) (AIP) and siRNA approaches to confirm the results obtained using chemical inhibitors. Transfection of A-10 VSMC for 48h with this AIP markedly reduced ET-1-induced phosphorylation of ERK1/2 and PKB (Fig.4). In addition, we also used siRNA of CaMKIIα to knock-down CaMKIIα expression in A-10 VSMC, to evaluate its role in ET-1-induced responses. As shown in Fig.5, transfection of A-10 VSMC with siRNA of CaMKIIα resulted in about 70% reduction in the expression of CaMKIIα protein, whereas control siRNA to CaMKIIα was without effect. siRNA of CaMKIIα also had no effect on the expression of β-actin (Fig.5). The cells with siRNA-induced knock-down of CaMKIIα showed a significant inhibition in the phosphorylation of ERK1/2 (Fig.6B) and PKB (Fig.6A) in response to ET-1.

ET-1-induced CaMKII phosphorylation in A-10 VSMC

Ang II has been shown to activate CaMKII (37) in VSMC, therefore, we wished to investigate if ET-1 would exert a similar effect on CaMKII. This was tested by determining the Thr^{286} phosphorylation of CaMKII, which has been shown to enhance its catalytic activity. As shown in Fig.7 treatment of A-10 VSMC with ET-1 resulted in a rapid and sustained phosphorylation of CaMKII, which was detectable within 2 min and remained elevated for up to 30 min.

CaM and CaMKII inhibitors attenuated ET-1-stimulated [^{3}H]leucine and [^{3}H]thymidine incorporation.

Since activation of ERK1/2 and PKB signaling has been implicated in mediating the hypertrophic and proliferative responses of ET-1 (38), we next examined whether there was a correlation between CaMKII-induced inhibition and ET-1-induced PKB and ERK1/2 phosphorylation and protein and DNA synthesis. As shown in Fig.8, ET-1 increased both [^{3}H]leucine (Fig.8A) and [^{3}H]thymidine (Fig.8B) incorporation into cellular protein and DNA, respectively, by about 50% over control. However, the inhibition of CaM by using W-7, or the inhibition of CaMKII by KN-93 reduced ET-1-induced [^{3}H]leucine and [^{3}H]tymidine incorporation to almost basal level. KN-92 was without any effect on protein and DNA synthesis induced by ET-1in A-10 VSMC.

Discussion

Since ET-1 rises intracellular Ca^{2+} concentration via PLC/DAG/IP_3 pathway, in VSMC (19) and Ca^{2+} exerts many of its effect through CaM and CaMKII, we investigated the role of CaMKII in mediating the responses of ET-1 in VSMC. By using three different approaches, i.e. use of pharmacological inhibitors, a CaMKII AIP and siRNA techniques here, we have demonstrated that CaMKIIα mediates the effect of ET-1 on ERK1/2 and PKB phosphorylation in A-10 VSMC. By using pharmacological inhibitor alone such as, KN-93, earlier studies have reported that AngII and Ca^{2+} elevation agents, such as ionomycin, exert their effects on ERK1/2 phosphorylation via CaM-dependent pathways in VSMC (34, 37). However, our studies, by using multiple approaches, have provided the first evidence to suggest an involvement of CaMKIIα in mediating the effect of ET-1 on ERK1/2 and PKB phosphorylation in A-10 VSMC.

We have also shown that ET-1 induced the phosphorylation of CaMKII in Thr^{286}. The phosphorylation of this site in AID of CaMKII has been shown to activate the catalytic activity of CaMKII (39). Since Ca^{2+} ionophore ionomycin has also been shown to increase the phosphorylation of CaMKIIδ in VSMC (40), it is possible to ET-1-induced Ca^{2+} release (through Ca^{2+}/CaM binding to AID) contributes to this autophosphorylation reaction in A10 VSMC. However, the precise mechanism by which activated CaMKIIα participates in the activation of ERK1/2 and PKB remains to be elucidated. ET-1 has been shown to activate a Ca^{2+}-dependent PTK, which has been linked with the activation of ERK1/2 and PKB pathways (41, 42). Since ET-1 activates Pyk-2 in A10 VSMC, it is possible that CaMKIIα, through some yet unidentified

intermediary protein, contributes to the activation of Pyk-2 and/or other PTKs, which eventually activate ERK1/2 and PKB signalling pathways.

It is well known that ERK1/2 and PKB play a crucial role in mediating VSMC hypertrophy and proliferation (43). Interestingly, the present study shows an inhibitory effect of W-7 and KN-93 on ET-1-induced DNA and protein synthesis. It may therefore be suggested that the ability of CaMKII to stimulate ERK1/2 and PKB pathways may serve as a potential mechanism by which ET-1 induces growth and proliferative responses in VSMC.

A role of CaMKII in several pathophysiological events in different cell type, such as VSMC (44) and cardiomyocytes (45) has been suggested and in heart, ET-1-induced cardiomyocyte hypertrophy has been linked to CaMKII activation (45). Moreover, studies showing that inhibition of CaMKII by using KN-93 improved vascular dysfunction (46) in animal models of diabetes or in AngII-induced hypertension (47), suggesting that abberant activation of CaMKII may be involved in these vascular pathologies.

In conclusion, we have shown that CaMKIIα plays an important intermediary role in activating ET-1-induced signalling of ERK1/2 and PKB pathways, which are also intimately linked to its hypertrophic and proliferative responses. It may be suggested that by regulating the growth promoting and hypertrophic events, CaMKII plays an important role in vascular pathophysiology.

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Figures legends

Figure 1. Effect of BQ-123 and BQ-788 inhibitors of ET_A and ET_B receptors, respectively on ET-1-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated with or without BQ-123 or BQ-788 10μM for 30 min followed by 100nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Thr^{202} -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, (bar diagrams) in each section 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%. *$P< 0.05$ considered as statistically significance versus ET-1 stimulation alone.

Figure 2. Dose-dependent effect of W-7 and calmidazolium, two calmodulin inhibitors, on ET-1-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated with or without the indicated W-7 (section A and B) or calmidazolium (section C and D) concentrations for 30 min followed by 100nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Ser^{473}-PKB antibodies (A and C) and phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 antibodies (B and D), 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 (bar diagrams) in each section 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%. *P< 0.05 considered as statistically significance versus ET-1 stimulation alone.

Figure 3. Dose-dependent effect of KN-93, CaMKII inhibitor, on ET-1-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated with or without the indicated KN-93 concentrations for 30 min followed by 100nM of ET-1 for 5 min. Cell lysates were immunoblotted by phospho-specific-Ser^{473}-PKB antibodies (A) and phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 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 (bar diagrams) in each section 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%. *P< 0.05 considered as statistically significance versus ET-1 stimulation alone.

Figure 4. Effect of an inhibitory peptide of CaMKII (AA 281-309) on ET-1-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. A-10 VSMC were transfected, using lipofectamin, with an inhibitory peptide for 48h prior stimulation of ET-1 (100nM, 5min). The effect of lipofectamin on ET-1-induced ERK1/2 and PKB phosphorylation was used as a control. Cell lysates were immunoblotted by phospho-specific-Ser^{473}-PKB antibodies (A) and phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 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 (bar diagrams) in each section 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%. *P< 0.05 considered as statistically significance versus ET-1 stimulation alone.

Figure 5. CaMKII siRNA knocks down the expression of CaMKII in A-10 cells.

A-10 VSMC were transfected, using Santa Cruz transfection reagent (sc-37007), with CaMKII siRNA (sc-29901) or control siRNA (sc-37007)for 24hrs, and cell lysates were immunoblotted with CaMKIIα or β-actin antibodies. Values are the means ± SE of 3 independent experiments and are expressed as % expression over basal. *P< 0.05 versus control siRNA.

Figure 6. Effect of CaMKII siRNA on ET-1-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. A-10 VSMC were transfected with CaMKIIα siRNA (cat #sc-37007) (70nM) or control siRNA for 24h prior to stimulation with ET-1 (100nM, 5min). Cell lysates were immunoblotted by phospho-specific-Ser^{473}-PKB antibodies (A) and phospho-specific-Tyr^{204}-ERK1/2 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 (bar diagrams) in each section represent average data quantified by densitometric scanning of pERK1/2 or pPKB 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%. *P< 0.05 versus ET-1 alone.

Figure.7. Time course of ET-1-induced CaMKII phosphorylation in A-10 VSMC. Serum-starved quiescent A-10 cells were treated with ET-1 100nM for indicated times. Cell lysates were immunoblotted with phospho-specific-Thr^{286}-CaMKII antibodies

(middle panel) and total CaMKII (Bottom panel). Top panels (bar diagrams) represent average data quantified by densitometric scanning of p-CaMKII immunoblots. Values are the means ± SE of at least 3 independent experiments and are expressed as fold increase. *$P < 0.05$ versus time 0 min.

Figure 8. Effects of W-7 and KN-93 on ET-1-induced [^{3}H]leucine and [^{3}H]thymidine incorporation. Serum-starved quiescent A-10 cells were stimulated with ET-1 (100 nM). Cells were pretreated or not with 10 μM of W-7 or KN-93 for 30 min before ET-1 stimulation, then the cells were labeled to equilibrium with [^{3}H]leucine or [^{3}H]thymidine 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 (A) or [^{3}H]thymidine (B) incorporated into total cellular proteins or DNA over the basal values. *$P < 0.05$ versus control and † $p < 0.05$ versus ET-1 alone.

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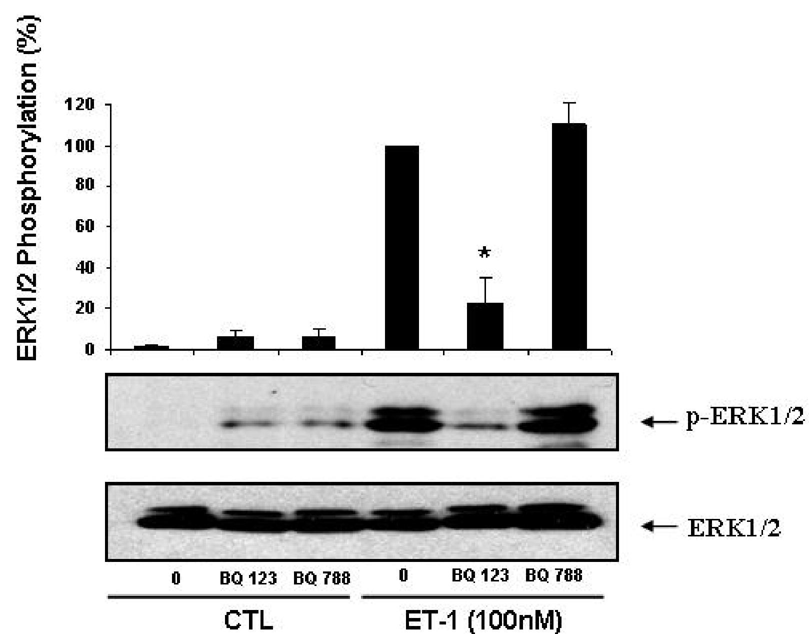
FIGURE 1

A



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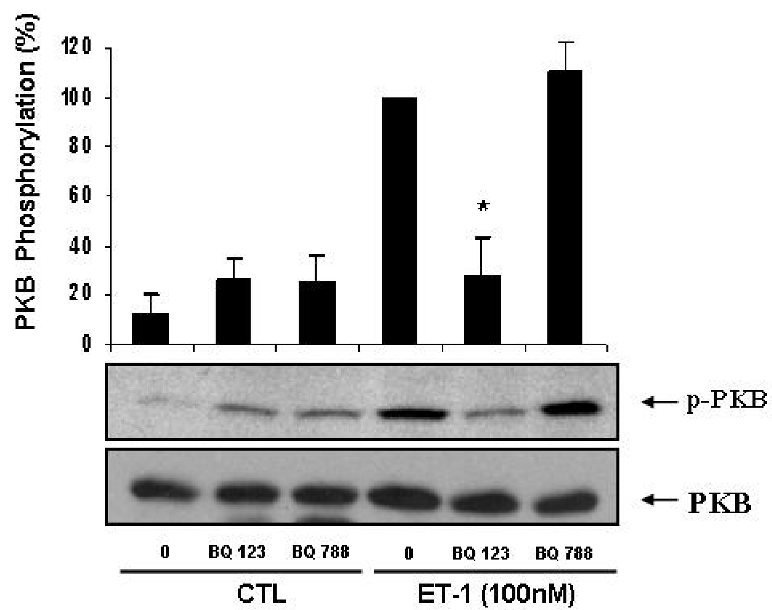


FIGURE 2

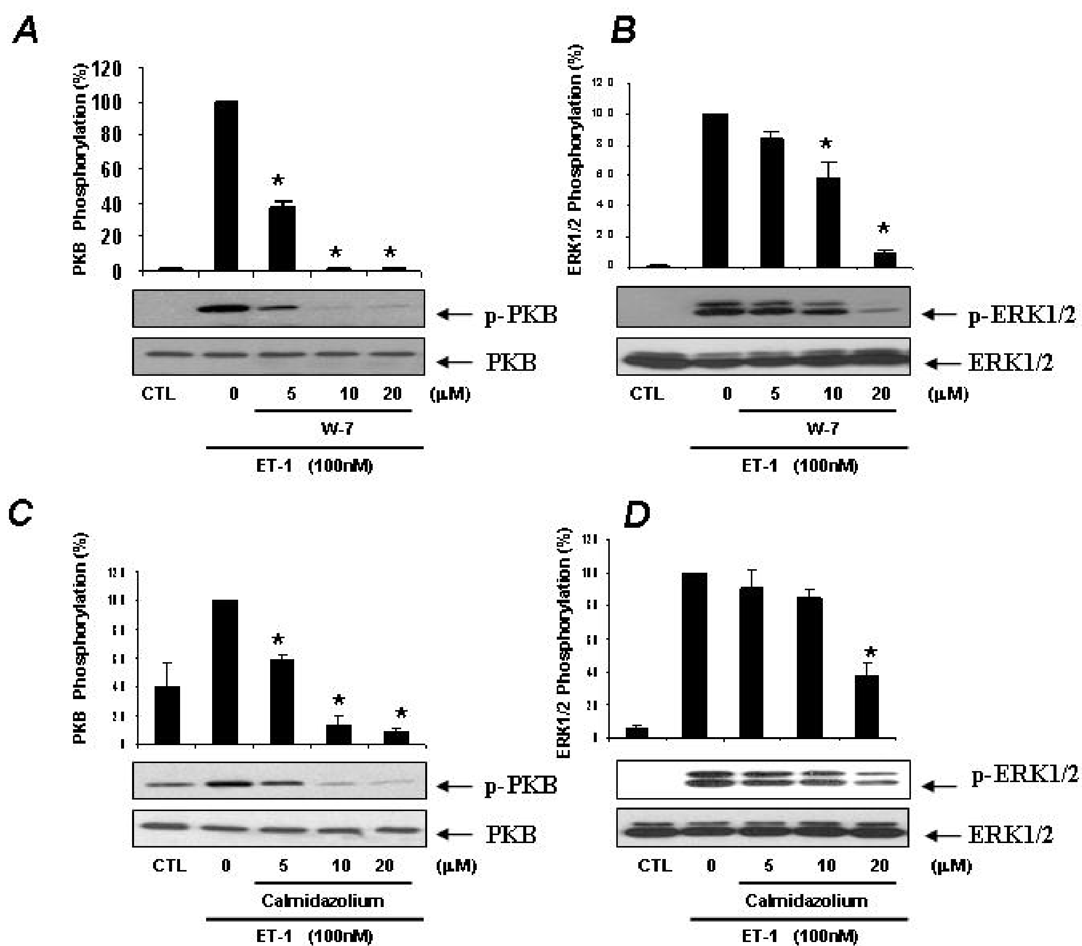


FIGURE 3

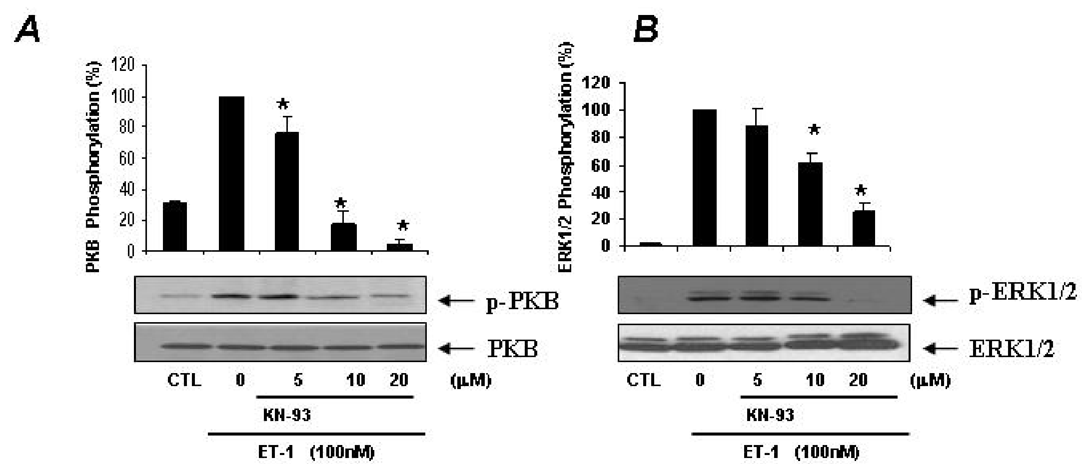
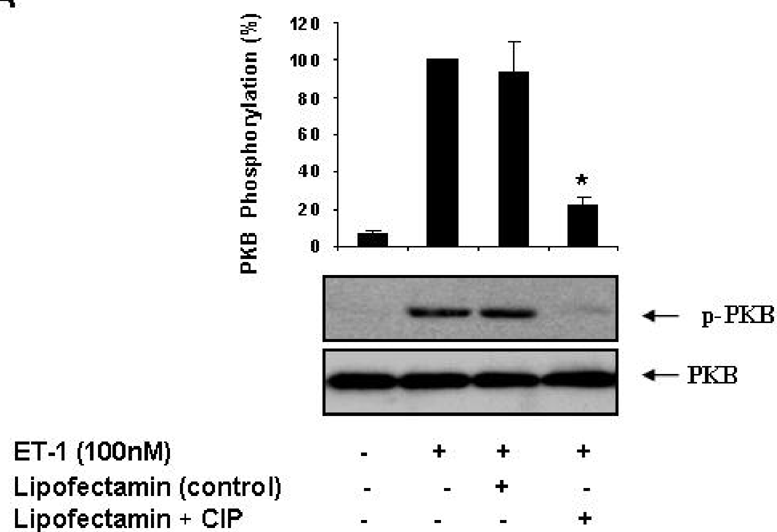


