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Upstream Mechanisms Responsible for H_2O_2-induced Activation of MAPK and PKB in Vascular Smooth Muscle Cells

par

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Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé:

« Upstream Mechanisms Responsible for H_2O_2-induced Activation of MAPK and PKB in Vascular Smooth Muscle Cells »

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RÉSUMÉ

Les espèces réactives oxygénées (ROS) sont produites de façon naturelle dans les cellules. Cependant, une production excessive ou une insuffisance dans le processus de leur élimination est associée à différentes maladies. Ainsi, les dernières années ont vu les ROS émerger comme des joueurs importants dans la pathogenèse des maladies cardiovasculaires, comme l'hypertension, l'athérosclérose et l'hypertrophie cardiaque, ainsi que les complications vasculaires du diabète. Il est connu que les ROS peuvent oxyder certains constituants cellulaires et qu'elles activent les voies de signalisation impliquées dans la croissance cellulaire, l'hypertrophie, la prolifération et la migration, ainsi que la survie, qui sont des événements caractéristiques du remodelage cardiovasculaire. Les voies de signalisation les plus importantes sont celles des MAPK et de la phosphatidylinositol-3-kinase (PI3-K)/protéine kinase B (PKB). Cependant, les mécanismes moléculaires par lesquels les ROS déclenchent l'activation de ces voies restent à être clarifiés. Bien qu'un rôle des récepteurs et non récepteurs protéines tyrosines kinases (« PTKs ») dans la médiation de la phosphorylation des ERK1/2 par le H_2O_2 soit suggéré, une implication possible des tyrosines kinases dans l'activation de PKB et de Pyk2 par le stress oxydatif n'a pas encore été clarifié dans les cellules du muscle lisse vasculaire (« VSMC »). Donc, l'objectif principal de notre étude était d'examiner le rôle des récepteurs et non récepteurs PTK dans l'activation de la PKB par le H_2O_2 dans la lignée cellulaire de « VSMC » A10. Ces cellules sont isolées de l'aorte thoracique de l'embryon de rat.

La phosphorylation de la PKB induite par le H_2O_2 a été complètement inhibée par le prétraitement des cellules avec l'AG1024, un inhibiteur pharmacologique spécifique du IGF-1R-PTK (« insulin-like growth factor type1 receptor PTK»), alors que l'AG1478, un inhibiteur spécifique du EGFR-PTK (« epidermal growth factor receptor PTK ») n'a pas eu d'effet inhibiteur sur la phosphorylation de la PKB. De même, la phosphorylation de Pyk2, Src et ERK1/2 induites par le H_2O_2 ont été complètement inhibées par AG1024

et non par AG1478. L'implication du « IGF-1R » dans la signalisation induite par H_2O_2 a aussi été démontrée par le fait que le H_2O_2 augmente la phosphorylation en tyrosine des sous unités β du « IGF-1R » et que le prétraitement au AG1024 a inhibé cette phosphorylation. De plus, l'inhibition pharmacologique de Src a diminué de façon significative la phosphorylation de la PKB et de Pyk2 par le H_2O_2. Tout comme l'effet de l'AG1478, l'AG1295, un inhibiteur spécifique du PDGFR-PTK («platelet-derived growth factor receptor PTK») n'a pas inhibé la phosphorylation de PKB ni de ERK1/2 induite par le H_2O_2, alors qu'il a supprimé complètement la réponse au «PDGF». En conclusion, nos résultats suggèrent que le «IGF-1R», et non pas le «EGFR» ni le «PDGFR» joue un rôle critique dans la médiation des évènements induits par le H_2O_2 et qui sont responsables de l'hypertrophie et de la croissance des « VSMC ».

Mots clés: ROS, H_2O_2, MAPK, PKB, Src, Pyk2, récepteurs et non récepteurs PTK, IGF-1R, VSMC.

ABSTRACT

Reactive oxygen species (ROS) are produced normally in the cells, but their excessive production or a lack in their elimination process is associated with various diseases. Thus, ROS have emerged in the recent years as important players in the pathogenesis of cardiovascular disorders, such as hypertension, atherosclerosis and cardiac hypertrophy as well as in the vascular complications of diabetes. ROS are known to modify the cell components by oxidation and to activate signaling pathways responsible of cell growth, hypertrophy, proliferation, and migration as well as survival, which are characteristic events in cardiovascular remodeling. The most important of these pathways are the mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol-3-kinase (PI3-K)/protein kinase B (PKB) pathway. However, the precise molecular mechanisms by which ROS trigger the activation of these pathways remain to be clarified. Although a role for receptor and non-receptor protein tyrosine kinases (PTKs) in mediating H_2O_2-induced ERK1/2 phosphorylation has been suggested, a possible involvement of tyrosine kinases in PKB and Pyk2 activation by oxidative stress has not yet been clarified in VSMC. Therefore, the main objective of our studies was to investigate the role of receptor and non-receptor tyrosine kinases in H_2O_2-induced PKB activation in A10 VSMC. These cells are obtained from rat embryonic thoracic aorta.

H_2O_2-induced PKB phosphorylation was completely abolished by pretreatment of the cells with AG1024, a specific pharmacological inhibitor of the insulin-like growth factor type 1 receptor PTK (IGF-1R-PTK), whereas AG1478, the specific inhibitor of the epidermal growth factor receptor PTK (EGFR-PTK) did not have any inhibitory effect on PKB phosphorylation under the same conditions. Similarly, H_2O_2-induced phosphorylation of Pyk2, Src and ERK1/2 phosphorylation were completely blocked by AG1024 but not by AG1478. The involvement of the IGF-1R in H_2O_2-induced signaling

was also demonstrated by the results showing that H_2O_2 enhanced the tyrosine phosphorylation of the β-subunit of IGF-1R and that AG1024 pretreatment markedly inhibited this phosphorylation. Moreover, pharmacological inhibition of Src significantly decreased H_2O_2-induced PKB and Pyk2 phosphorylation. Similar to the effect of AG1478, AG1295, the specific inhibitor of the platelet-derived growth factor receptor PTK (PDGFR-PTK) failed to inhibit H_2O_2-induced PKB and ERK1/2 phosphorylation, while it abrogated the PDGF-induced response under the same conditions. Taken together, our data suggests that IGF-1R, but not EGFR nor PDGFR plays a critical role in mediating H_2O_2-induced signaling events which are important mediators for hypertrophy and growth in VSMC.

Key words: ROS, H_2O_2, MAPK, PKB, Src, Pyk2, receptor and non-receptor PTK, IGF-1R, VSMC.