FIGURE 4



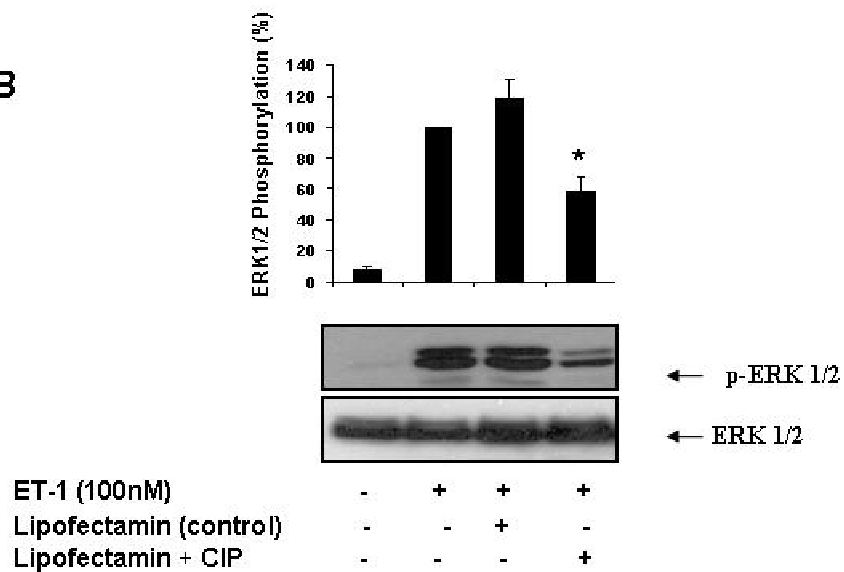


FIGURE 5

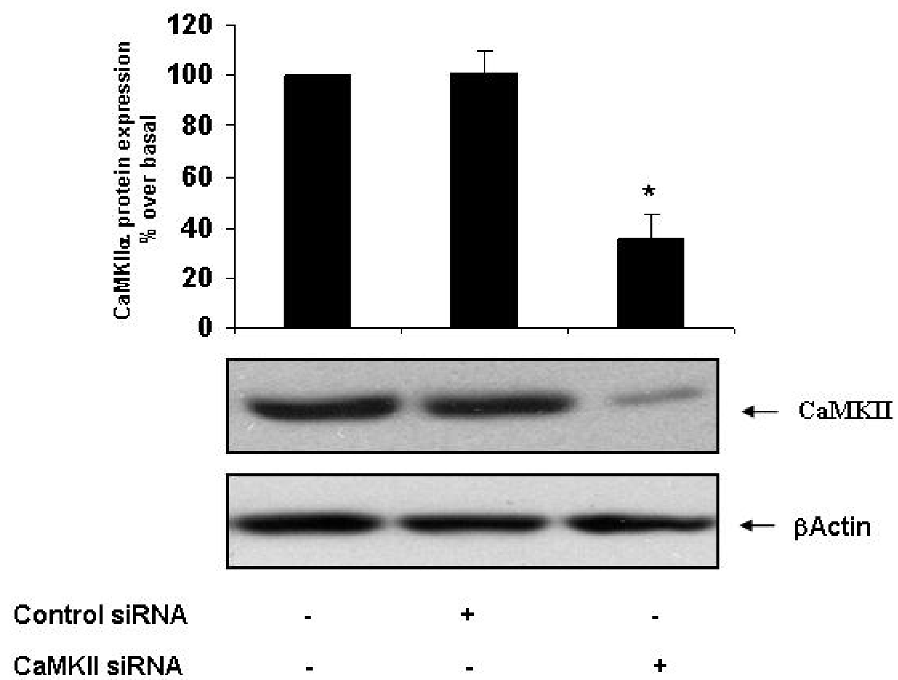
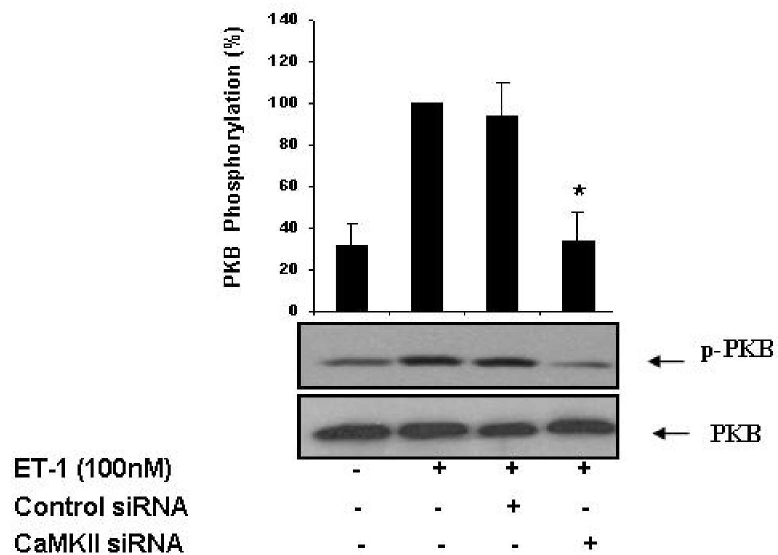


FIGURE 6

A



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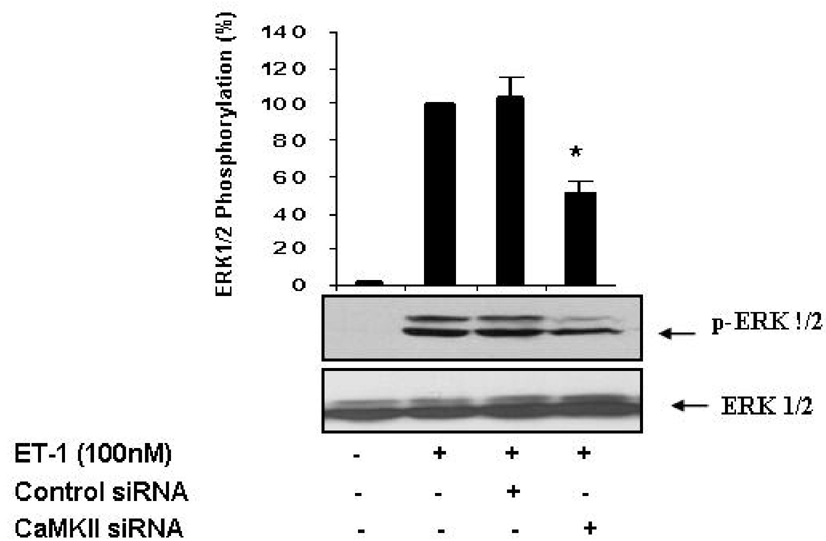


FIGURE 7

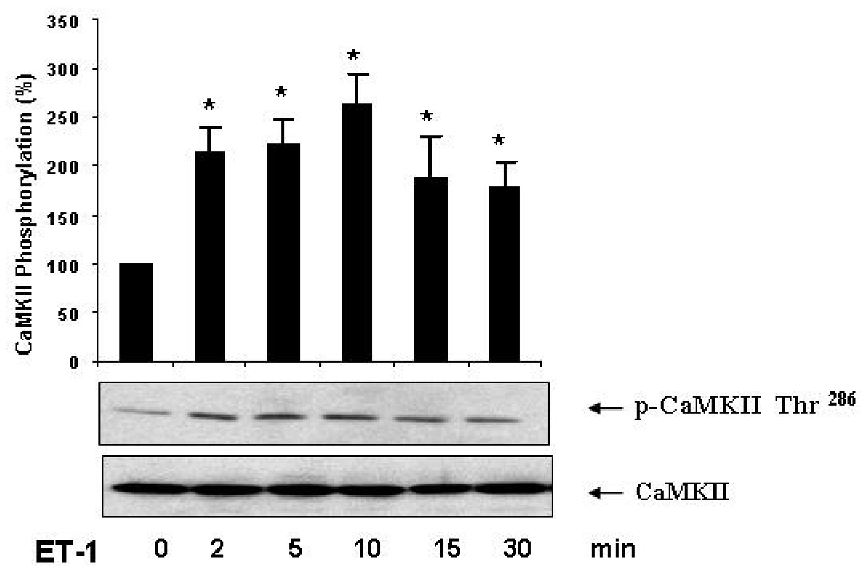
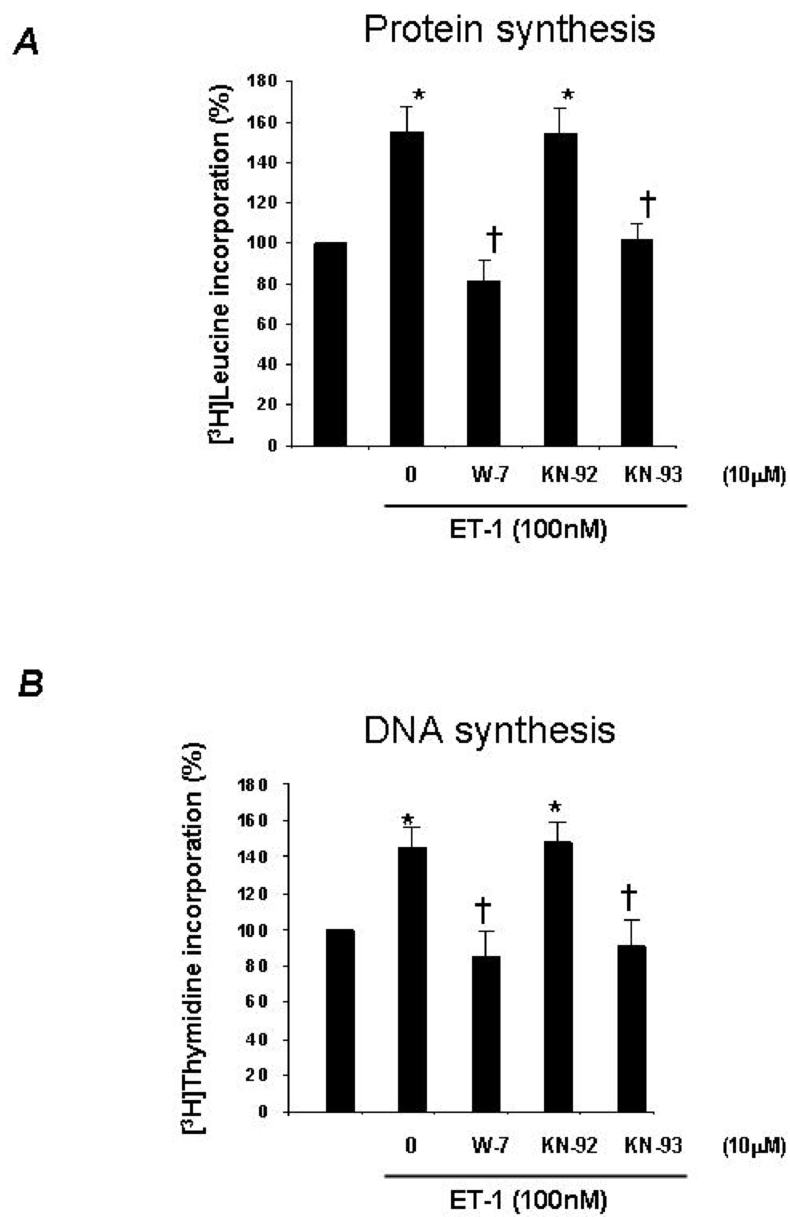


FIGURE 8



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CHAPTER-3

CaMKII-Knockdown Attenuates H_2O_2-Induced Phosphorylation of ERK1/2, PKB/Akt and IGF-1R In Vascular Smooth muscle cells

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Running title: CaMKII in H_2O_2-induced vascular signaling

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Abstract

We have shown earlier a requirement of Ca^{2+} and calmodulin (CaM) in H_2O_2-induced activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and protein kinase B (PKB), key mediators of growth promoting, proliferative and hypertrophic responses in vascular smooth muscle cells (VSMC). Since the effect of CaM is mediated through CaM-dependent protein kinaseII (CaMKII), we have investigated here the potential role of CaMKII in H_2O_2-induced ERK1/2 and PKB phosphorylation by using pharmacological inhibitors of CaM and CaMKII, a CaMKII inhibitor peptide and siRNA knockdown strategies for CaMKIIα. Calmidazolium and W-7, antagonists of CaM as well as KN-93, a specific inhibitor of CaMKII, attenuated H_2O_2-induced responses on ERK1/2 and PKB phosphorylation in a dose-dependent fashion. Similar to H_2O_2, calmidazolium and KN-93 also exhibited an inhibitory effect on glucose/glucose oxidase (G/GO)-induced phosphorylation of ERK1/2 and PKB in these cells. Transfection of VSMC with CaMKII auto-inhibitory peptide (AIP) corresponding to auto-inhibitory domain (AA 281-309) of CaMKII and with siRNA of CaMKIIα, attenuated H_2O_2-induced phosphorylation of ERK1/2 and PKB. In addition, calmidazolium and KN-93 blocked H_2O_2-induced Pyk2 and insulin-like growth factor-1 receptor (IGF-1R) phosphorylation. Moreover, treatment of VSMC with CaMKIIα siRNA abolished the H_2O_2-induced IGF-1-R phosphorylation. H_2O_2 treatment also induced Thr^{286} phosphorylation of CaMKII which was inhibited by both calmidazolium and KN-93. These results demonstrate that CaMKII plays a critical upstream role in mediating the effect of H_2O_2 on ERK1/2, PKB and IGF-1R phosphorylation. Key words: Oxidative stress signaling, H_2O_2, VSMC, CaMKII, ERK1/2, PKB, Pyk-2, IGF-1R.

Introduction

Oxidative stress is believed to play a critical role in the pathogenesis of several diseases such as cancer [1], diabetes [2,3] and cardiovascular pathophysiology, including hypertension and atherosclerosis[4-6]. Excessive endogenous formation of reactive oxygen species (ROS) overcomes cellular antioxidant defense mechanisms, leading to oxidative stress. Superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are among the most important ROS molecules. In VSMC, NAD(P)H oxidase is one of the primary enzymes responsible for the generation of $O_2^{-\bullet}$ [7]. NAD(P)H oxidase catalyzes $O_2^{-\bullet}$ production by the one electron reduction of O_2 where NAD(P)H is the electron donor. Under physiological conditions, $O_2^{-\bullet}$ undergoes dismutation either spontaneously or by a reaction catalyzed by superoxide dismutase (SOD) to produce H_2O_2 which is more stable than $O_2^{-\bullet}$and is a freely diffusible ROS molecule [8]. Normally H_2O_2 is scavenged by catalase and glutathione peroxidase to produce H_2O [9,10]. However, an aberration either in generation or scavenging of H_2O_2 or other ROS molecules has been suggested to contribute the pathophysiology of various diseases including cardiovascular diseases[4-6]. A direct role of H_2O_2 in angiotensin (AngII)-induced vasculature hypertrophy has also been suggested recently in a model of hypertensive vascular disease [11]. Exogenous H_2O_2 activates several signaling protein kinases such as mitogen activated protein kinases (MAPK) and protein kinase B (PKB) [12-15] which have been proposed to play key roles in mediating the hypertrophic response in VSMC [16]. Although the precise mechanism and intermediary steps by which H_2O_2 activates these signaling pathways remain poorly characterized, our earlier studies have reported that tyrosine phosphorylation of β-subunit IGF-1R is an important step in tranducing the effect of H_2O_2 on the phosphorylation of

ERK1/2, PKB and Pyk-2 in VSMC [17,18]. In addition, we have also demonstrated that Ca^{2+} and Calmodulin (CaM) play an important role in mediating H_2O_2-induced ERK1/2 and PKB phosphorylation in VSMC [13]. CaMKII is a multifunctional serine/threonine protein kinase which is believed to transduce the downstream effects of Ca^{2+}/CaM [19]. CaMKII holoenzyme is a multimeric protein which contains 12 subunits arranged in two sets of six subunits that form a stacked hexagonally shaped rings [19]. Each subunit of CaMKII contains three main regions: a N-terminal catalytic region responsible for catalyzing the phosphotransferase reaction, a regulatory region that contains Ca^{2+}/CaM binding sites and autoinhibitory domain (AID) and the C-terminal subunit association region responsible for assembling the multimeric holoenzyme [19]. In the absence of bound Ca^{2+}/CaM, the CaMKII is maintained in an inactive state because of an interaction of the AID with the catalytic domain of its own subunit [20]. The Ca^{2+}/CaM complex binds to a sequence that overlaps the AID and causes a conformational change, thereby relieving the inhibitory effect of AID on the catalytic activity and inducing the phosphorylation of the CaMKII in thr^{286} and enhancing its kinase activity [19]

AngII, which transduces its effect through ROS generation [21], has been shown to phosphorylate and activate CaMKII in VSMC, and AngII-induced activation of ERK1/2 has been shown to be blocked by pharmacological inhibitor of CaMKII [22]. However, a role of CaMKII in mediating H_2O_2-induced phosphorylation of ERK1/2, PKB, Pyk2 and IGF-1R in VSMC remains unexplored. Therefore, in the present studies, by using a series of pharmacological inhibitors and molecular approaches, we have investigated the involvement of CaMKII and associated pathways in H_2O_2-induced phosphorylation of these signaling components in A-10 VSMC.

Materials and Methods

Materials

H_2O_2 , glucose and glucose oxidase were procured from Sigma (St. Louis, MO, USA). Calmidazolium, W-7, KN-93, KN-92 and CaMKII autoinhibitory domain (AID) specific peptide inhibitor (AA 281-309, autoinhibitory peptide (AIP)) were obtained from Calbiochem and Lipofectamine was form Invitrogen, Canada. Monoclonal phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 antibody, polyclonal ERK1/2 antibody, CaMKII α siRNA (catalogue number sc-29901), control siRNA (catalogue number sc-37007), transfection reagent for siRNA (catalogue number sc-29528), phospho-specific-Thr^{286}-CaMKII, anti-CaMKIIα, anti-IGF-1R and β-actin antibodies were from Santa Cruz Biotech (Santa Cruz, CA). CaMKIIα siRNA is a pool of 3 target-specific, 20-25 nucleotide siRNAs designed to knock down gene expression of CaMKIIα. The non-specific siRNA (scrambled) consist of non-targeting 21 nucleotides with no homology to rat genes. The phospho-specific-Ser^{473}-PKB, total PKB, phospho-specific-Tyr^{402}-Pyk2, total Pyk2 antibodies as well as horseradish peroxidase-conjugated anti-rabbit antibodies were procured from New England Biolabs (Beverly, MA). Anti-pIGF-1R was obtained from Biosource. The enhanced chemiluminescence (ECL) detection system kit was from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada).

Cell culture

VSMC derived from embryonic rat thoracic aorta A-10 cells were maintained in culture with Dulbecco's modified eagle medium (DMEM)containing 10% fetal bovine serum at 37^0C in a humidified atmosphere of 5% CO_2 as described earlier [23]. The cells were

grown to 80-90% confluence in 60-mm plates and incubated in serum-free DMEM 5h prior to the treatments.

Transfection with CaMKII auto- inhibitory peptide

A-10 VSMCs at 80-90 % confluence were transfected with a CaMKII-specific (281-309) auto-inhibitory peptide (AIP) (2ng/ml) by using lipofectamine (4µg/ml) [24]. Transfections were performed in serum and antibiotics-free DMEM for 2h then serum was added to reach a final concentration of 10%. H_2O_2-induced signaling responses in peptide-transfected or control cells were examined 48h later.

Transfection with siRNA

A-10 VSMCs at 80-90 % confluence were transfected with CaMKIIα siRNA or control scrambled siRNA (final concentration of CaMKIIα or scrambled, non-specific siRNA was 70 nM) according to the manufacturer's protocol. Transfections were performed in serum and antibiotics-free DMEM. A mixture of CaMKIIα siRNA or control scrambled siRNA and transfection reagent was added to cells and incubated for 6h at $37^{o}C$ then serum was added to reach 10% of FBS. Cells were incubated for an additional 48h before stimulation with H_2O_2

Cell treatment, lysis and Immunoblotting

A-10 VSMCs, made quiescent by serum deprivation for 5 h, were treated in the absence or presence of various agents (e.g. H_2O_2, W-7, calmidazolium, KN-93, AIP or siRNA) at $37^{o}C$ as indicated in the legends to each figure. None of these treatments altered the viability of cells. Following these treatments the cells 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 12,000 g for 10 min at 4^0C. Protein concentrations were measured by Bradford assay. Equal amounts of protein were subjected to 7.5% SDS-polyacrylamide gel (SDS-PAGE), transferred to PVDF membranes (Millipore, MA, USA) and incubated with respective primary antibodies, (monoclonal phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 antibody (1:4,000), polyclonal phospho-specific-Ser^{473}-PKB antibody, phospho-specific-Tyr^{402}-Pyk2 and phospho-specific-IGF-1R antibody (1:1,000). phospho-specific-Thr^{286}-CaMKII antibody (1:2,000). The antigen-antibody complex was detected by a horseradish peroxidase-conjugated second antibody (1:2000), and protein bands were visualized by ECL. The intensity of specific bands was quantified by NIH Image software as described previously [25].

Statistics

The data are means ± SE of at least three individual experiments. Statistical significance was determined with paired or unpaired Student's *t* test, and $p<0.05$ was considered significant.

Results

Pharmacological inhibitors of CaM abolish H_2O_2-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. We have reported earlier that Ca^{2+}-dependent events are essential to tranduce the signals of H_2O_2 to enhance the phosphorylation of ERK1/2 and PKB in A-10 VSMC [13]. Since CaM mediates various actions of Ca^{2+}, we examined the involvement of CaM in H_2O_2-induced ERK1/2 and PKB phosphorylation. As shown in Fig.1, pretreatment of A-10 VSMCs with W-7 and calmidazolium, specific inhibitors of CaM, dose-dependently attenuated H_2O_2-induced phosphorylation of both ERK1/2 and PKB. However, as compared to ERK1/2, PKB appeared to be more sensitive to the inhibitory effect of both W-7 and calmidazolium, and exhibited almost complete attenuation of H_2O_2-stimulated phosphorylation of PKB at 5 μM of W-7 and 10 μM of calmidazolium (Fig.1 A and C). In contrast, a higher concentration of these inhibitors was required to attain the same level of inhibition of H_2O_2-enhanced phosphorylation of ERK1/2 (Fig.1, B and D).

CaMKII inhibitor attenuates H_2O_2 -induced ERK1/2 and PKB phosphorylation in A-10 VSMC. CaMKII is a downstream signaling molecule that participates in mediating the effects of CaM [19]. Therefore, we examined whether CaMKII mediates the effect of H_2O_2 in enhancing the phosphorylation of ERK1/2 and PKB in A-10 VSMC. As shown in Fig.2, pretreatment of VSMC with KN-93, a specific CaMKII inhibitor which inhibits CaMKII by competitively binding to the CaM binding domain of the enzyme, dose-dependently blocked the stimulatory effect of H_2O_2 on both ERK1/2 and PKB phosphorylation.

Calmodulin and CaMKII inhibitors attenuate glucose/glucose oxidase -induced ERK1/2 and PKB phosphorylation in A-10 VSMC. In order to investigate if endogenously generated H_2O_2 would exert a similar effect on ERK1/2 and PKB phosphorylation in A-10 VSMC as was observed with the exogenously added H_2O_2, we examined the effect of glucose (G) and glucose oxidase (GO), known to generate H_2O_2 extracellulary [26], on ERK1/2 and PKB phosphorylation in these cells. As shown in Fig.3 A and B, G/GO treatment dose-dependently enhanced the phosphorylation of both ERK1/2 and PKB. However, as compared to PKB, G/GO caused a more potent phosphorylation of ERK1/2. Time course of G/GO response using 0.64 U/ml GO revealed that phosphorylation of both ERK1/2 and PKB by G/GO treatment was rapid (Fig.3 C and D) and was detectable at 5min. However, as compared to the phosphorylation of ERK1/2 which remained elevated during the 30 min of treatment (Fig.3D), PKB phosphorylation was transient and was decreased to the basal levels within 15 min of treatment (Fig.3C).

Next, we determined whether similar to exogenously H_2O_2, pharmacological blockade of CaM or CaMKII will exert an inhibitory effect on G/GO-induced phosphorylation of ERK1/2 and PKB. As shown in Fig.4, pretreatment of cells with either calmidazolium or KN-93 prior to the addition of G/GO significantly inhibited G/GO-evoked phosphorylation of both PKB (Fig.4A) and ERK1/2 (Fig.4B).

CaMKII inhibitory peptide (AIP) and CaMKII siRNA attenuates H_2O_2 -induced ERK1/2 and PKB phosphorylation. In addition to the use of classical pharmacological inhibitors of CaM and CaMKII, we also confirmed the involvement of CaMKII in

mediating H_2O_2 responses by two additional approaches. In approach one, we utilized a peptide (AIP) corresponding to the AID of CaMKII (AA 281-309). As shown in Fig.5 transfection of A-10 VSMC for 48-h with AIP, markedly reduced H_2O_2-induced phosphorylation of both ERK1/2 and PKB (Fig.5). Lipofectamin, the transfection reagent, had no effect on H_2O_2-induced ERK1/2 and PKB phosphorylation (Fig.5). In the second approach, CaMKIIα expression was suppressed by siRNA-induced knock down of CaMKIIα . As shown in Fig. 6A, siRNA-induced knock-down of CaMKIIα decreased the expression of CaMKIIα by about 90% but had no effect on the expression of β-actin. The cells treated with siRNA to CaMKIIα exhibited a significantly attenuated effect of H_2O_2 on the phosphorylation of both PKB and ERK1/2 (Fig. 6B and C respectively). However, control siRNA was without any effect on CaMKIIα expression (Fig.6 A) or on H_2O_2-induced ERK1/2 and PKB phosphorylation (Fig.6 B,C)

H_2O_2 induces CaMKII phosphorylation in A-10 VSMC. Since Thr^{286} phosphorylation is critical for the activation of CaMKII activity, therefore, we wished to determine the effect of H_2O_2 on CaMKII phosphorylation in A-10 VSMC. To determine the effect of H_2O_2 on Thr^{286} autophosphorylation, A-10 VSMC were treated for different time periods with H_2O_2 and the lysates were immunoblotted with a specific antibody that recognizes CaMKII phosphorylation on Thr^{286}. As shown in Fig.7A, H_2O_2 -induced the phosphorylation of CaMKII in a time-dependent fashion which peaked within 2 min of exposure with H_2O_2 and then declined to basal values at 10 min. Furthermore, both calmidazolium and KN-93 inhibited the phosphorylation of CaMKII induced by H_2O_2, whereas KN-92, an inactive analog of KN-93 was without any effect (Fig.7B) .

Calmidazolium and KN-93 blocked H_2O_2-induced phosphorylation of Pyk-2. We have demonstrated that Pyk-2, a non-receptor Ca^{2+}-dependent proline rich tyrosine kinase is phosphorylated in response to H_2O_2 in A-10 VSMC [17]. A potential role of Pyk-2 in AngII-induced PKB signaling has been suggested [27]. Therefore, we investigated the effect of pharmacological blockade of CaM or CaMKII on H_2O_2- induced phosphorylation of Pyk-2. As shown in Fig.8 both calmidazolium and KN-93 prevented the phosphorylation of Pyk-2 induced by H_2O_2. In contrast, KN-92 was without effect on H_2O_2-induced response.

Calmidazolium, KN-93 and CaMKIIα siRNA blocked H_2O_2-induced phosphorylation of IGF-1R. We have previously demonstrated that H_2O_2-induced the tyrosine phosphorylation of the β–subunit of IGF-1R and pharmacological blockade of the tyrosine kinase activity of IGF-1R inhibited H_2O_2-stimulted phosphorylation of ERK1/2, PKB and Pyk-2 in A10 VSMC [17,18]. Therefore, we examined the effect of CaM or CaMKII blockade on H_2O_2–induced tyrosine phosphorylation of IGF-1R. As shown in Fig.9,A,C,D both calmidazolium and KN-93 significantly reduced H_2O_2-stimulated phosphorylation of IGF-1R. Similarly, siRNA-induced suppression of CaMKIIα also exerted an inhibitory effect on the H_2O_2-evoked IGF-1R phosphorylation (Fig.9, B).

Discussion

H_2O_2 has been shown to increase the intracellular Ca^{2+} concentration [28] and we have reported in earlier studies that both Ca^{2+} and CaM play a critical role in transducing H_2O_2-induced signaling events in VSMC [13]. In the studies presented here, we demonstrated that activation of CaMKII, a downstream effector of Ca^{2+}/CaM responses, is required to enhance H_2O_2-induced phosphorylation of ERK1/2 and PKB. This conclusion is based on the use of highly selective pharmacological inhibitors of CaM and CaMKII. We have also confirmed the participation of CaMKIIα in mediating the H_2O_2-induced phosphorylation of ERK1/2 and PKB by using siRNA to specifically knock down CaMKIIα

Although, the involvement of CaMKII in H_2O_2-induced responses on NF-kappaB activation in T-lymphocytes [29], on ERK1/2 and $p38^{MAPK}$ activation and in eNOS expression in endothelial cells [24,30], and on JNK and $p38^{MAPK}$ activation in VSMC [31], have been demonstrated , to our knowledge, the data presented here are the first to show a role of CaMKIIα in mediating the effect of H_2O_2 on ERK1/2 and PKB phosphorylation in A10 VSMC.

In addition to H_2O_2 other agonists such as AngII, norepinephrine, epidermal growth factor, ionomycin and ATP have also been shown to activate MAPK signaling via CaMKII [22,32]. Our findings showing that H_2O_2 enhanced the phosphorylation of CaMKII in Thr^{286}, further supports a role of activated CaMKII in H_2O_2-induced responses in VSMC. These data are also consistent with H_2O_2–induced increase in the phosphorylation of CaMKII reported in astrocytes [33] and T-Jurkat-lymphocytes [34]. In contrast, our results do not support the observation of Robison et al, who showed a

decrease in CaMKII activity in response to H_2O_2 in neurons [35]. It should be noted that in addition to CaMKII, other receptor and non-receptor tyrosine kinase such as IGF-1R and Pyk-2 have been implicated in H_2O_2-induced signaling events in VSMC [17,36,37]. Our earlier studies have demonstrated that H_2O_2 enhanced the tyrosine phosphorylation of IGF-1R β subunit and this event contributed to the initiation of H_2O_2-evoked PKB phosphorylation with an intermediary role of Pyk-2 [17]. It has been shown that phosphorylated form of Pyk-2 interacts with several signaling molecules such as Shc and Grb2 implicated in ERK1/2 activation, and with p85 subunit of phosphatidyl inositol 3-kinase which is involved in PKB activation [38]. Pyk-2 also mediates the effect of AngII in inducing ERK1/2 and PKB activation in VSMC [27]. Since ROS generation is critical in transducing AngII response, it is possible that Pyk-2 plays a similar role in H_2O_2-induced effects on ERK1/2 and PKB phosphorylation. Our findings that inhibition of either CaM or CaMKII significantly attenuated H_2O_2-induced phosphorylation of Pyk-2 indicates a requirement of CaMKII in enhancing Pyk-2 phosphorylation and suggest the participation of Pyk-2 in the signaling cascade leading to the activation of ERK1/2 and PKB in response to H_2O_2 in VSMC. The results showing that blockade of CaMKIIα either by pharmacological approach or by siRNA-induced silencing of CaMKIIα inhibited H_2O_2–induced tyrosine phosphorylation of IGF-1R, suggests an upstream role of CaMKIIα in this process. Although ionomycin-induced increase in EGF-R phosphorylation had earlier been shown to be mediated through CaMKII-dependent pathway [32], our current studies are the first to report that CaMKIIα plays a key role in mediating H_2O_2-induced phosphorylation of IGF-1R, Pyk-2, ERK1/2 and PKB.