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LIST OF ABREVIATIONS

ACE : Angiotensin converting enzyme

AngII : Angiotensin II

BAD : Bcl-2-associated death

CAKβ : Cell adhesion kinase beta

DNA : Deoxyribonucleic acid

DOCA-salt : Deoxycorticosterone acetate-salt

EGF : Epidermal growth factor

EGFR : Epidermal growth factor receptor

ERK1/2 : Extracellular signal-regulated kinases 1 and 2

ET-1 : Endothelin-1

FAK : Focal-adhesion kinase

FKHR : Forkhead transcription factor

Gab1 : Grb2-associated binding 1

GPCR : G protein-coupled receptor

Grb2 : Growth factor receptor binding protein 2

GSH : Glutathione

GSK-3β : Glycogène synthase kinase 3 beta

GSSG : Glutathione disulfide

GTP : Guanosine triphosphate

HB-EGF	: Heparin-binding epidermal growth factor
H_2O_2	: Hydrogen peroxide
IGF-1R	: Insulin-like growth factor type 1receptor
IL-1β	: Interleukin-1 beta
IRS	: Insulin receptor substrate
JNK	: c-Jun NH2-terminal kinase
LDL	: Low density lipoprotein
MAPK	: Mitogen-activated protein kinase
MAPKK/MEK	: Mitogen-activated protein kinase kinase
MAPKKK/Raf	: Mitogen-activated protein kinase kinase kinase
mTOR	: mammalian target of Rapamycin
NADP+	: Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	: Nicotinamide adenine dinucleotide phosphate, reduced form
NF-κB	: Nuclear factor-kappaB
NO	: Nitric oxide
O_2	: Molecular oxygen
$^{\cdot}O_2^-$	: Superoxide anion
$^{\cdot}OH$	: Hydroxyl radical
$ONOO^-$	: Peroxynitrite
PDGF	: Platelet-derived growth factor
PDGFR	: Platelet-derived growth factor receptor

PDK1/2	: Phosphoinositide-dependent kinases 1 and 2
PI	: Phosphatidylinositol
PI3-K	: Phosphatidylinositol-3-kinase
PIP_2	: Phosphatidylinositol-4,5-biphosphate
PIP_3	: Phosphatidylinositol-3,4,5-triphosphate
PKB	: Protein kinase B
PL	: Phospholipase C
PTK	: Protein tyrosine kinase
Pyk2	: Proline-rich tyrosine kinase
RAFTK	: Related adhesion focal tyrosine kinase
ROS	: Reactive oxygen species
SFKs	: Src family tyrosine kinases
SH2	: Src homology 2
SHP-2	: Src Homology 2 domain-containing tyrosine phosphatase
SHR	: Spontaneously hypertensive rat
SOD	: Superoxide dismutase
Sos	: Son of sevenless
TGF-beta1	: Transforming growth factor beta 1
TNF-α	: Tumor necrosis factor alpha
VSMC	: Vascular smooth muscle cell

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CHAPTER 1

INTRODUCTION

Evidence generated during the recent years has suggested a crucial role for reactive oxygen species (ROS) in the pathophysiology of cardiovascular disorders including hypertension, atherosclerosis and restenosis after angioplasty [1-3] as well as in diabetes (reviewed in [4]) and cancer (reviewed in [5]). Therefore, there is a lot of interest to understand the mechanism of ROS generation, its elimination and identification of its cellular targets. Thus, the objective of this section is to provide a brief overview on these aspects of ROS and its relationship in the pathophysiology of cardiovascular disease.

1.1 The multiple sources of ROS

ROS are formed as intermediates in redox reactions, leading from molecular oxygen (O_2) to water (H_2O) [6]. They are small, quickly diffusible and highly reactive molecules [7] and are classified into superoxide anion ($^{\cdot}O_2^-$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) [8]. Their major intracellular source is the mitochondria which converts 1-2% of consumed O_2 into $^{\cdot}O_2^-$ [7]. $^{\cdot}O_2^-$ and/or H_2O_2 can also be derived from NAD(P)H oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, heme oxygenases, peroxidases, as well as hemoproteins such as heme and hematin (reviewed in [9]). The univalent reduction of O_2 leads to $^{\cdot}O_2^-$, which is relatively unstable and short-lived because of its unpaired electron [10] (**Fig. 1.1**).

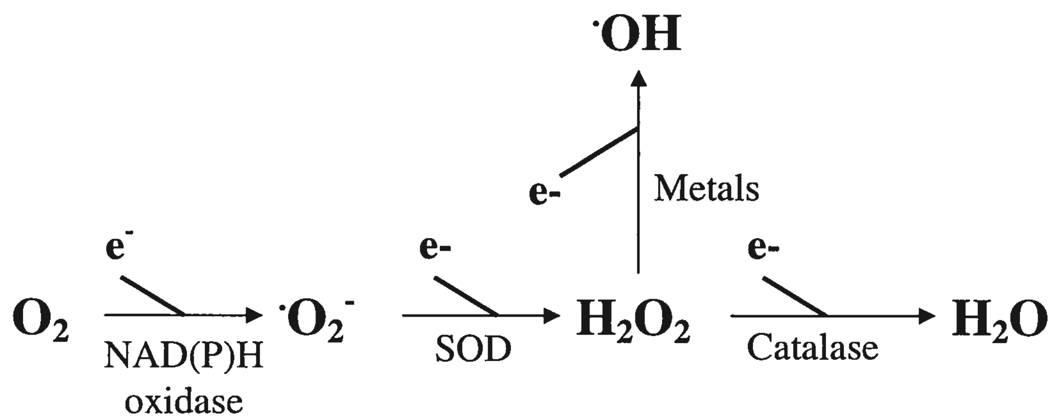
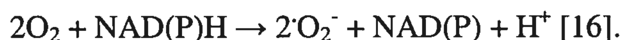


Figure 1.1: Simplified scheme showing key steps in the production of reactive oxygen species (ROS). The most important ways of ROS generation are shown here. One electron from NAD(P)H is transferred to molecular Oxygen (O_2) by NAD(P)H oxidase to generate superoxide anion ($^{\cdot}O_2^-$), which can be converted to hydrogen peroxide (H_2O_2) either spontaneously or by superoxide dismutase (SOD). In presence of metals, H_2O_2 can be converted to Hydroxyl anion ($^{\cdot}OH$). (Adapted from Ref. [11]).

1.2 The NAD(P)H oxidase

The major source of ROS in the vascular wall is the NAD(P)H oxidase [12], which is a complex enzyme system composed of many subunits including p22phox, p47phox, the GTPase Rac and the recently identified Nox1 and Nox4 [12-15]. The NAD(P)H oxidase catalyzes $^{\cdot}O_2^-$ production by the one electron reduction of O_2 where NAD(P)H is the electron donor:



$2O_2 + NAD(P)H \rightarrow 2^{\cdot}O_2^- + NAD(P) + H^+$ [16].

NAD(P)H oxidase is activated in hypertensive animals [17;18] as well as in human hypertensive subjects [19;20] and it is believed to act as a major player in the development of atherosclerosis [21]. Particularly, p22phox was shown to play an important role in agonist-induced ROS generation and gene expression in VSMC in response to AngiotensinII (AngII), Tumor necrosis factor α (TNF-α) and thrombin [12;22-24]. Moreover, Nox1 seems to be upregulated by proliferative stimuli, such as AngiotensinII (AngII) and platelet-derived growth factor (PDGF) in VSMC [25].

1.3 The ROS scavenging systems

Under physiological conditions, $^{\cdot}O_2^-$ undergoes dismutation either spontaneously or by a reaction catalyzed by superoxide dismutase (SOD) to produce H_2O_2. Dismutation of $^{\cdot}O_2^-$ by SOD is favored at low concentrations of $^{\cdot}O_2^-$ and at high concentrations of SOD, which happens under physiological conditions. H_2O_2 is much more stable than $^{\cdot}O_2^-$, can cross cell membranes and has a longer half-life. Normally it is scavenged by catalase and glutathione peroxidase to produce H_2O [10]. In the presence of metal-containing molecules such as Fe^{2+}, H_2O_2 can also be reduced to generate the extremely active

hydroxyl radical ($^{\cdot}OH$) that causes damage to the cell components [6]. In the glutathione peroxidase reaction, glutathione (GSH) is oxidized to glutathione disulfide (GSSG), which can be converted back to GSH by glutathione reductase in a NADPH-consuming process (**Fig. 1.2**). Several forms of SOD are known: copper-zinc SOD (Cu/Zn SOD), mitochondrial or manganese SOD (Mn SOD) and iron-containing SOD (Fe SOD) [26;27].

Normally, the rate of ROS production is balanced by the rate of their elimination. However, in pathological conditions, a disequilibrium between ROS generation and elimination results in increased ROS bioavailability leading to oxidative stress [28]. Furthermore, in case of excessive production, $^{\cdot}O_2^-$ reacts with nitric oxide (NO) to produce the very harmful $ONOO^-$ [29].

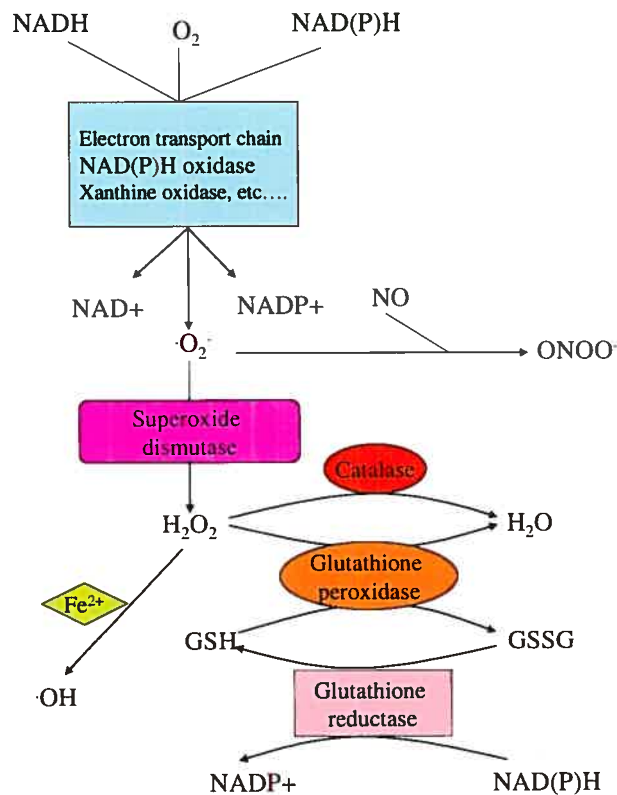


Figure 1.2: Production and elimination of $^{\cdot}O_2^-$ and H_2O_2. $^{\cdot}O_2^-$ can undergo dismutation by superoxide dismutase (SOD) to produce H_2O_2. H_2O_2 is scavenged by catalase or Glutathione peroxidase to produce water (H_2O) while in the same reaction, Glutathione (GSH) is converted to Glutathione disulfide (GSSG), which can be converted back to GSH by Glutathione peroxidase. In presence of metals, H_2O_2 can be converted to Hydroxyl anion ($^{\cdot}OH$). It is noteworthy that high levels of $^{\cdot}O_2^-$ may lead to the generation of peroxynitrite anion ($ONOO^-$) since $^{\cdot}O_2^-$ can react with nitric oxide (NO).

1.4 Regulation of ROS production by vasoactive peptides and growth factors

An increasing number of studies have demonstrated that vasoactive peptides mediate their responses through the generation of ROS. The potent vasoconstrictor endothelin-1 (ET-1) has been shown to activate NAD(P)H oxidase resulting in ROS generation in endothelial cells [30] and to increase H_2O_2 levels via its subtype A receptor (ET_A receptor) in pulmonary smooth muscle cells [31]. Similarly, a role for ROS generation in mediating ET-1-induced activation of various signaling pathways, such as ERK1/2, PKB and Pyk2 [32] as well as JNK and p38mapk [33] has also been demonstrated. Moreover, ET-1, through ET_A receptor, has also been implicated in the increased vascular levels of $^{\cdot}O_2^-$ in both low-renin hypertension and chronic ET-1-infused rats models of hypertension [34;35].

Another important vasoactive peptide, AngII is also known to induce ROS generation in many cell types including cardiomyocytes [36], endothelial cells [37] and VSMC [12]. In hypertension, AngII activates the NAD(P)H oxidase thereby enhancing the ROS generation [12;38]. Furthermore, H_2O_2 appears to play a direct role in AngII-induced vasculature hypertrophy [39;40] (**Fig. 1.3**).

Growth factors such as the epidermal growth factor (EGF) [41], the PDGF [42] and cytokines were also shown to induce the generation of ROS such as $^{\cdot}O_2^-$ and H_2O_2 in nonphagocytic cells [43]. Transforming growth factor-beta1 (TGF-beta1), which is abundantly expressed in pulmonary hypertension, induced expression of Nox4 and likewise increased production of ROS, in freshly isolated human pulmonary artery smooth muscle cells [44]; this is believed to be an important mechanism in pulmonary vascular remodeling.

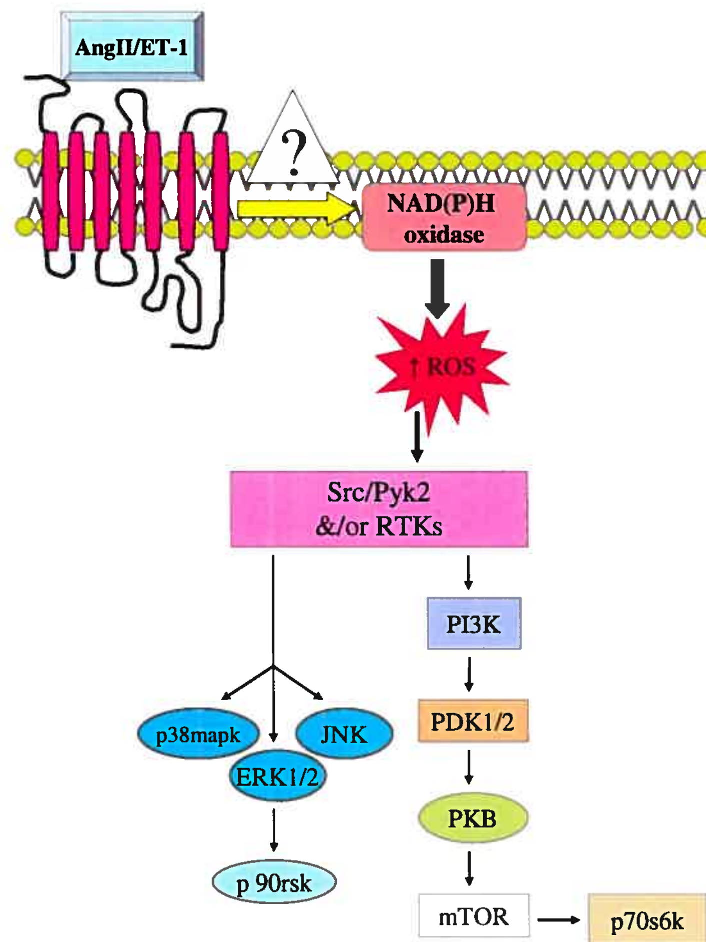


Figure 1.3: Regulation of ROS generation by vasoactive peptides. The vasoactive peptides AngII and ET-1 are known to increase the ROS generation in many cell types, particularly in VSMC, by activating the NAD(P)H oxidase through an as yet unknown mechanism. Endogenously produced ROS activate many protein tyrosine kinases, such as c-Src, Pyk2 and receptor tyrosine kinases (RTKs) that act as upstream regulators of MAPK as well as PI3-K/PKB pathway.