An involvement of CaMKII in regulating the proliferation, migration and differentiation of VSMC has been shown in several studies [39-43]. Since a hightened proliferation and migration of VSMC are hallmarks of vascular disease, and excessive generation of ROS has been suggested to play an important role in the pathogenesis of vascular diseases [5-7,11] it may be suggested that ROS-induced upregulation of CaMKII, through IGF-1R, ERK1/2 and PKB signaling pathways may contribute to aberrant VSMC functions associated with these disorders. This notion is further supported by studies showing that pharmacological blockade of CaMKII by KN-93 improved vascular hyperplasia and hypertension in AngII-induced hypertensive rats [44], and normalized aberrant vascular reactivity in diabetes-induced vascular dysfunction [45].

In conclusion, we have shown that CaMKIIα serves as a critical upstream component in triggering the H_2O_2-induced signaling cascade resulting in the phosphorylation of IGF-1R, ERK1/2 and PKB in VSMC. It may be suggested that through the activation of these signaling events CaMKII contributes to the regulation of various cellular processes including cell growth, proliferation, hypertrophy and survival in VSMC, and a dysregulation of CaMKII activity may play an important role in the pathogenesis of vascular disease.

Acknowledgements

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Author contributions:

AB and NRP performed research. AB and AKS analyzed the data, AB and AKS wrote the paper, AKS designed the research.

Abbreviation:

AngII–angiotensin
AID–autoinhibitory domain peptide

AIP–autoinhibitory peptide (corresponding to the AID of CaMKII)

CaM–calmodulin

CaMKII–CaM-dependent protein kinaseII

DMEM–Dulbecco's Modified Eagle medium

ERK1/2–extracellular signal-regulated kinases 1 and 2

H_2O_2–hydrogen peroxide

G–glucose

GO–glucose oxidase

IGF-1R– insulin-like growth factor-1 receptor

MAPK–mitogen activated protein kinases

$O_2^{\cdot -}$ –superoxide anion

PKB–protein kinase B

Pyk2– Proline-rich tyrosine kinase 2

ROS–reactive oxygen species

siRNA– Small interfering RNA

VSMC–vascular smooth muscle cells

Figures legends

Figure 1. Pharmacological inhibition of CaM abolishes H_2O_2-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated in the absence (0) or presence of the indicated concentration of W-7 (section A and B) or calmidazolium (section C and D) for 30 min followed by stimulation with 250 μM of H_2O_2 for 5 min. Cell lysates were immunoblotted with phospho-specific-Ser^{473}-PKB antibodies (A and C) and phospho-specific-Thr^{202}-Tyr^{204}-ERK1/2 antibodies (B and D), 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 (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots showing in the middle panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage of control, taken as 100%. $P< 0.05$ considered as statistically significance versus H_2O_2 stimulation alone.

* indicates that $P < 0.05$, ** indicates that $P <0.005$, and # indicates that $P <0.0005$.

Figure 2. CaMKII inhibitor attenuates H_2O_2-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were pretreated in the absence (0) or presence of the indicated KN-93 concentrations for 30 min followed by stimulation with 250 μM of H_2O_2 for 5 min. Cell lysates were immunoblotted with phospho-specific-Ser^{473}-PKB antibodies (A) and phospho-specific- Thr^{202} -Tyr^{204}-ERK1/2 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 (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots showing in the middle panel. Values are the means ± SE of at least 3 independent experiments and

are expressed as percentage of control, taken as 100%. $P < 0.05$ considered as statistically significance versus H_2O_2 stimulation alone. * indicates that $P < 0.05$, ** indicates that $P < 0.005$ and # indicates that $P < 0.0005$.

Figure 3: Dose response and time course of glucose/glucose oxidase-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Serum-starved quiescent A-10 cells were treated without (0) or with 6mM glucose (G) and the indicated concentrations of glucose oxidase (GO) for 10 min (A and B) and without (0) or with G/GO (6mM/0.64 U/ml) for the indicated time periods (C and D). Cell lysates were immunoblotted with phospho-specific-Ser^{473}-PKB antibodies (A and C) and phospho-specific-Thr^{202}-Tyr^{204}-ERK1/2 antibodies (B and D), 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 (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the middle panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage of control, taken as 100%. $P < 0.05$ considered as statistically significance versus control.

* indicates that $P < 0.05$, ** indicates that $P < 0.005$, and # indicates that $P < 0.0005$.

Figure. 4. Calmodulin and CaMKII inhibition blocks ERK1/2 and PKB phosphorylation induced by G/GO.

Serum-starved quiescent A-10 cells were pretreated in the absence (0) or presence of CMZ, KN-92 or KN-93 (10 M) for 30 min followed by stimulation with G/GO (6mM/0.64U/ml) for 10 min. Cell lysates were immunoblotted with phospho-specific-Ser^{473}-PKB antibodies (A) and phospho-specific-Thr^{202}-Tyr^{204}-ERK1/2 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 (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots shown in the middle panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage of control, taken as 100%. $P < 0.05$ considered as statistically significance versus G/GO stimulation.

* indicates that $P < 0.05$, ** indicates that $P < 0.005$, and # indicates that $P < 0.0005$.

Figure 5. CaMKII AIP peptide (AA 281-309) attenuates H_2O_2-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. Cells were transfected, using lipofectamin, with CaMKII autoinhibitory peptide (AIP) for 48-h prior to stimulation with H_2O_2 (250 μM, 5min). The cells treated with lipofectamin alone were used as control. Cell lysates were immunoblotted with phospho-specific-Ser^{473}-PKB antibodies (A) and phospho-specific-Thr ^{202}-Tyr^{204}ERK1/2 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 (bar diagrams) in each section represent average data quantified by densitometric scanning of immunoblots showing in the middle panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage of control, taken as 100%. $P < 0.05$ considered as statistically significance versus H_2O_2 stimulation alone. # indicates that $P < 0.0005$.

Figure 6. CaMKIIα siRNA attenuates H_2O_2-induced ERK1/2 and PKB phosphorylation in A-10 VSMCs. A-10 VSMC were transfected with CaMKII siRNA or control siRNA for 48h prior to stimulation with H_2O_2 (250 μM, 5min). Cell lysates were immunoblotted with CaMKIIα antibodies (A), phospho-specific-Ser^{473}-PKB antibodies (B) and phospho-specific-Thr^{202}-Tyr^{204}-ERK1/2 antibodies (C). Blots were also analyzed for total PKB

and ERK1/2 (Bottom panels of B and C). Top panels (bar diagrams) in B and C represent average data quantified by densitometric scanning of immunoblots of p-Proteins showing in the middle panel. Blots in panel A was also blotted with β actin. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage of control, taken as 100%. $P< 0.05$ considered as statistically significance versus H_2O_2 stimulation alone. ** indicates that $P < 0.005$ and # indicates that $P < 0.0005$.

Figure.7. H_2O_2 induces the phosphorylation of CaMKII which is blocked by CaM and CaMKII inhibitors in A-10 VSMC.

(A): Serum-starved quiescent A-10 cells were treated with H_2O_2 (250 μM) for indicated time periods. (B): Quiescent A-10 cells were pretreated in the absence (0) or presence of calmidazolium(CMZ), KN-92 or KN-93 (10μM) for 30 min followed by stimulation with 250 μM of H_2O_2 for 5 min. Cell lysates were immunoblotted with phospho-specific-Thr^{286}-CaMKII antibodies (middle panel in each section) and total CaMKIIα antibodies (Bottom panel in each section). Top panels in each section (bar diagrams) represent average data quantified by densitometric scanning of immunoblots of p-CaMKII. Values are the means ± SE of at least 3 independent experiments are expressed as percentage of control, taken as 100%. $P< 0.05$ considered as statistically significance versus control for (A) and versus H_2O_2 stimulation alone for (B). * indicates that $P < 0.05$, ** indicates that $P <0.005$ and # indicates that $P <0.0005$.

Figure.8. CaMKII inhibition blockes Pyk-2 phosphorylation induced by H_2O_2

Serum-starved quiescent A-10 cells were pretreated in the absence (0) or presence of CMZ, KN-92 or KN-93 (10μM) for 30 min followed by stimulation with 250 μM of

H_2O_2 for 5 min. Cell lysates were immunoblotted with phospho-Pyk-2 antibodies (middle panel) and total Pyk-2 antibodies (Bottom panel). Top panels in each section (bar diagrams) represent average data quantified by densitometric scanning of immunoblots of p-Pyk-2. Values are the means ± SE of at least 3 independent experiments are expressed as percentage of control, taken as 100%. $P< 0.05$ considered as statistically significance versus H_2O_2 stimulation alone. * indicates that $P < 0.05$, ** indicates that $P <0.005$.

Figure. 9. CaMKIIα inhibition attenuates IGF-1R phosphorylation induced by H_2O_2

Serum-starved quiescent A-10 cells were pretreated in the absence (0) or presence of CMZ, KN-92 or KN-93 (10μM) for 30 min followed by stimulation with 250 μM of H_2O_2 for 5 min (A). In (B), A-10 VSMCs were transfected with either control siRNA or CaMKII siRNA, as described in the legend to Fig 4, 48-h prior to stimulation with 250 μM of H_2O_2 for 5 min. In (C) and (D): Quiescent A-10 VSMC were pretreated with the indicated concentrations of CMZ (C) and KN-93 (D) for 30 min followed by stimulation with 250 μM of H_2O_2 for 5 min. Cell lysates were immunoblotted with p-IGF-1R antibodies that recognize the phosphorylated form of IGF-1R (phospho-$Tyr^{1131/1135/1136}$) (middle panel in each section) and total IGF-R antibodies (Bottom panel in each section). Top panels in each section (bar diagrams) represent average data quantified by densitometric scanning of immunoblots of p-IGF-1R. Values are the means ± SE of at least 3 independent experiments are expressed as percentage of control, taken as 100%. $P< 0.05$ considered as statistically significance versus H_2O_2 stimulation alone. * indicates that $P < 0.05$, ** indicates that $P <0.005$.