1.5 ROS in vascular diseases

There is increasing evidence that oxidative stress is involved in the initiation and progression of cardiovascular disease. An enhanced ROS generation is observed at sites of vascular injury and has been related to the development of restenosis/ atherosclerosis [45]. A key role for vascular NAD(P)H oxidases in the development of human atherosclerosis was reported recently [46-48]. Originally, it was thought that ROS are involved in low density lipoproteins (LDL) oxidation, a key step in the initiation and progression of atherosclerosis [49]. More recently, studies have shown that ROS are also implicated in endothelial dysfunction, increased contractility, VSMC migration, growth and apoptosis, inflammation and increased depositions of extracellular matrix proteins, which are important factors in hypertensive damage [50].

The decrease in NO bioavailability has been correlated to an increase in $^{\cdot}O_2^-$ in hypertension and hypercholesterolemia (reviewed in [51]). $^{\cdot}O_2^-$ reacts with NO and forms $ONOO^-$, leading to a decreased NO bioavailability. Elevation in $^{\cdot}O_2^-$ levels contributes to impaired endothelial function associated with atherosclerotic disease [52].

1.5.1 ROS in genetic and experimental hypertension

A considerable number of studies have shown that hypertension is associated with an elevated level of ROS and also with an impairment of endogenous antioxidant defense mechanisms (reviewed in [53]). ROS, such as H_2O_2 and $^{\cdot}O_2^-$ are increased in vessels, heart and kidneys of the spontaneously hypertensive rats (SHR) [16;54], the obesity-prone and the Dahl salt-induced sensitive rats [55;56]. In SHR, the increased NAD(P)H-induced $^{\cdot}O_2^-$ generation appears to be associated with NAD(P)H oxidase subunit Nox4

overexpression and enhanced oxidase activity [17;57]. Polymorphisms in the promoter region of the p22phox gene have also been identified in SHR [58]. Renal NAD(P)H oxidase upregulation has been reported in young SHR [17]. Vascular $^{\cdot}O_2^-$ and oxidant markers are also increased in aortas of mice with deoxycorticosterone acetate-salt (DOCA-salt)-induced hypertension [18] as well as in hypertensive models created by infusion of ET-1 [34;59;60]. In a study performed on Nox1-deficient mice, Matsuno et al. demonstrated that Nox1- derived ROS are involved in AngII-induced hypertension and this occurred by reducing the bioavailability of NO [61]. Recently, Callera et al. demonstrated that ET-1-induced oxidative stress in DOCA-salt hypertension not only involves NAD(P)H oxidase, but also mitochondria-derived ROS [62].

Experimentally, generators of $^{\cdot}O_2^-$ were shown to abolish endothelium-dependent relaxation of aortic rings isolated from SHR [63], induce contraction of mesenteric beds [64] and to increase chloride reabsorption by isolated thick ascending limbs of the loop of Henle [65]. Makino et al. demonstrated that infusion of H_2O_2 in the renal medulla of whole rats increased blood pressure and decreased urine flow as well as sodium excretion [66]. In accord with this study, Kopkan et al. demonstrated that enhanced $^{\cdot}O_2^-$ generation in AngII-induced hypertensive rats modulates renal hemodynamic and tubular reabsorptive function which leads possibly to sodium retention [67]. It was also shown that H_2O_2 as well as $^{\cdot}O_2^-$ regulated vascular contraction by increasing intracellular calcium concentration ($[Ca^{2+}]_i$) in pig coronary artery SMC [68;69] and in rat mesenteric arteries with greater effects in SHR versus normotensive Wistar Kyoto (WKY) rats [69;70].

1.5.2 ROS in human hypertension

Recent clinical studies have demonstrated increased oxidative stress and reduced antioxidant status in patients with essential hypertension, renovascular hypertension and malignant hypertension. In these studies, the levels of plasma thiobarbituric acid-reactive substances and 8-epi-isoprostames, biomarkers of lipid peroxidation and oxidative stress, were shown to be increased [19;20;71;72]. In mild-to-moderate hypertension, lipid peroxidation and oxidative stress are not increased [73], suggesting that ROS may not be critical in the early stages of human hypertension, but could be more important in severe hypertension. Activation of the renin-angiotensin system has been proposed as a mediator of NAD(P)H oxidase activation and ROS production [16;74-76]. In fact, some of the therapeutic blood pressure-lowering effects of AT_1 receptor blockers and angiotensin-converting enzyme (ACE) inhibitors have been attributed to NAD(P)H oxidase inhibition and decreased ROS production [77;78]. An association between a p22phox gene polymorphism and NAD(P)H oxidase-mediated $^{\cdot}O_2^-$ production in the vascular wall of patients with hypertension and atherosclerosis has also been described [79].

1.5.3 ROS in heart failure

Experimental and clinical studies have suggested an increased production of ROS in animals and in patients with acute and chronic heart failure (reviewed in [80]). ROS are released during ischemia and ischemic reperfusion (reviewed in [81]). Moreover, oxidants production is increased by AngII and catecholamine, two endocrine factors known to induce cardiac remodeling [82;83]. The possible sources are many and include xanthine and NAD(P)H oxidoreductases, cyclooxygenases, mitochondrial electron

transport chain and activated neutrophils. Excessive NO derived from NO Synthase seems to be implicated in the pathogenesis of chronic heart failure: NO reacts with $^{\cdot}O_2^-$ to form $ONOO^-$, a reactive oxidant which impairs cardiac function. Increased oxidative and nitrosative stress activates also the nuclear enzyme poly-ADP-ribose polymerase which is known to contribute to pathogenesis of cardiac and endothelial dysfunction associated with myocardial infarction, congestive heart failure, hypertension, atherosclerosis and diabetes (reviewed in [80]).

1.5.4 ROS in vascular complications of diabetes

Oxidative stress can induce abnormal changes in intracellular signaling and result in chronic inflammation and insulin resistance. It is known that hyperglycemia as well as free fatty acids stimulate ROS production [84]. In diabetes, plasma levels of ROS are significantly elevated [85]. Inflammation and oxidative stress have been linked to insulin resistance *in vivo*, not only in type 2 diabetes, but also in obese, nondiabetic individuals and in patients with metabolic syndrome (reviewed in [4]). Moreover, NAD(P)H oxidase components are upregulated in vascular tissues of animal models and patients suffering from diabetes and obesity (reviewed in [86]). High levels of ROS trigger the activation of serine/threonine kinase cascades such as c-Jun N-terminal kinase, nuclear factor-kappaB (NF-κB), and others that in turn phosphorylate multiple targets, including the insulin receptor and the insulin receptor substrate (IRS) proteins. Increased serine phosphorylation of IRS reduces its ability to undergo tyrosine phosphorylation and may accelerate the degradation of IRS-1 leading to an aberrant insulin signaling and insulin resistance (reviewed in [4]).

Studies performed *in vitro* could also explain in part the molecular mechanism by which ROS can contribute to insulin resistance. H_2O_2 as well as a ROS inducer, diamide was found to inhibit insulin signaling in cultured rat VSMC by blocking the insulin-induced protein kinase B (PKB) phosphorylation on Serine 473 (Ser^{473}) [87]. In addition, H_2O_2 pretreatment also attenuated the insulin receptor binding and insulin receptor autophosphorylation in this study [87]. The negative regulation of PKB could contribute to insulin resistance because its activation by insulin is critical in stimulating glucose transport, glycogen synthase, protein synthesis, antilipolysis and suppression of hepatic gluconeogenesis [88]. Moreover, studies have suggested that insulin might protect VSMC from remodeling by inhibiting apoptosis [89] and migration [90]. Taken together, it is possible that ROS, through their effects on the insulin signaling pathway, may contribute to cardiovascular disease.

1.6 The intracellular effects of ROS

Cells respond to oxidant injury with the activation of multiple signal transduction pathways that serve to coordinate the cellular response and determine the outcome. High levels of ROS result in severe damage to the cell components (lipids, proteins, carbohydrates and DNA). ROS-induced oxidative modification of proteins and lipids can modify the structure and function of proteins and lipid bilayers resulting in cellular dysfunction. In the vasculature, $^{\cdot}O_2^-$ and H_2O_2 are particularly important since they act as inter- and intracellular signaling molecules. $^{\cdot}OH$ induces local damage, whereas $^{\cdot}O_2^-$ and H_2O_2 can travel some distance from their site of generation (reviewed in [9]). The biochemical events by which ROS mediate cellular dysfunction and contribute to

pathogenesis of vascular diseases include changes in gene expression and cell signaling pathways. Two major signaling pathways, the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3-K)/protein kinase B (PKB) pathways, have been identified as important targets of ROS (**Fig. 1.3**). Aberrant activation of these pathways in the pathogenesis of vascular diseases has been suggested (**Fig. 1.6**).

1.7 The MAPK pathway

MAPK are a family of ubiquitous serine/threonine protein kinases, classically associated with cell growth, differentiation and death [91]. MAPK are activated in response to a variety of external stimuli such as growth factors, hormones and stress, as well as vasoactive peptides and ROS (reviewed in [92;93]). Of the major mammalian MAP kinases, extracellular signal-regulated kinases (ERK1/2), p38MAP kinase (p38mapk), c-Jun N-terminal kinases (JNK) and ERK5 are the best characterized.

MAPK are activated by MAPK kinase (MAPKK also known as MEK) which, in turn are activated by MAPKK kinase (MAPKKK also known as MEKK or Raf). The MAPKKK/Raf are serine/threonine kinases and are activated by phosphorylation and/or by their interaction with small GTP-binding protein of Ras/Rho family through adaptor proteins Son of sevenless/Growth factor receptor binding protein 2 ((Sos)/Grb2)). Activated MAPKKK/Raf phosphorylate and activate MAPKK/MEK, which then phosphorylate MAPK on Thr and Tyr residues located in the activation loop of the kinases (**Fig. 1.4**). Activated MAPKs phosphorylate target substrates on Ser or Thr residues followed by a proline, such as p90rsk, and transcription factors such as c-Jun, CHOP, CREB and MEF-2 (reviewed in [94]). ERK1/2, phosphorylated by MEK1/2

(MAP/ERK kinase), is a major growth signaling kinase, whereas p38mapk and JNK, phosphorylated by MEK3/6 and MEK4/7 respectively, influence cell survival, apoptosis, differentiation and inflammation [91]. ERK5, regulated by MEK5, is involved in protein synthesis, cell cycle progression and cell proliferation [95-97].

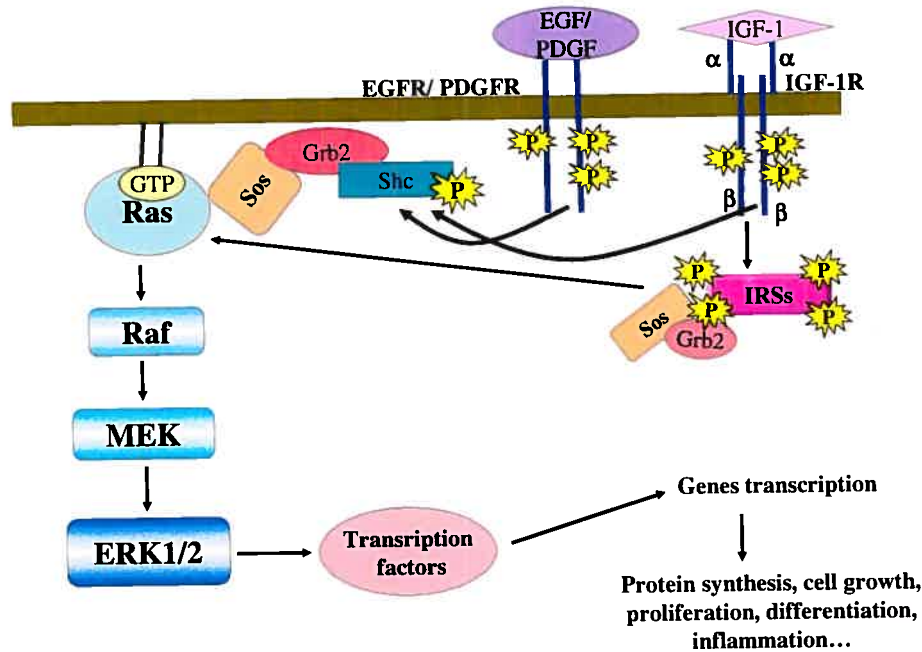


Figure 1.4: Schematic model showing the signaling cascade induced by receptor tyrosine kinases leading to ERK1/2 activation. Insulin-like growth factor (IGF-1), by binding to its receptor (IGF-1R) enhances tyrosine phosphorylation of the insulin receptor substrates (IRSs). Phosphorylated IRSs recruit Src homology 2 (SH2) domain containing proteins, such as Grb2/Sos. This complex formation triggers the activation of Ras/Raf/MEK/ERK1/2 signaling pathway, which is important for cell growth, proliferation and differentiation. Similarly, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) activate ERK1/2 signaling cascade. However, in this case, Grb2/Sos interacts directly with tyrosine phosphorylated EGFR or PDGFR, triggering thereby the activation of ERK1/2 pathway.