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FIGURE 1

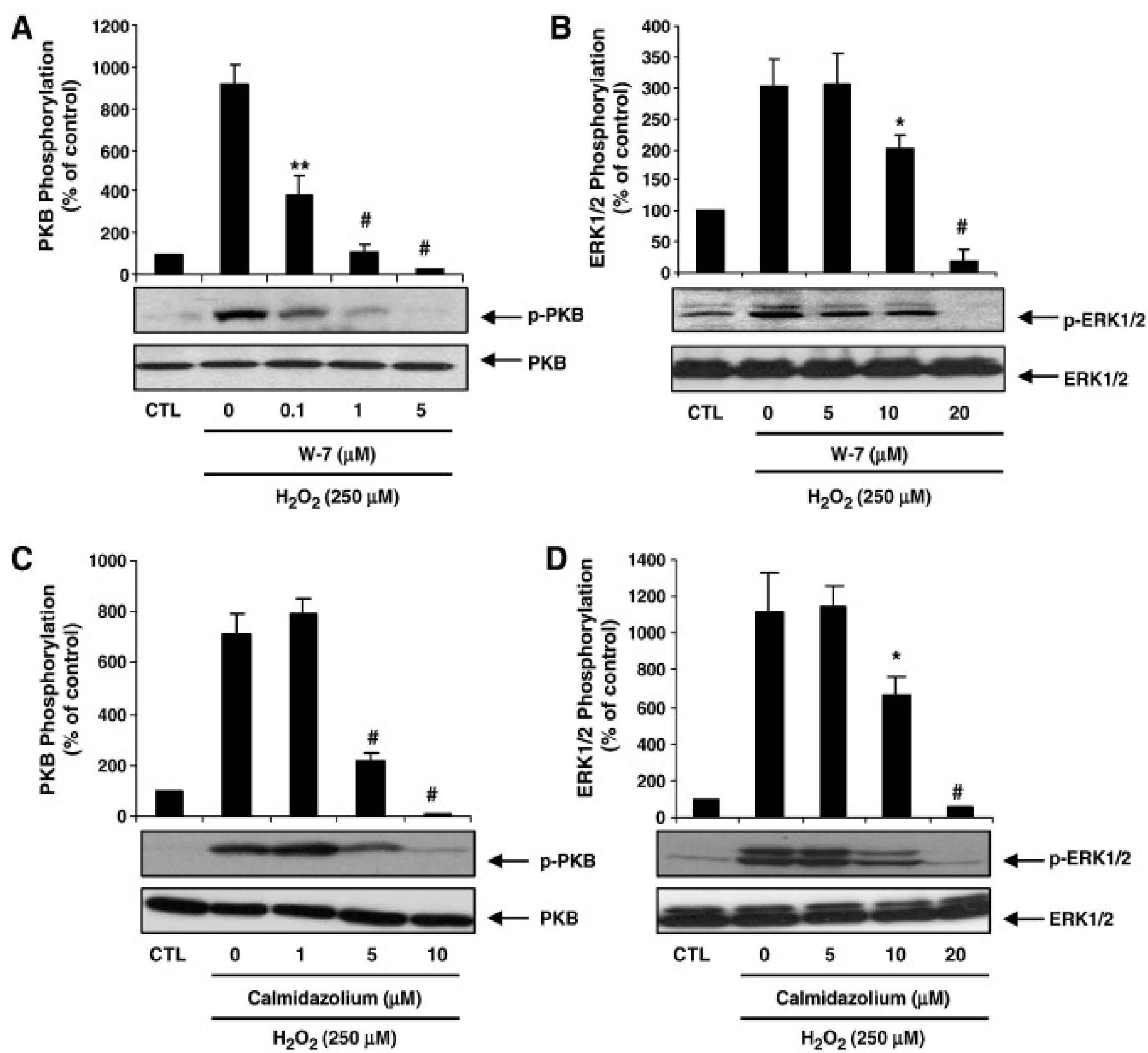


FIGURE 2

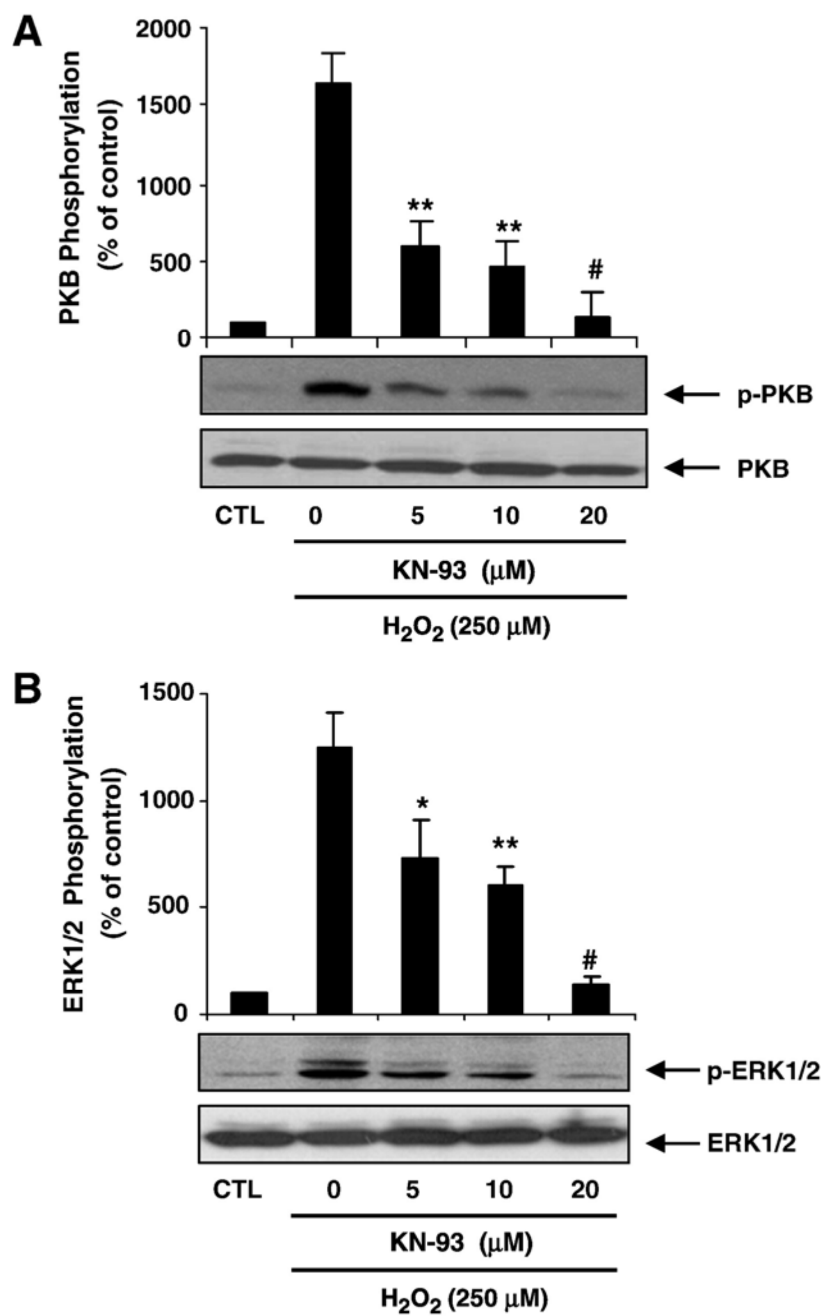


FIGURE 3

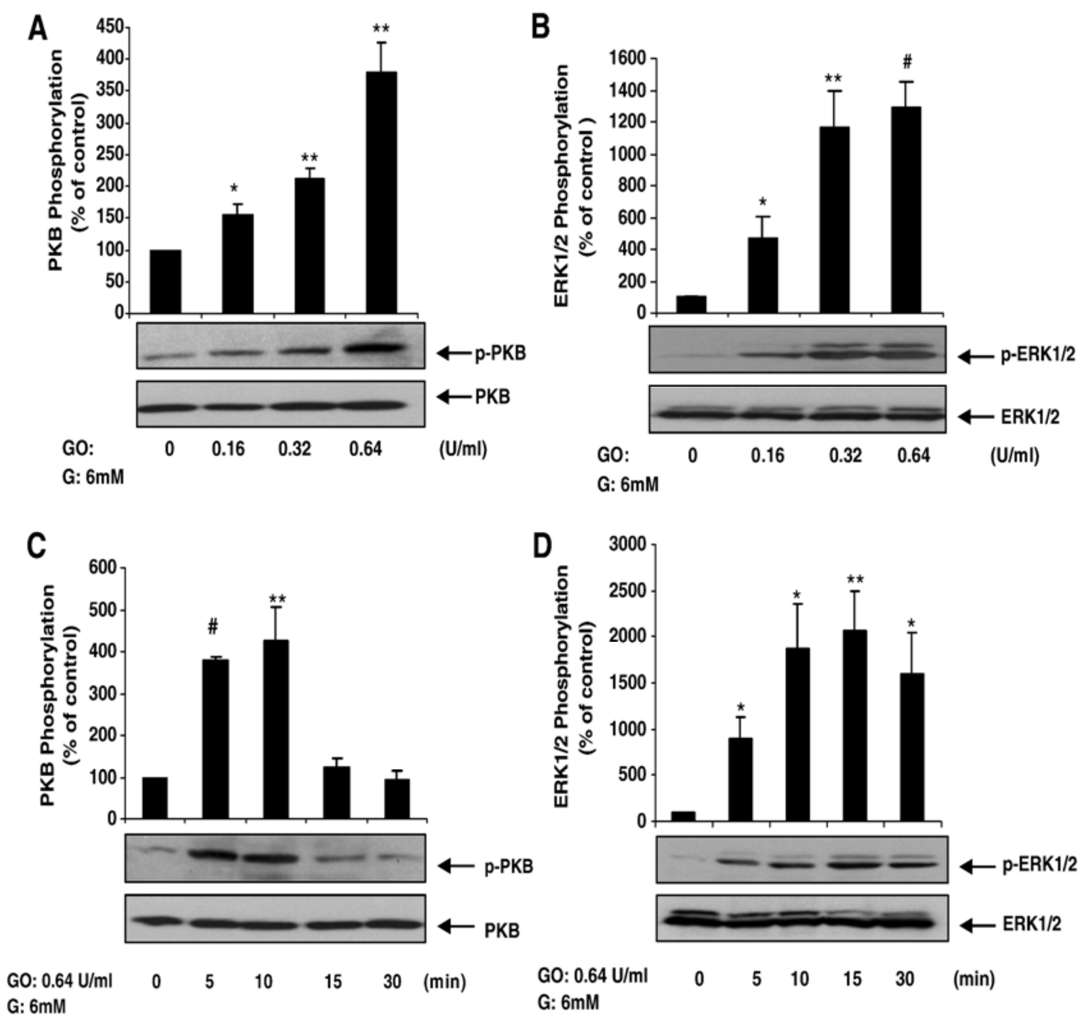


FIGURE 4

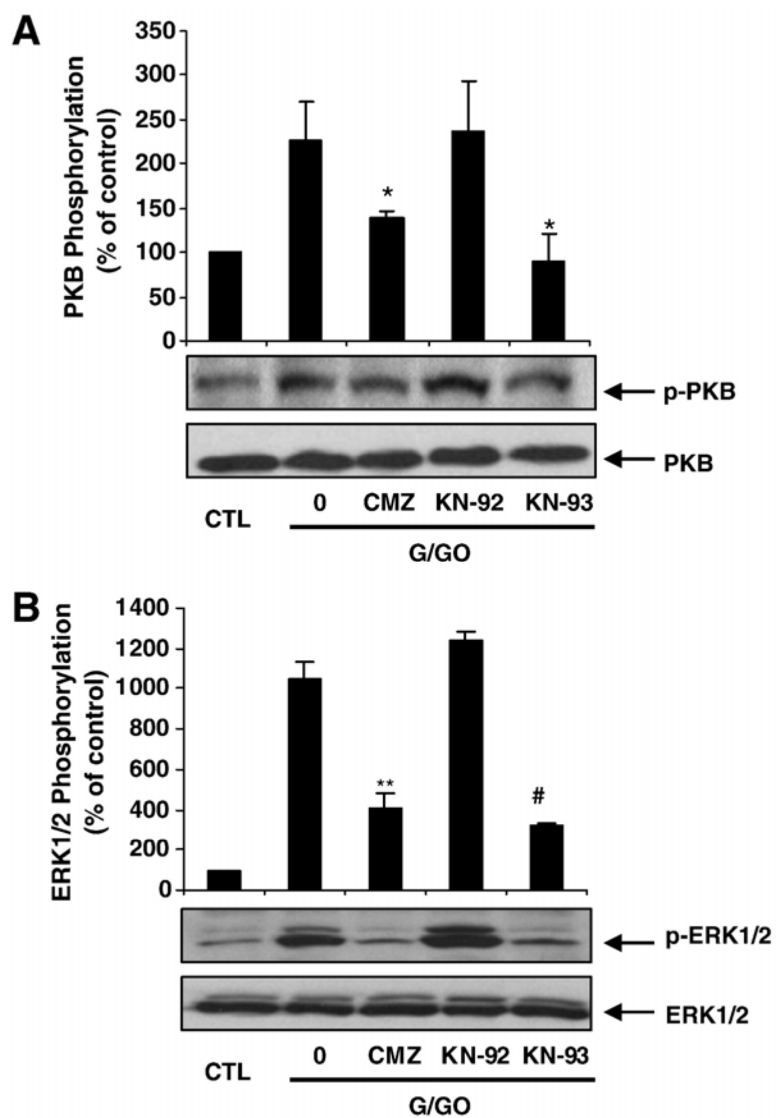


FIGURE 5

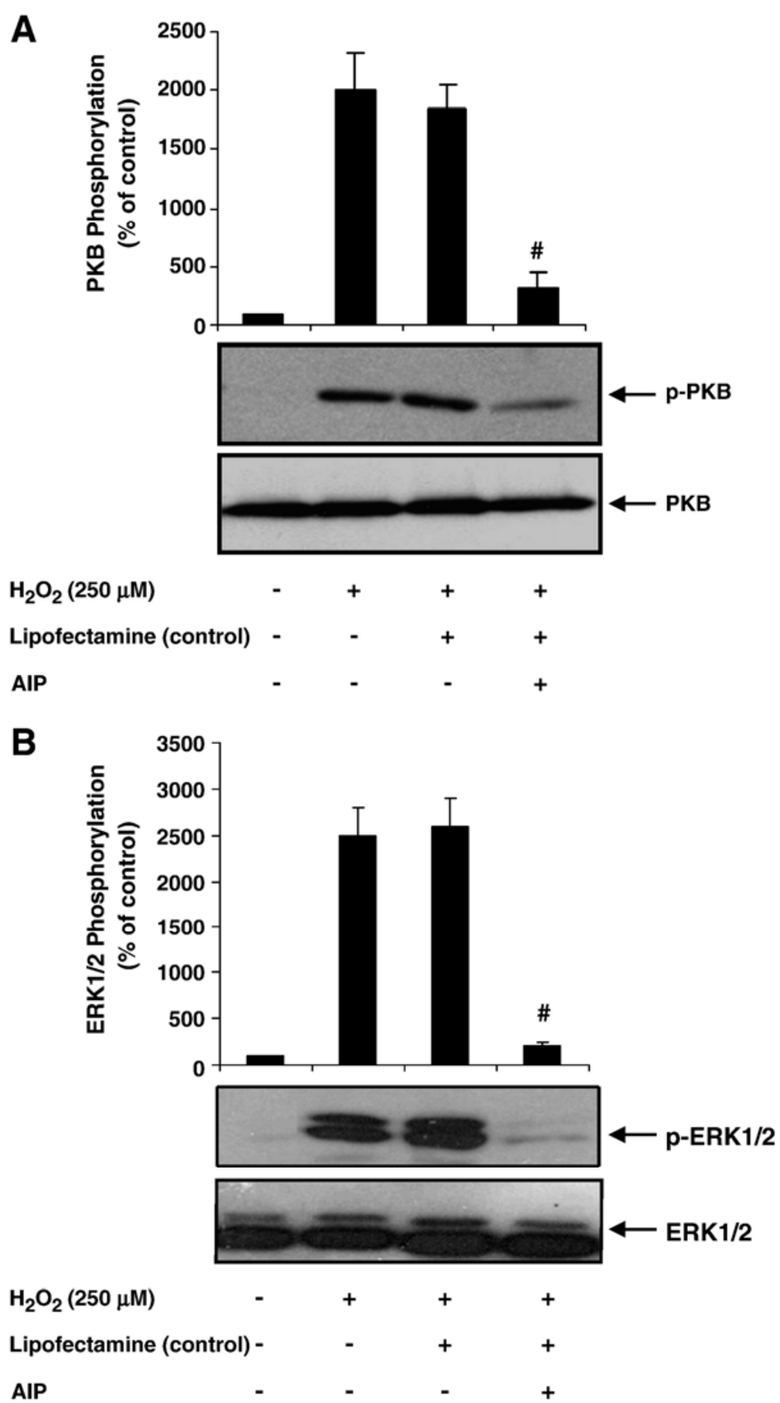


FIGURE 6

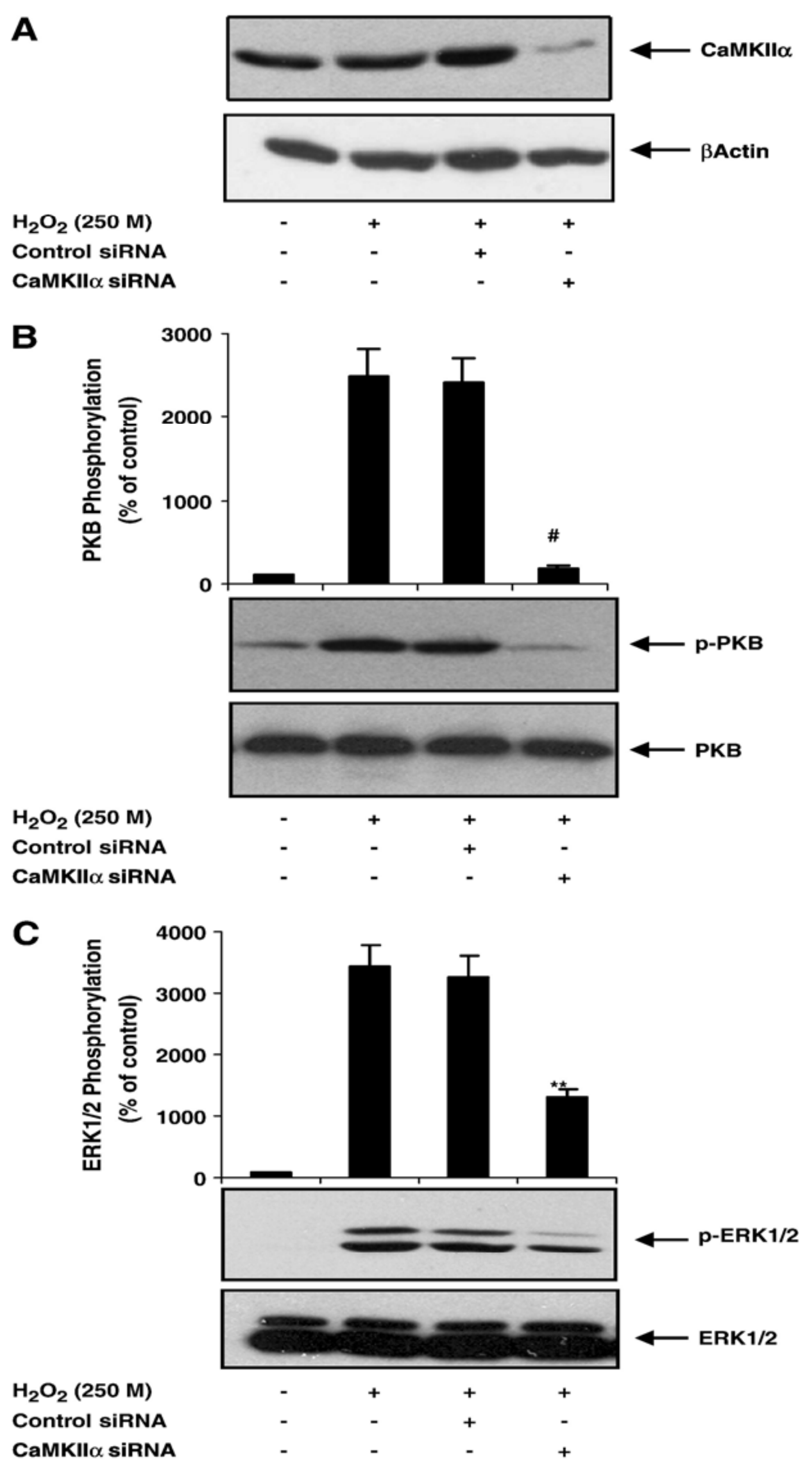


FIGURE 7

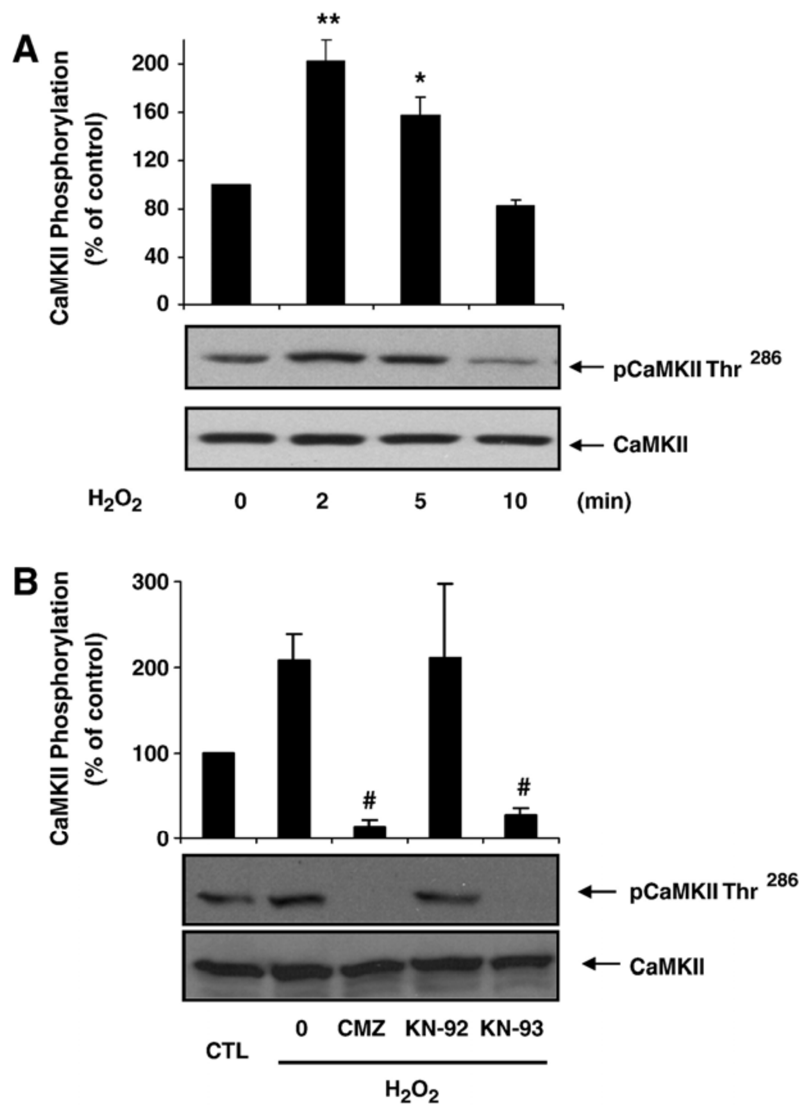


FIGURE 8

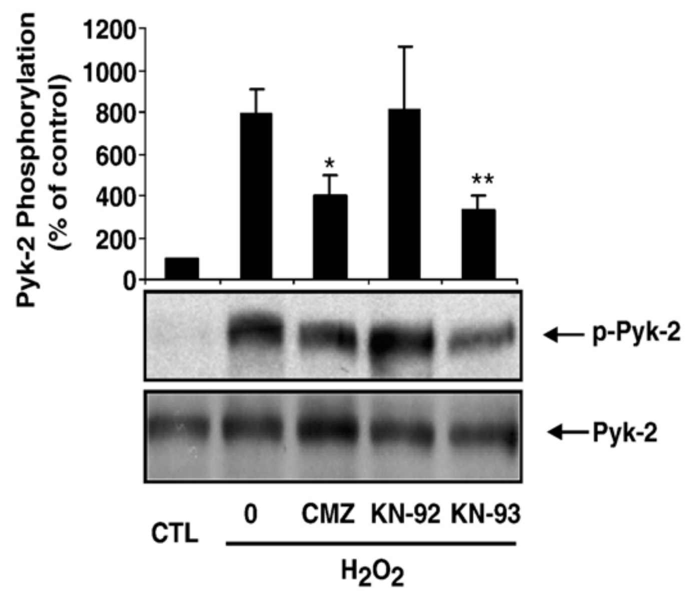
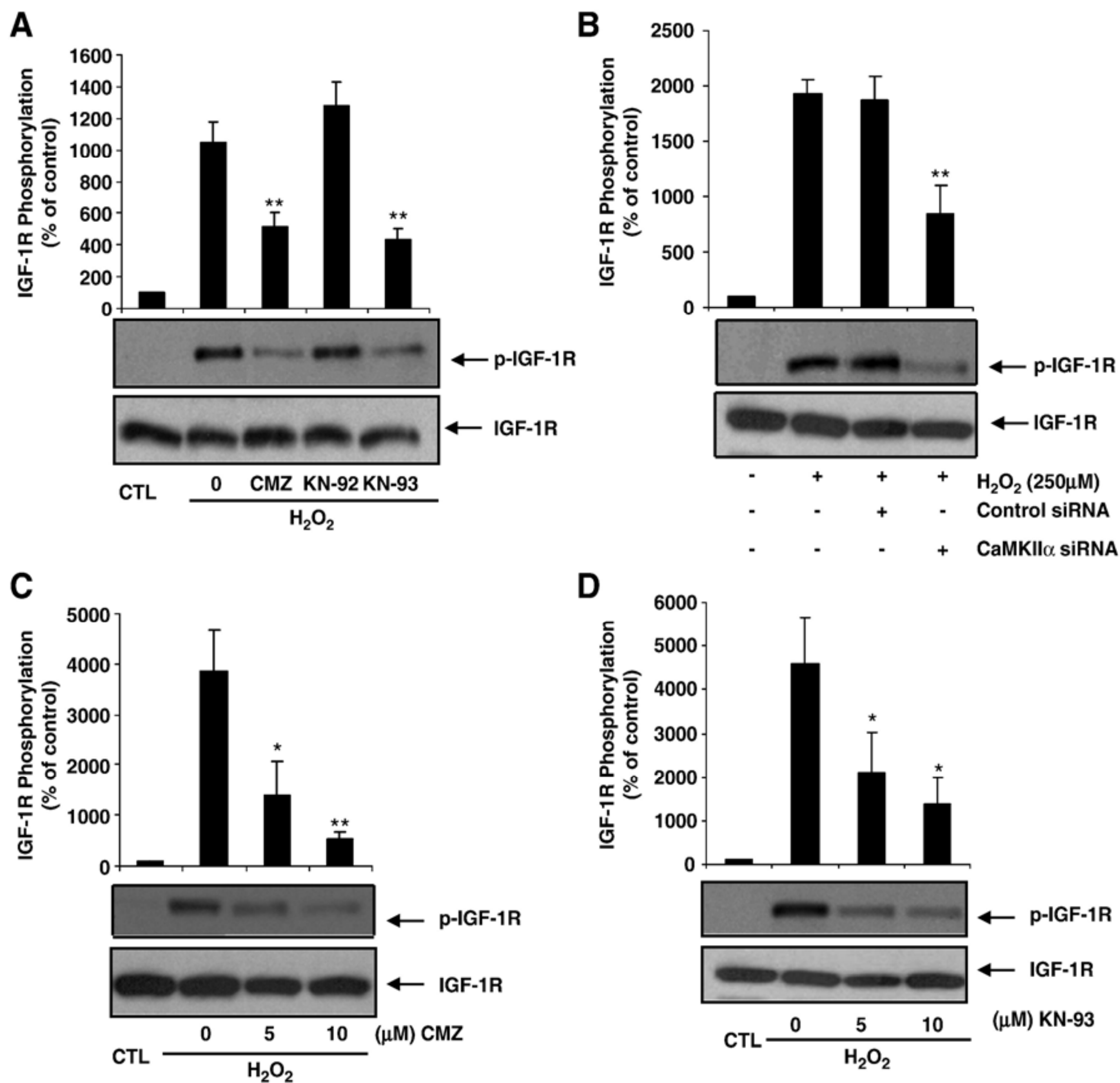


FIGURE 9



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CHAPTER-4

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

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Running title: Regulation of ET-1 signaling by NO

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Abstract

Nitric oxide (NO), in addition to its vasodilator action, has also been shown to antagonize the mitogenic and hypertrophic responses of growth factors and vasoactive peptides such as endothelin-1 (ET-1) in vascular smooth muscle cells (VSMCs). However, the mechanism by which NO exerts its anti-mitogenic and anti-hypertrophic 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, proliferation and hypertrophy in A-10 VSMC. Treatment of A-10 VSMCs with S-nitroso-N-acetylpenicillamine (SNAP) or sodium nitroprusside (SNP), two NO donors, attenuated the ET-1-enhanced phosphorylation of several key components of growth promoting and hypertrophic signaling pathways such as ERK1/2, PKB and Pyk2. On the other hand, the inhibition of the endogenous NO generation by using N-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, increased the ET-1-induced phophosphorylation of these signaling components. Since, NO mediates its effect principally through a cyclic GMP/soluble guanylyl cyclase (sGC) pathway, we investigated the role of these molecules in NO action. 8-Br-cGMP, a non-metabolizable and cell permeable analogue of cGMP, exhibited a similar effect to that of SNAP and SNP. Furthermore, oxadiazole quinoxalin (ODQ), an inhibitor of sGC, reversed the inhibitory effect of NO on ET-1-induced responses. SNAP treatment also decreased the protein synthesis induced by ET-1. Taken together, these data demonstrate that NO, in a cGMP-

dependent manner, attenuated ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2 and also antagonized the hypertrophic effects of ET-1. It may be suggested that NO-induced generation of cGMP contributes to the inhibition of ET-1-induced mitogenic and hypertrophic responses in VSMCs. (260words)

Key words: Cell signaling, protein synthesis, endothelin-1, NO.

Introduction

Endothelin-1 (ET-1) is a 21-amino acid peptide and is considered as a potent vasoconstrictor (47). It also exhibits mitogenic activity in vascular smooth muscle cells (VSMC) (5; 23; 24), suggesting a possible role for ET-1 in the pathogenesis of many diseases, such as atherosclerosis (29), hypertension (18) and restenosis after angioplasty (11).

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 (13), Ca^{2+} (32), mitogen-activated protein kinases (MAPKs) including extracellular signal-regulated kinases 1/2 (ERK1/2), c-Jun-NH_2-terminal kinase (JNK) and p38mapk (7; 8; 39; 50; 51) and phosphatidylinositol 3-kinase (PI-3K) (8; 17). Activation of receptor and non-receptor protein tyrosine kinases (PTKs) in transducing ET-1-induced signaling responses have also been suggested (16; 26; 27; 39; 49). PTKs activated by ET-1 include epidermal growth factor (EGF) (27), c-Src (16; 26; 38) and a Ca^{2+}-dependent PTK, Pyk2 (26; 39). Of particular interest, ET-1 mediates Pyk2 activation which contributes to ERK1/2 (26) and JNK (27) signaling in cardiomyocytes and p38mapk (39) in mesangial cells.

Nitric oxide (NO) is a free radical that has been suggested to play an important role in cardiovascular function (38). NO mediates relaxation principally through the stimulation of soluble guanylyl cyclase (sGC), leading to enhanced

production of intracellular cGMP, which in turn, activates cGMP-dependent protein kinases (PKG) (30). NO can also influence cellular events by a PKG-independent mechanism (14; 22), and is also able to react with superoxide anion to form the reactive peroxynitrite radical (25), a potent oxidant with the potential to disrupt protein structures by nitrating the tyrosine residues in protein (48). In addition to its vasodilating effect, NO has been suggested to antagonize the physiological and pathophysiological effects of several growth factors such as EGF (52), angiotensin II (AII) (46) as well as ET-1 (1). This is probably achieved by inhibiting one or more serine/ threonine/ tyrosine kinases implicated in the signaling events induced by these factors. Several studies using AII, have shown that NO suppressed the activation of ERK1/2, p38mapk and JNK (45) as well as Pyk2 (46) in cardiac fibroblasts. It has also been recently reported that in rat neonatal pulmonary vascular smooth muscle cells a NO donor inhibited ET-1-induced ERK1/2 phosphorylation (3). However, to our knowledge, a possible contribution of NO on ET-1-induced activation of other signaling 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, the key mediators of growth-promoting, proliferative, hyperthrophic survival responses. In addition, we have also examined whether NO acts via a cGMP-dependent mechanism in eliciting these responses.

Materials and Methods

Materials

ET-1 was purchased from Peninsula Laboratories (Belmont, CA, USA), and S-nitroso-N-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), 8-Bromo-guanosine 3', 5'-cyclic monophosphate (8-Br-cGMP) and 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), were obtained from Calbiochem (San Diego, CA). L-NAME was purchased form Sigma Aldrich (St-Louis, MO). Monoclonal phospho-specific-Tyr^{204}-ERK1/2 antibody, polyclonal ERK1/2 antibody, eNOS antibody, iNOS antibody and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were from Santa Cruz Biotech (Santa Cruz, CA). 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). The enhanced chemiluminescence (ECL) detection system kit and L-(4,5-^{3}H) leucine were from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada). Human umbilical vein endothelial cells (HUVEC) was a gift from Dr Eric Thorin, Montreal Cardiology Institute.

Methods

Cell culture

A-10 VSMC derived from embryonic rat thoracic aorta 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 (40). 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 Immunoblotting