1.7.1 Activation of MAPK pathway by ROS

In the last few years, many reports have shown that ROS activate the MAPK pathway in different cell types, including Rat-2 fibroblasts [98], rabbit renal proximal tubular cells [99], Chinese hamster ovary (CHO) cells [100], rat cardiomyocytes and heart fibroblasts [101] as well as VSMC [11;102;103]. Moreover, ROS generation was shown to be critical in the activation of MAPK by AngII [104;105] and ET-1 [32;105] in VSMC. Despite an overwhelming evidence supporting an involvement of ROS in vasoactive peptide-induced ERK1/2 signaling, ROS-independent mechanism of this response has also been documented in some cell types [106].

1.7.2 MAPK activation in cardiovascular diseases

Several lines of evidence have indicated that an aberrant activation of the MAPK is often associated with vascular remodeling in cardiovascular diseases. For example, the enhanced activation of vascular MAPK has been demonstrated in different models of hypertension [107;108]. Activation of MAPK by AngII and ET-1 in VSMC was shown to be involved in vascular changes associated with hypertension [109;110] and a role of MAPK in VSMC contraction and migration has also been suggested [111;112]. Thus, a heightened activation of MAPK may be responsible for aberrant vascular remodeling and muscle contractility, which is a hallmark of vascular disease.

1.8 The phosphatidylinositol-3-kinase (PI3-K)/protein kinase B (PKB) pathway

Another key cellular signaling pathway that plays an important role in cell growth, survival, proliferation and gene expression is PI3-K/PKB pathway. PI3-K is a heterodimeric lipid kinase, which is divided into three classes that are different in structure and mechanism of regulation [113].

1.8.1 Classification of PI3-Ks

Class I PI3-K are activated by receptor tyrosine kinases and G protein-coupled receptors (GPCRs).They catalyze the phosphorylation of phosphatidylinositol (PI), PI 4-phosphate and PI 4,5-biphosphate in the 3' position of the inositol ring. This reaction promotes the generation of PI 3'-P, PI 3, 4-biphosphate (PI-3,4-P_2) and PI-3, 4, 5-triphosphate (PIP_3) [114]. Class II PI3-K possesses a lipid binding domain and generates PI 3'-P as well as PI-3,4-P_2 whereas class III PI3-K generates only PI 3'-P [114]. Class I PI3-Ks represent the dominant form in cardiovascular tissues [115] and are divided into class IA and IB. The 110kDa catalytic subunit of class IA exists in three isoforms: p110α, p110β and p110δ. Similarly, the 85kDa regulatory subunit of class IA exists in three isoforms: p85α, p85β and p55γ, which are products of three different genes [116]. Class IA of PI3-Ks are typically activated in response to tyrosine kinase-coupled stimuli [115]. The p85 subunit contains the Src homology-2 (SH-2) domain and is able to interact with phosphorylated tyrosine residues on receptor or other docking proteins, leading to the stimulation of the 110 kDa catalytic subunit, then p110 catalyzes the phosphorylation of PI substrates into PI 3'-P, PI-3',4-P_2 and PI-3,4,5-P_3 (PIP_3). PIP_3 binds to Pleckstrin homology (PH) domain of several proteins which are downstream

targets of PI3-K, such as PKB. In contrast, class IB consist of a catalytic subunit (p110γ) and a regulatory subunit (p101) and are usually activated by GPCR activation [115].

PKB, also known as Akt, which mediates several effects of PI3-K, exists as three isoforms: PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3; each isoform possesses an amino-terminal PH domain, a kinase domain and a carboxy-terminal regulatory domain. PKB is rapidly activated in response to insulin [117;118], AngII [119], ET-1 [32] and many other growth factors [120-123] in a phosphatidylinositol-3-kinase (PI3-K)-dependent manner.

Binding of PIP_3 to PKB recruits it to the plasma membrane for phosphorylation by phosphoinositide-dependent kinases 1 (PDK-1) and 2 (PDK-2). PDK-1 phosphorylates PKB at Threonine 308 (Thr^{308}) in the catalytic domain while putative PDK-2 phosphorylates it at Serine 473 (Ser^{473}) in the C-terminal regulatory domain of PKB (reviewed in [124]). Activated PKB phosphorylates several downstream substrates, such as glycogen synthase kinase-3β (GSK-3β), Forkhead transcription factor (FKHR), Bcl-2-associated death (BAD), Caspase 9, mammalian target of rapamycin (mTOR), Mdm2, nuclear factor-κB (NF-κB) and endothelial nitric oxide synthase (eNOS) (reviewed in [124;125]). Phosphorylated form of these substrates regulates diverse cellular functions, such as glucose transport, cell growth, gene expression, cell survival and death as well as protein synthesis [119](reviewed in [126]) (**Fig. 1.5**).

1.8.1 Activation of PKB by ROS

Exogenously added H_2O_2 was shown to enhance the phosphorylation of PKB on Ser^{473} in many cell types including NIH3T3, MCF-7 and malignant HeLa cells [127], Rat-2 fibroblasts [98], CHO cells [100], renal cells [99], cardiomyocytes and heart fibroblasts [101] as well as VSMC [11;102]. Moreover, an intermediary role of ROS in mediating PKB activation by AngII [128] and ET-1 [32] in VSMC has also been demonstrated.