Cells incubated in the absence or presence of various agents were washed twice with ice-cold PBS and lysed in 200 μl of buffer (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 1 mM Na orthovanadate, 10 mM Na fluoride, 10 mM Na pyrophosphate, 2 mM benzamidine, 2 mM ethylenebis(oxyethylenenitrolo)-tetraacetic acid, 2 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 0.5 μg/ml leupeptin) on ice. The cell lysates were centrifuged at 12,000g for 10 min at 4^{0}C. Protein concentrations were measured by Bradford assay. Equal amounts of protein were subjected to either 7.5% or 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), eNOS or iNOS antibodies (1:2,000)). 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 (31).

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 NO, cells were pretreated for 30 min with 10 or 100 μM of S-nitroso-N-acetylpenicillamine (SNAP) (Calbiochem, San Diego, CA) 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

Both SNAP and SNP 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 Fig. 1, pretreatment of A-10 VSMC with SNAP for 15 min dose-dependently attenuated ET-1-induced phosphorylation of all of these protein kinases. Among the kinases, PKB appeared to be more sensitive to the inhibitory effect of SNAP and exhibited almost complete attenuation of ET-1-stimulated phosphorylation at 10 μM (Fig. 1B). In contrast, ET-1-enhanced phosphorylation of ERK1/2 and Pyk2 was inhibited significantly only at 300 μM SNAP.

In addition, sodium nitroprusside (SNP), another NO donor also exhibited a similar effect and attenuated ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation to varying degrees (Fig. 2).

L-NAME potentiated ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2 in A-10 VSMCs.

To examine if decreasing the endogenous NO production by inhibition of nitric oxide synthase (NOS) activity would modify the effect of ET-1 on various signaling components, we investigated the effect of pretreatment of A-10 VSMC with L-NAME, a specific inhibitor of NOS on ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2. As shown in Fig.3, L-NAME treatment, at both the doses used, potentiated the response of ET-1 on all three signaling component examined. 100 μM L-NAME potentiated ET-1-induced ERK1/2 phosphorylation by 4 fold (Fig. 3A), whereas only 2 fold potentiation in PKB and Pyk2 phosphorylation was observed under these conditions (Fig. 3B,C). Although, it is generally believed that VSMC are devoid of NOS, however, the ability of L-NAME to potentiate ET-1-induced signaling suggested the presence of NOS in A10-VSMC. This possibility was evaluated by subjecting the total cellular lysates of A10-VSMC or HUVEC to western blotting using specific antibodies against eNOS and iNOS. As shown in Fig.4A, A10 cells exhibited a significant expression of eNOS in the basal state. However, as compared to HUVEC the expression level of eNOS in A10-VSMC was much less. Under these conditions iNOS could not be detected in A10-VSMC (data not shown). Furthermore, treatment of A10-VSMC with ET-1 or L-NAME did not alter the eNOS expression in these cells (Fig.4B)

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

Since SNAP-induced production of NO would cause an elevation in 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 by pretreating the cells with 8-Br-cGMP, a non-metabolizable and cell permeable analogue of cGMP. As shown in Fig.5, treatment of cells with 8-Br-cGMP decreased ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. 100 μM 8-Br-cGMP inhibited ET-1-induced ERK1/2 and Pyk2 phosphorylation almost completely , whereas, 50% inhibition in PKB phosphorylation was observed.

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 (sGC), which prevents the generation of cGMP from GTP. For these experiments, cells 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 Fig. 6, A-C, pretreatment with 10 μM ODQ resulted in a significant reversal in the inhibitory effect of SNAP on ET-1-stimulated ERK1/2, PKB and Pyk2 phosphorylation. Under these conditions however, pretreatment of cells with ODQ alone did not modify ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. (Fig.6D)

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 (42). Therefore, we next examined whether there was a correlation between the response of SNAP and ET-1-induced protein synthesis. As shown in Fig.7, ET-1 increased [^{3}H]leucine incorporation in total cellular proteins by about 50% over control. However, pretreatment of cells with SNAP dose-dependently decreased ET-1-induced [^{3}H]leucine incorporation with almost complete attenuation observed at 100 μM of SNAP. SNAP alone did not significantly affect basal [^{3}H]leucine uptake.

Discussion

Here we have provided evidence showing that NO generation induced by SNAP and SNP significantly attenuated ET-1-enhanced phosphorylation of ERK1/2, PKB and Pyk2 in VSMC. We have also demonstrated that SNAP treatment was able to antagonize ET-1-induced total protein synthesis, an index of hypertrophy, in VSMC. Since both ERK1/2 and PKB pathways play critical role in mediating hypertrophic and cell survival responses (8), it is reasonable to suggest that the ability of NO donors to inhibit ET-1-induced activation of these pathways is responsible for the antihypertrophic and vascular protective effect of NO. Although NO donors have been found to attenuate EGF (52), Platelet-Derived Growth Factor (PDGF) (35) and AII (44) stimulated proliferation of VSMC and cardiac fibroblasts, studies reported here are the first to demonstrate an effect of NO on ET-1-induced phosphorylation of ERK1/2, PKB and Pyk2 in A-10 VSMC. These results are similar to the studies in neonatal pulmonary VSMC, in which SNP treatment was found to inhibit ET-1-induced ERK1/2 phosphorylation (3) and in rat cardiac fibroblasts, where AII-induced phosphorylation of ERK1/2 and Pyk2 was blocked by SNAP (45; 46). However, our work represents the first study demonstrating that NO antagonizes ET-1-induced PKB and Pyk2 activation as well as ET-1-induced protein synthesis in VSMC.

The demonstration that pharmacological inhibition of basal NO production by using L-NAME augmented ET-1 responses on ERK1/2, PKB and Pyk2

phosphorylation support an inhibitory role of NO on ET-1-induced signaling events in A10-VSMC. A similar increase in ET-1-induced phosphorylation of ERK1/2 in L-NAME treated pulmonary artery VSMC has also been demonstrated (3). It is generally believed that VSMC are devoid of NOS activity however, recently, both iNOS and eNOS immunoreactivity as well as NOS activities have been detected in isolated VSMC (9; 10; 34). Our results showing that A10-VSMC express eNOS in the basal state, further supports the presence of eNOS in VSMC. Thus it is possible that L-NAME-induced inhibition of eNOS by decreasing NO bioavailability potentiates ET-1-induced signaling events in these cells.