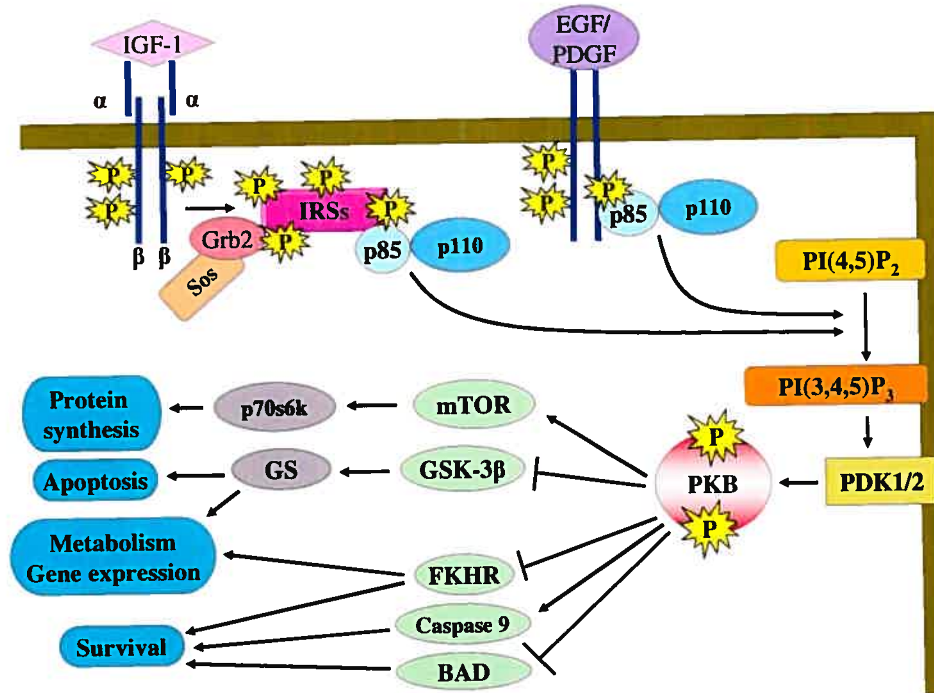


Figure 1.5: Schematic model showing the signaling cascade induced by receptor tyrosine kinases leading to PKB activation. Insulin-like growth factor (IGF-1), by binding to its receptor (IGF-1R) enhances tyrosine phosphorylation of the insulin receptor substrates (IRSs). Phosphorylated IRSs recruit Src homology 2 (SH2) domain containing proteins, such as Grb2/Sos and p85 subunit of the lipid kinase PI3-K, leading to activation of PI3-K. Activated PI3-K catalyzes the phosphorylation of PIP_2 into PIP_3. PIP_3 binds to Pleckstrin homology (PH) domain of PKB and recruits it to the plasma membrane for phosphorylation by PDK1/2. Activated PKB phosphorylates several targets such as mTOR, GSK-3β, FKHR, BA and Caspase that regulate metabolism, gene expression and cell survival. Similarly, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) activate PI3-K/PKB signaling cascade. However, in this case, p85 subunit interacts directly with EGFR or PDGFR, triggering thereby the activation of the pathway.

The precise upstream mechanisms by which ROS activate MAPK and PI3-K/PKB pathways remain unclear. However, a potential role for both growth factor receptors [99;101;103;127;129], and non-receptor protein tyrosine kinases [98-100;103] as signal transducers of H_2O_2-induced responses has been suggested.

1.9 The epidermal growth factor receptor (EGFR)

The EGFR is a receptor tyrosine kinase that is ubiquitously expressed in a variety of cell types, with the most abundant expression in epithelial cells and many cancer cells [130-132]. It contains an extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic tyrosine kinase autophosphorylation and regulatory domain (reviewed in [133]). EGFR belongs to a family containing three other members (ErbB2, ErbB3 and ErbB4) that undergo homodimerization or heterodimerization to induce autophosphorylation and receptor tyrosine kinase activation in response to ligand binding [131;134]. Dimerization activates the intrinsic tyrosine kinase activity of the intracellular domain at different residues, resulting in the recruitment of the SH-containing domain proteins, which trigger downstream events. The phosphorylation of EGFR on tyrosine 1068 (Tyr^{1068}) is followed by recruitment of the adaptor protein Grb2, leading to the activation of Ras/ERK1/2 pathway (**Fig. 1.4**).

1.9.1 Transactivation of the EGFR

The EGFR can also undergo phosphorylation in a ligand-independent manner by a process called transactivation and trigger the response of many agonists, such as AngII, ET-1, thrombin, lysophosphatidic acid, H_2O_2 and others. Thus, EGFR was identified as

an essential link in AngII-mediated activation of MAPK [135;136] and PKB [137;138] in VSMC. In the same context, Voisin et al. have reported that the activation of EGFR by AngII is necessary for increasing protein synthesis *in vitro* in cultured aortic smooth muscle cells and *in vivo* in the rat aorta as well as in small resistance arteries [139]. Similarly, ERK1/2 activation by ET-1 appears to be dependent on transactivation of EGFR in Rat-1 fibroblasts [140], VSMC [141], cardiomyocytes [142] and rat mesangial cells [143].

H_2O_2 has been shown to enhance tyrosine phosphorylation of EGFR in endothelial cells [144], renal cells [99], HeLa cells [127] as well as in VSMC [129;145;146]. Whether H_2O_2 directly activates the intrinsic tyrosine kinase activity of EGFR or modulates signaling molecules that transactivate the receptor is still unclear. There are some reports proposing that ROS may exert their effects through targeting the cysteine regions of the active sites of tyrosine phosphatases, which in turn activates tyrosine kinases [147]. In fact, H_2O_2 was shown to inhibit the dephosphorylation of the EGFR by inhibiting a tyrosine phosphatase [148]. Some other reports suggest that ROS may activate receptor tyrosine kinases by generating growth factors, such as heparin-binding EGF-like growth factor (HB-EGF) through metalloprotease-dependent cleavage [129]. ROS production [149] and metalloprotease-dependent HB-EGF generation are also implicated in EGFR transactivation by different GPCR agonists in many cell types [150;151]. Frank et al. have demonstrated that both mechanisms are necessary for EGFR transactivation by AngII in VSMC [152;153].

The tyrosine phosphorylation of the EGFR by ROS is accompanied by its association with Grb2 leading to activation of ERK1/2, which appears to be an important

mechanism in triggering MAPK activation induced by H_2O_2 in various cell types [99;101;103;145;154]. On the other hand, studies on the involvement of EGFR transactivation in H_2O_2-induced PKB phosphorylation gave discrepant results: for example, EGFR transactivation was implicated in H_2O_2-induced PKB phosphorylation in HeLa cells [127], but not in renal cells [99] or in PC12 cells [155]. However, no attempts have been made to investigate a possible role for EGFR in mediating the H_2O_2-induced phosphorylation of PKB in VSMC.

1.10 The insulin-like growth factor type 1 receptor (IGF-1R)

The IGF-1R is a transmembrane protein tyrosine kinase that shares structural and functional homology with the insulin receptor and is abundantly expressed in VSMC. The mature receptor is a tetramer consisting of 2 extracellular α-chains and 2 intracellular β-chains [156]. The β-chains include an intracellular tyrosine kinase domain that is believed to be essential for most of the receptor's biologic effects [157]. Binding of IGF-1 or Insulin (at very high, unphysiological concentrations) induces the activation of PTK domain of IGF-1Rβ subunit which in turn activates the autophosphorylation of the receptor (reviewed in [158]).

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), Shc and Grb2 [159;160]. IRS-1, an important substrate for both the insulin and the IGF-1 receptor, contains multiple tyrosine phosphorylation sites that recognize and bind SH2-containing signaling molecules, such as Grb2, Nck, the p85 subunit of PI3-K and the SH2 domain-containing tyrosine phosphatase-2 (SHP-2) [159]. Of these, the

binding of Grb2/Sos to tyrosine-phosphorylated IRS-1 activates Ras, which then stimulates the Raf-1/MAPK cascade [161]. Shc can also interact directly with IGF-1R [162]. After tyrosine phosphorylation of Shc, it recruits the Grb2/Sos complex and activates the Ras/Raf-1/MEK/ERK pathway [161] **(Fig. 1.4)**. The activated IGF-1R also triggers the PI3-K and its downstream targets PKB and p70s6k [163;164] **(Fig. 1.5)**. The Grb2-associated binding 1 (Gab1) can also function as an adaptor protein in mediating IGF-IR-induced signaling events [165] by interacting with Grb2, p85 subunit of PI3-K and SHP-2 [166]. As stated earlier, activation of MAPK pathway is critical for cell proliferation, whereas the PI3-K pathway mediates the metabolic and antiapoptotic response of IGF-1.