NO is believed to exert its physiological effect through activation of sGC, a heme containing protein (20). Binding of NO to the heme iron leads to allosteric modification of sGC, resulting in its enhanced catalytic activity to produce cGMP (21). cGMP, thus generated, elicits its downstream responses by interacting with its target proteins such as PKG (37). Additional non-sGC/cGMP-dependent mechanism of NO action has also been suggested, which include ONOO- catalyzed post-translational modification of protein via nitration of tyrosine residues (6). However, our results showing that 8-Br-cGMP mimicked the effect of SNAP and SNP in decreasing ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation suggest that an intermediary role of cGMP in exerting this inhibitory response. Further proof for the involvement of sGC in this processes has been provided by using ODQ, a specific inhibitor of sGC which can block SNAP-induced elevations in cGMP levels in rat aortic VSMC

(28), A-10 VSMC (4), endothelial cells (19) and cardiomyocytes (36). We found that ODQ treatment of A-10 VSMC was able to significantly reverse the inhibitory effect of SNAP on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation. A similar involvement of cGMP/PKG pathway in NO-induced inhibition of ERK1/2 phosphorylation by ET-1 in cardiomyocytes and in pulmonary artery VSMC has also been reported (3; 12). The fact that 8-Br-cGMP caused only partial inhibition of ET-1-induced PKB phosphorylation and ODQ was not able to completely reverse the SNAP-induced responses in our studies suggest a partial contribution of non-cGMP-dependent events in mediating the effect of NO donors in ET-1-induced responses. These cGMP-independent mechanisms include nitration of some upstream signaling components resulting in attenuation of their catalytic activity. Existence of cGMP-independent mechanism in mediating the antiproliferative effects of NO has also been suggested from other studies in which ODQ, despite lowering NO-induced cGMP levels, failed to reverse the antiproliferative effect of NO donors in pulmonary microvascular smooth muscle cells (41) or in human endothelial cells (19). In these studies , however the effect of ODQ on signaling pathways linked to proliferative responses was not investigated.

The precise mechanism by which cGMP inhibits ERK1/2 signaling remains elusive however, the ability of PKG, the downstream effector of cGMP action, to phosphorylate c-Raf kinase on Ser ^{43} and the resulting uncoupling between Ras-Raf might contribute to this effect (43). Since the upstream elements leading the PKB phosphorylation are different from that of ERK1/2

(8), the precise mechanism by which cGMP/PKG system attenuates PKB phosphorylation remain undefined.

Pyk2 is a Ca2+ -dependent proline rich-non receptor protein tyrosine kinase which plays an essential role in Ang II-induced ERK1/2 signaling and hypertrophy in VSMC (33) Pyk2 is activated by autophosphorylation in Tyr[402] located in its catalytic domain (2). It thus may also be possible that SNAP/cGMP-induced decrease in Pyk2 phosphorylation observed in our studies contributed to the attenuating effect of SNAP on ET-1-induced signaling in A-10 VSMC. NO generation has been shown to attenuate IGF-1 and insulin-induced elevation in H_2O_2 levels through a cGMP dependent event in VSMC (53). ET-1–induced ERK1/2 and PKB signaling is known to require activation of NADPH-oxidase system and resultant H_2O_2 generation (15). Thus, it is possible that a NO/cGMP induced reduction in H_2O_2 generation contributes to the decrease in ET-1 response observed in our studies.

Taken together, we have demonstrated that SNAP and SNP, a NO donors, inhibit ET-1-stimulated increase of ERK1/2, PKB and Pyk2 phosphorylation through a cGMP/sGC-dependent mechanism in A-10 VSMC. We have also provided evidence showing that ET-1-stimulated protein synthesis, a hallmark of hypertrophic response, is also attenuated by NO donor SNAP in A-10 VSMC. Since ERK1/2, PKB and Pyk2 plays a crucial role in 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 growth promoting and hypertrophic responses of ET-1 in VSMC.

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Author contributions:

AB and GBD performed research. AB, GBD and AKS analyzed the data, AB, GBD and AKS wrote the paper, AKS designed the research.

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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 top panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (middle panels of each section). bottom panels, (bar diagrams) in each section 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, †$P< 0.0001$ vs ET-1. (C) *$P< 0.002$ vs control, †$P< 0.0003$ vs ET-1.

Figure 2. Dose-dependent effect of the NO donor, SNP 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 SNP 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 top panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (middle panels of each section).

Bottom panels (bar diagrams) in each section 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) *$P< 0.0003$ vs control, †$P< 0.0001$ vs ET-1. (B) *$P< 0.001$ vs control, †$P< 0.0006$ vs ET-1. (C) *$P< 0.0003$ vs control, †$P< 0.0002$ vs ET-1.

Figure 3. Effect of L-NAME, a NO synthase inhibitor, 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 L-NAME 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) and phospho-specific-Ser^{473}-PKB antibodies (B), and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the top panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (middle panels of each section). Bottom panels (bar diagrams) in each section 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. (C) *$P< 0.0001$ vs control, †$P< 0.0005$ vs ET-1.

Figure 4. Expression of eNOS in A10-VSMC. Total cellular lysates from HUVEC (15µg) or A10-VSMC (60µg) were subjectd to SDS-PAGE followed by immunoblotting using eNOS antibody as described in the materials and methods (A); Serum-starved quiescent A-10 cells were pretreated without or with the indicated L-NAME concentrations for 30 min followed by 10 nM of ET-1 for 5 min. Cell lysates were immunoblotted by using eNOS specific antibody (B).

Figure 5. Effect of a stable analogue of cGMP, 8-Br-cGMP 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 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), and phospho-specific-Tyr^{402}-Pyk2 antibodies (C), as shown in the top panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (middle panels of each section). Bottom panels (bar diagrams) in each section 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. (C) $^*P < 0.001$ vs control, $^†P < 0.005$ vs ET-1.

Figure 6. 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 top panels of each section. Blots were also analyzed for total ERK1/2, PKB and Pyk2 (middle panels of each section). Bottom panels (bar diagrams) in each section 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. Section (D) shows the effect of ODQ on basal or on ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation.

Figure 7 Effect of different concentrations of SNAP on ET-1-induced $[^3H]$leucine incorporation into proteins. Serum-starved quiescent A-10 cells were pretreated with 10 and 100 μM of SNAP for 30 min before ET-1 (10 nM) stimulation , then the cells were labeled to equilibrium with $[^3H]$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 into total cellular proteins over the basal values. *$P <$ 0.002 vs control, †$P <$ 0.004 vs ET-1.

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FIGURE 1

A

ET-1 - + + + + +
SNAP - - 1 10 100 300 (μM)

p-ERK1/2

ERK1/2

P-ERK1/2 (%)

125 100 75 50 25 0

ET-1 - + + + + +
SNAP - - 1 10 100 300 (μM)

B

ET-1 - + + + + +
SNAP - - 1 10 100 300 (μM)

p-PKB

PKB

P-PKB (%)

125 100 75 50 25 0

ET-1 - + + + + +
SNAP - - 1 10 100 300 (μM)

C

ET-1 - + + + + +
SNAP - - 1 10 100 300 (μM)

p-Pyk2

Pyk2

P-Pyk2 (%)

125 100 75 50 25 0

ET-1 - + + + + +
SNAP - - 1 10 100 300 (μM)

FIGURE 2

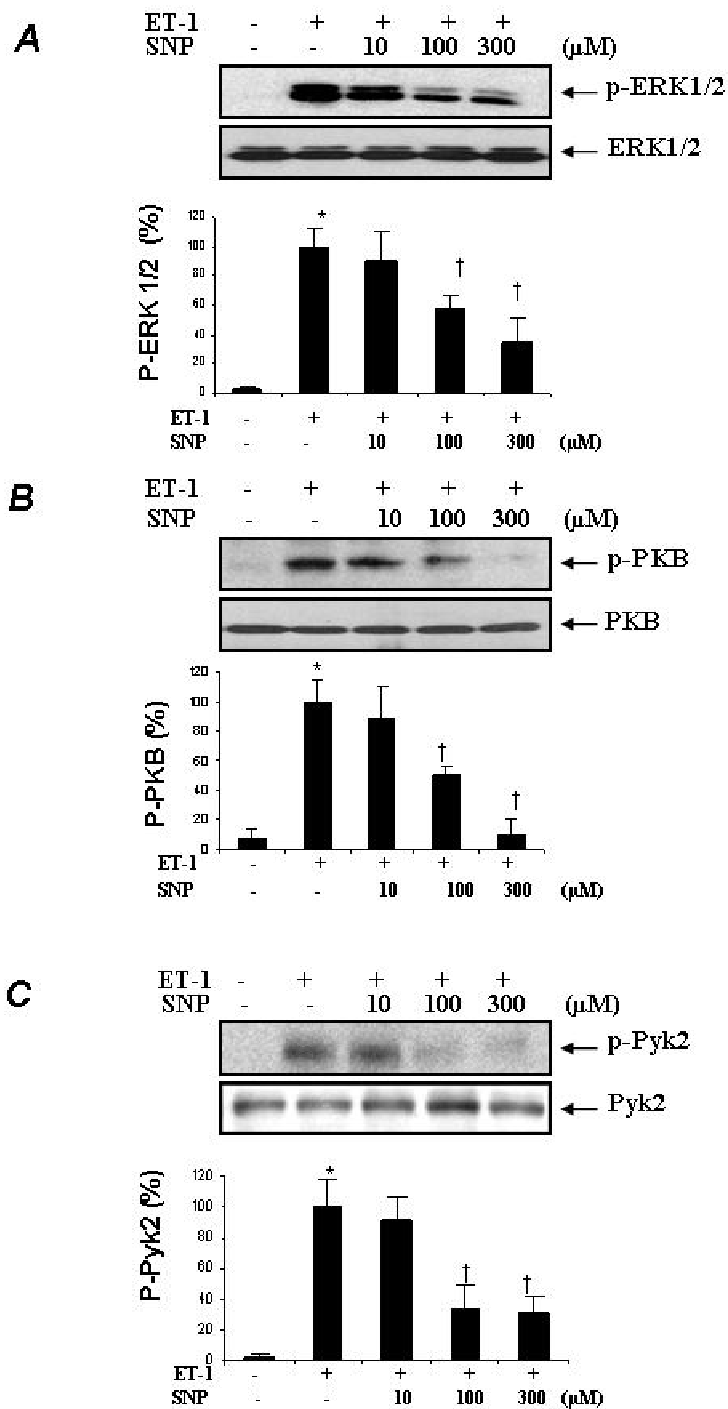


FIGURE 3

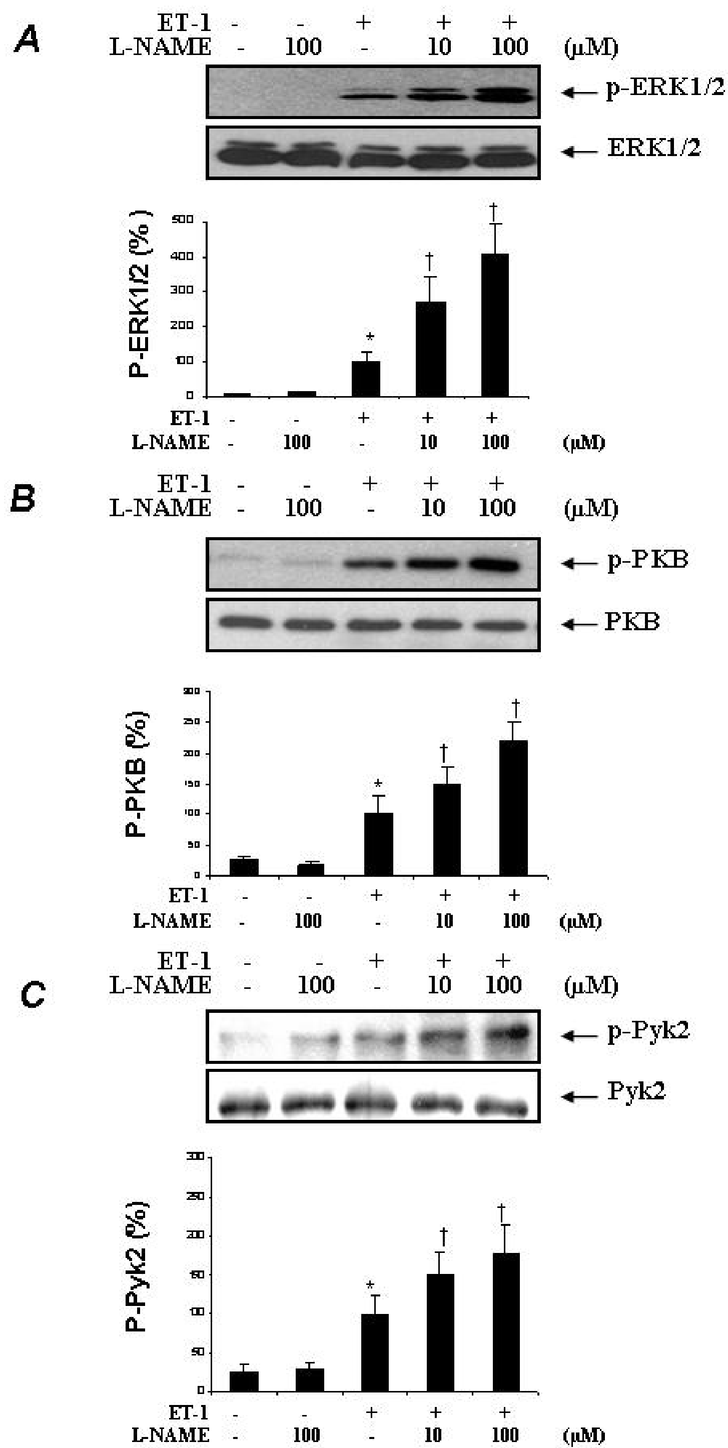
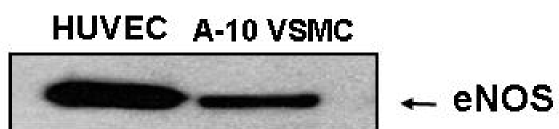


FIGURE 4

A



B

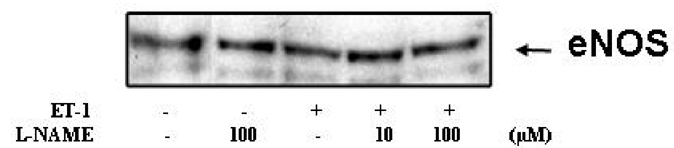


FIGURE 5

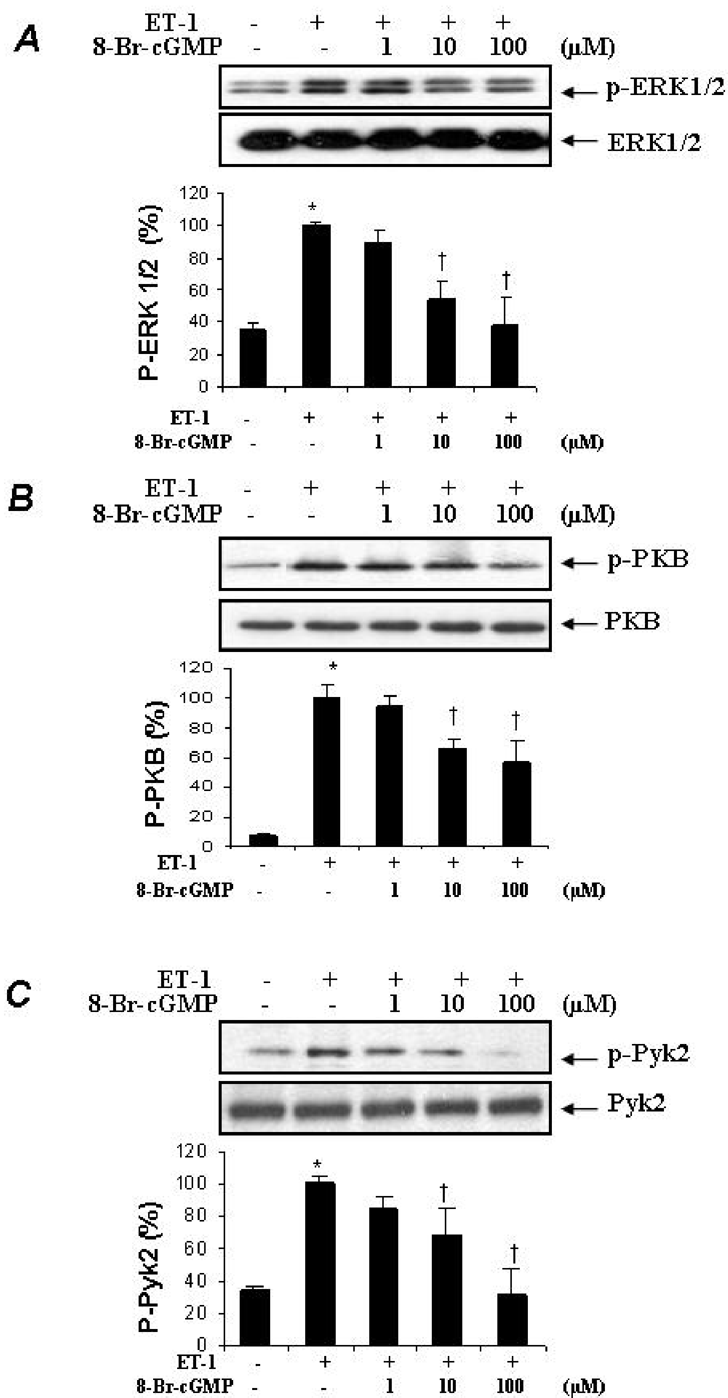


FIGURE 6

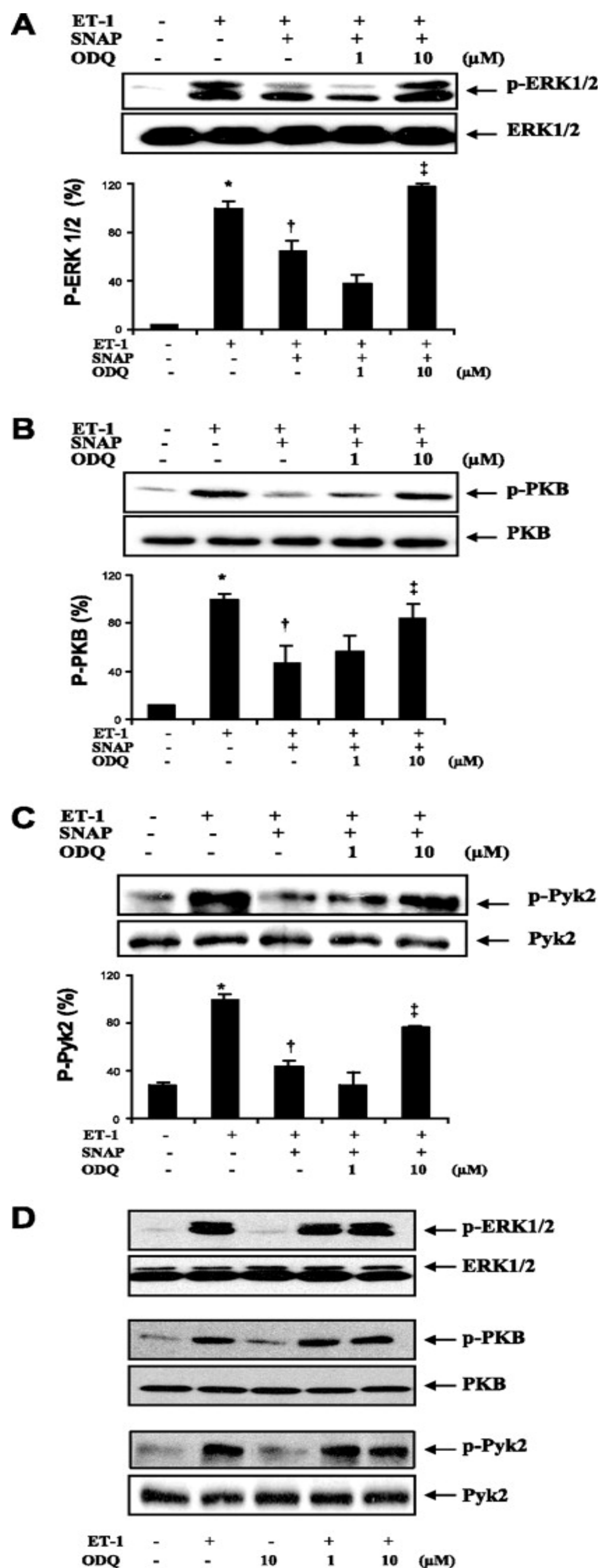
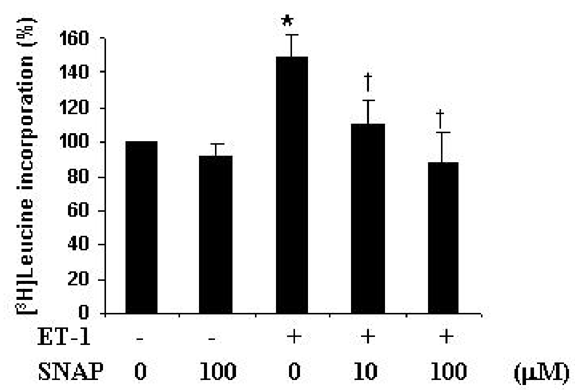


FIGURE 7



CHAPTER-5

GENERAL DISCUSSION

ET-1, the most potent vasoconstrictor peptide was discovered only 20 years ago. Although studies done during the last 2 decades have clarified the physiological and pathophysiological role of ET-1 in multiple systems, many questions in this field still remain unresolved.

In the vascular system, ET-1 has a basal vasoconstriction role (tonic role) and an upregulated ET-1 system has been suggested among the contributing factors in to the development of vascular disease such as hypertension and atherosclerosis. ET-1 contributes also to myocardial contractility, chronotropy and arrhythogenesis, as well as congestive heart failure, renal disorders and pulmonary diseases. Based on recent experimental data, treatment with newly available ET-1 antagonists is likely to inhibit ET-1-induced functional 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 its G-protein-coupled receptors (GPCR) which leads to the recruitment of multiple signaling pathways which includes protein kinases of the Raf family and lipid kinases of the phosphatidylinositol 3-kinase (PI-3K)

family. Both pathways are shown to be involved in cellular growth and proliferation two important phenomenons by which ET-1 triggers some of pathophysiological states. In parallel, it is also known that ET-1 increases intracellular Ca^{2+} concentration in many cell types including VSMCs and induces vascular contractility via the activity of Ca^{2+} and calmodulin (CaM) system. CaMKII is a multifunctional serine/threonine protein kinase which is believed to transduce the downstream effects of Ca^{2+}/CaM. A role of CaMKII in mediating various physiological functions in several systems and pathophysiological events in different cell types, such as VSMCs (405) and cardiomyocytes (207) has been suggested and in heart, ET-1-induced cardiomiocyte hypertophy has been linked to CaMKII activation (207). Recently, Tiel et al have suggested a proarrhythmic effect of CaMKII by showing that CaMKII activation is a part of arrhythmia mechanism of Timothy syndrome(406). Moreover, studies showing that inhibition of CaMKII by KN-93 prevents arrhythmic activity in rabbit pulmonary veins (407), decreases early afterdepolarizations in rabbit heart (408) and improves vascular dysfunction in animal models of diabetes (222) or in Ang II-induced hypertension (221), suggesting that aberrant activation of CaMKII may be involved in these cardiovascular pathologies.

The involvement of CaMKII system in ET-1 induced signaling was not studied before, therefore we investigated the role of CaMKII in ET-1-induced 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 these studies, we used three different strategies: classical pharmacological inhibitors, transfection of cells with CaMKII inhibitory peptide (AA 281-309) corresponding to auto-inhibitory domain of CaMKII and finally use of small interfering RNA (siRNA) technique. These studies are

the first to report an activation of CaMKII by ET-1 in VSMCs in Fig.2 article 1. This finding supports the idea that CaMKII plays a role in ET-1-induced signaling in VSMC. We further confirm this idea by showning that CaMKII phosphorylation was required for ET-1-induced ERK1/2, PKB and Pyk2 phosphorylation in VSMC. We have also shown in these studies that ET-1 was able to induce an increase in both protein and DNA synthesis which is consistent with other studies done in VSMC. However, these studies are the first to report an inhibitory effect of both CaM and CaMKII antagonists on ET-1-induced both growth and proliferation in VSMC which suggests a probable role of CaMKII in vascular diseases related to ET-1. Thus, CaMKII inhibition may be a good strategy to prevent or treat these pathologies.

Several studies have indicated that ROS play an essential role in propagating the signals of many growth factors, peptide hormones and cytokines such as EGF, Ang- II (409) and tumor necrosis factor-α (TNF-α) (410). Increased ROS generation has been associated with a variety of cardiovascular pathologies including hypertension and atherosclerosis. In our laboratory, we have shown earlier that ROS is generated by ET-1 in VSMC and these ROS are needed to mediate ET-1-induced activation of ERK1/2, PKB, and Pyk2 signaling as well as protein synthesis (10). We have also reported earlier a requirement of Ca^{2+} and CaM in H_2O_2-induced activation ERK1/2 and PKB (411). On the other hand, CaMKII have been shown to be upstream of H_2O_2-induced ERK1/2 phosphorylation in endothelial cells (412). Thus, the contribution of CaMKII in H_2O_2-induced ERK1/2, PKB and Pyk2 in VSMC remains obscure and needs to be clarified. Therefore, in the second article of this thesis, we investigated the role of CaMKII in H_2O_2-induced ERK1/2, PKB and Pyk2 phosphorylation in VSMC and since we have shown earlier the ability of H_2O_2

to activate Pyk2 and IGF-1R (324), we extended these studies to examine the role of CaMKII in Pyk2 and IGF-1R phosphorylation induced by H_2O_2. Our results provided evidence that CaMKII is upstream of Pyk2 phosphorylation induced by H_2O_2 and also CaMKII phosphorylation is needed for H_2O_2 evoked IGF-1R transactivation in VSMCs. In this article we have shown a time course of H_2O_2 induced CaMKII phosphorylation (Fig.5 Article 2). This data shows for the first time a phosphorylation of CaMKII induced by H_2O_2 in VSMC. Overall, our data reveal that CaMKII serves as a critical upstream component in triggering the H_2O_2-induced signaling cascade resulting in the phosphorylation of IGF-1R, Pyk2, ERK1/2 and PKB in VSMC. It may be suggested that through the activation of these signaling events CaMKII contributes to the regulation of various cellular processes including cell growth, proliferation, hypertrophy and survival in VSMC. Dysregulation of CaMKII activity may thus play an important role in the pathogenesis of vascular disease. NO is another important free radical that has been shown to contribute to the regulation of several hormone-mediated responses such as attenuation of the proliferative stimulation of VSMCs and cardiac fibroblasts induced by EGF (387) and Ang II (413). 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 (387) and Ang II (404) as well as PKB in response to PDGF (392). NO was also shown to attenuate Ang II-stimulated Pyk2 phosphorylation in cardiac fibroblasts (393). Recently, ET-1-induced cardiomyocyte hypertrophy also has been fund to be attenuated by NO through cGMP production (366). 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 VSMCs is available. The only study to investigate a role of NO in ET-1-induced responses in VSMCs was from Sand's laboratory in which they have been investigating the effect of NO on ET-1-induced vascular contraction in human placental arteries. In these studies, they have reported an inhibitory effect of NO in arteries contraction induced by ET-1(414).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 donors, SNAP or SNP decreased the phosphorylation level of ERK1/2, PKB and Pyk2 induced by ET-1. On another hand inhibition of NO generation by a pretreatment with NO inhibitor, L-NAME, increases these responses. These results are similar to previous studies in which Ang II-induced phosphorylation of ERK1/2 and Pyk2 was blocked by SNAP. This work represents the first detailed investigation on the effect of NO system on multiple signaling systems induced by ET-1 in VSMC. This study demonstrated that NO antagonizes ET-1-induced signaling in VSMC. Pyk2 has also been implicated in Ang II 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. In these studies, we have also shown that A10 VSMC express eNOS as compared to endothelial cells which support our finding by detecting the source of NO in these cells.

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, PKB and Pyk2 phosphorylation induced by ET-1and it thus mimicked the inhibitory effect of SNAP and SNP. 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 (415), endothelial cells (367) and cardiomyocytes (416). 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-6

Conclusion and Perspectives

Overall, the studies presented in this thesis (Fig. 13) demonstrate that CaMKII activation is needed for both ET-1 and H_2O_2 (which is known to play an essential role in propagating the signals of ET-1) -induced phosphorylation of ERK1/2, PKB and Pyk2. Also our work demonstrates a critical role of CaMKII in mediating H_2O_2-induced IGF-1R transactivation in VSMCs. On the other hand, we have demonstrated that NO antagonizes ET-1-induced activation of above signaling pathways. Finally, we have demonstrated that CaMKII activation and NO generation have opposite effect on hypertrophic and proliferative responses induced by ET-1 in VSMCs which are associated to several cardiovascular diseases. The results presented in this thesis have uncovered a key upstream role of CaMKII in triggering ET-1 and H_2O_2-induced signaling events in VSMC. The results presented in this thesis highlight the protective role of NO and the pro-pathogenic role of CaMKII in vasculature. Since both NO and CaMKII are involved in ET-1-induced signaling pathways and since both of these components are Ca/CaM dependent, it will be important to test the effect of NO on ET-1-induced CaMKII activation as well as its effect on ET-1 and H_2O_2-induced growth factor receptors transactivation because role of NO in growth factor receptors transactivation has not been studied before. Clarifying these points will help us to better understand the vasoactive peptide and redox-induced signaling in VSMCs.

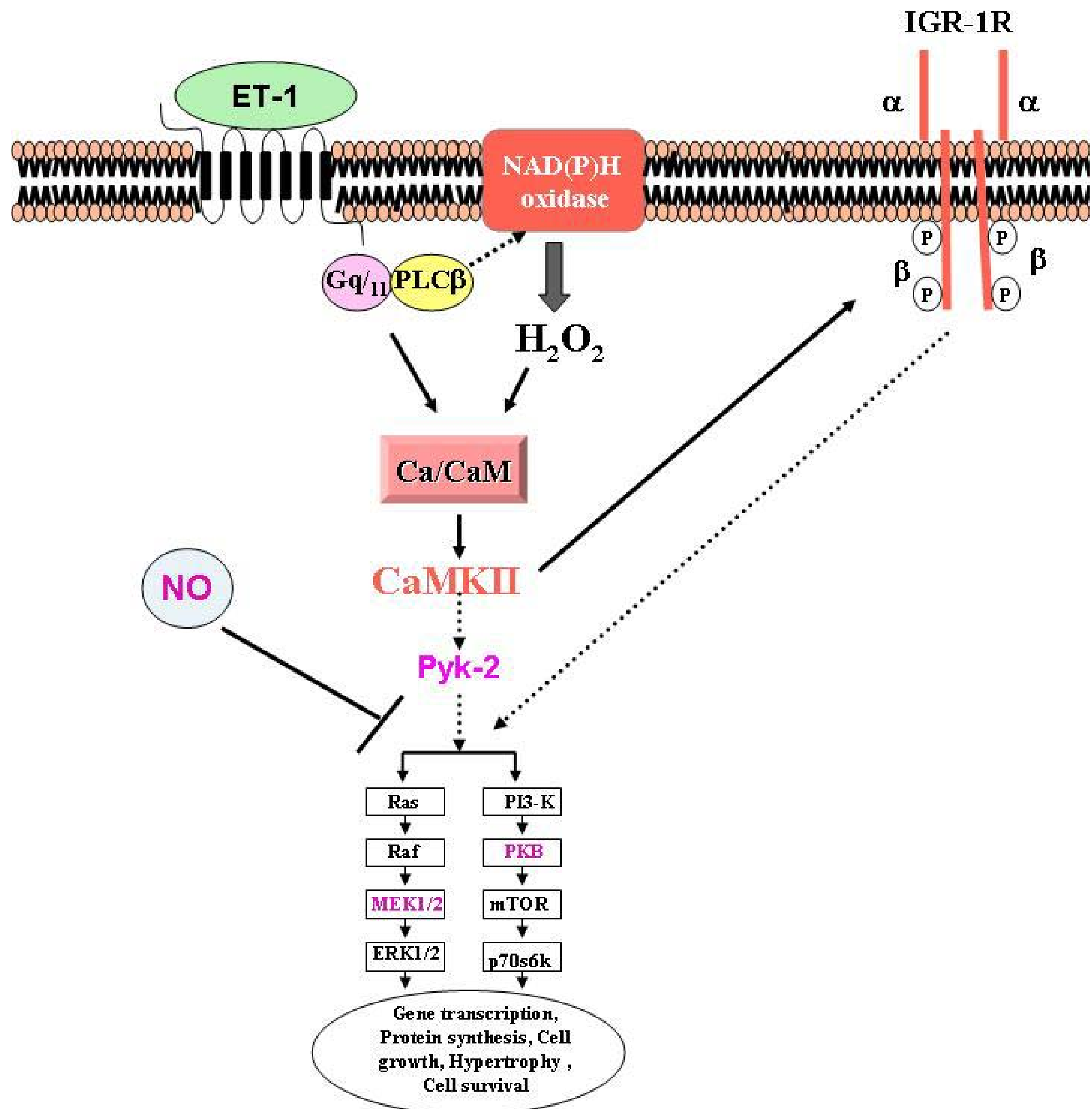


Fig.13. A model summarizing mechanism by which ET-1 and H_2O_2-induced signaling in VSMC with implication of CaMKII and NO.

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