1.10.1 Transactivation of the IGF-1R

An important role for IGF-1R transactivation in triggering AngII-induced responses in VSMC was reported recently [167;168]. Pharmacological inhibition of IGF-1R kinase was found to markedly inhibit AngII-induced ERK1/2 phosphorylation in rat VSMC [167] and PI3-K/PKB activation in VSMC derived from the left anterior descending coronary artery of porcine hearts [168]. It was also demonstrated that AngII enhances the phosphorylation of the IGF-1Rβ subunit [168] and that this phosphorylation was prevented by AG538 and AG1024, two selective inhibitors of IGF-1R tyrosine kinase [169;170]. Interestingly, AngII-induced activation of MAPK appeared to be IGF-1R independent [168]. It should also be noted that the IGF-1R expression in VSMC is regulated by several factors, including AngII [171] and ROS [172]. Moreover, the up

regulation of IGF-1R expression by AngII and basic fibroblast growth factor in VSMC appears to be a critical determinant for their mitogenic effects [171].

Recently, Tabet et al. have demonstrated that H_2O_2-induced ERK1/2 was blocked by AG1024 in cultured VSMC from mesenteric arteries, suggesting a role for IGF-1R transactivation in mediating the H_2O_2 response [103]. Whether H_2O_2 enhances the tyrosine phosphorylation of IGF-1R or utilizes this receptor to activate other downstream signaling components in VSMC has not yet been investigated.

1.11 The platelet-derived growth factor receptor (PDGFR)

The PDGFR is a receptor tyrosine kinase expressed in many cell types including VSMC [173] and its expression can be stimulated by AngII [174]. It exists in two isoforms: PDGFRα and PDGFRβ that display affinity for the different isoforms of PDGF family PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. PDGFRα can be activated by PDGF-AA, PDGF-AB, PDGF-BB and PDGF-CC, while PDGF-BB and PDGF-DD bind and activate PDGFRβ (reviewed in [175]). Tissue culture and in vivo mouse models studies have suggested that PDGFRα and PDGFRβ activate distinct signaling pathways. Downstream targets of the PDGFRβ include ERK, PKB and small G proteins including Rho and Rac-1, which ultimately mediate PDGF-induced responses such as cell cycle progression, migration, and survival (reviewed in [176]). Similar to EGF, binding of PDGF to its receptor on the cell surface induces its dimerization and autophosphorylation of the tyrosine kinase domain which in turn recruits and activates SH2 domain containing proteins such as Grb2, Src, p85 subunit of PI3-K and phospholipase C γ (PLCγ). The Tyr^{751} in the kinase domain of PDGFRβ is the docking

site for PI3-K. Tyr[740] is also important for the activation of PI3-K by PDGFRβ while PDGF-stimulated PLCγ signaling is dependent on the phosphorylation at the two sites Tyr[1009] and Tyr[1021] (reviewed in [175]) (**Fig. 1.4** and **1.5**).

1.11.1 Transactivation of the PDGFR

Several studies have shown that PDGFR undergoes tyrosine phosphorylation in response to AngII [177] as well as ROS in different cell types such as MCF10A [98], NIH3T3 fibroblasts [178] and VSMC prepared from the thoracic aorta of Sprague-Dawley rats [146]. H_2O_2 was found to enhance the Tyr[1021] phosphorylation of PDGFRβ in a Src- and PKCδ-dependent manner [146]. On the other hand, unlike the EGFR transactivation by ROS, PDGFR transactivation was shown to be independent of metalloproteases effect [146]. An implication of PDGFR in ROS-induced activation of MAPK pathway in VSMC derived from mesenteric arteries has been studied by using AG1295, a selective inhibitor of PDGFR kinase, and it was found that this inhibitor blocked only the phosphorylation of ERK1/2 but had no effect on p38mapk phosphorylation in response to H_2O_2 [103]. However, a similar involvement of PDGFR in H_2O_2-induced phosphorylation of PKB has not been investigated to date.

1.12 The Src-family tyrosine kinases (SFKs)

The SFKs, a family of non-receptor tyrosine kinases, consists of nine structurally-related peptide members: c-Src, Fyn and Yes are widely expressed in most tissues, whereas the others (Lck, Lyn, Hck, Fgr, Blk, Yrk) have a more restricted distribution (reviewed in [179]). SFK are 52–62 kDa proteins composed of six distinct functional

regions: the SH4 domain, the unique region, the SH3 domain, the SH2 domain, the catalytic domain (SH1), and a short C-terminal tail containing a negative regulatory tyrosine residue (Tyr^{530} in humans) (reviewed in [179]). The SH2 and SH3 domains are important for molecular interactions that regulate Src catalytic activity, Src localization, and recruitment of substrates, whereas the SH1 kinase domain contains autophosphorylation site, which is important for regulation of its catalytic activity. This site of phosphorylation corresponds to Tyr^{416} in mouse c-Src and Tyr^{419} in human's, which is not phosphorylated in inactive wild type Src, but is constitutively phosphorylated in activated Src (reviewed in [179]).

1.12.1 Activation of SFKs and its implication in signal transduction pathways

SFKs can be activated by various extra cellular stimuli, such as antigens, growth factors, integrins [179], AngII [180], ET-1 [181] and oxidative stress [99;100;182] in various cell types (**Fig. 1.3**). Among the SFKs, c-Src is the major isoform in the vascular wall [183] and appears to be involved in contraction [184], proliferation [185], growth [186] and cytoskeletal reorganization [187].

In the last few years, much attention has been given to the critical role of c-Src as a mediator of GPCR- and H_2O_2-induced responses: transactivation of EGFR by GPCR agonists [140;188;189] as well as by H_2O_2 [99] in different cell types were shown to be c-Src-dependent. Similarly, PDGFR transactivation by ROS seems to be Src-dependent in VSMC [146] and in NIH3T3 fibroblasts [178].

Src is not only involved in GPCR-induced transactivation of RTK, but also in triggering the activation of MAPK and PI3-K/PKB pathways in many cell types. Several

studies reported that GPCR-mediated activation of the Ras/MAPK pathway involves Src function [190-192]. Particularly in VSMC, AngII-induced activation of ERK1/2 pathway, which is a potential contributor pathway to vascular remodeling, appears to be mediated by Src [186]. Similarly, Src was shown to be a critical link in ET-1-induced ERK1/2 activation in rat myometrial cells [181] and in ET-1-induced cardiomyocyte hypertrophy [193].

Pharmacological approaches have suggested a role of c-Src in mediating H_2O_2-induced ERK1/2 phosphorylation in mesenteric arteries VSMC [103], in CHO-IR cells [100] and in renal cells [99;154]. Moreover, c-Src-PTK activity appears to be required in mediating H_2O_2-induced PKB phosphorylation in different cell types such as CHO-IR [100], renal cells [99] and in Rat-2 fibroblasts [98]. In contrast, diethylmaleate, which is known to induce an increase in intracellular ROS levels, has enhanced ERK1/2 phosphorylation in a Src-independent fashion in Rat-2 fibroblasts [98]. Conversely, a recent report has shown that H_2O_2 markedly inactivates Src *in vivo*, but not *in vitro* and that a reduced phosphorylation of the tyrosine residue in the activation loop is responsible for the inactivation *in vivo* [194]. Therefore, it appears that H_2O_2 exerts both activation and inhibitory effect on Src and triggers the responses of several agonists in a cell-specific manner and differently *in vitro* and *in vivo*.

1.13 The Proline rich tyrosine kinase 2 (Pyk2)

Pyk2 is a non-receptor tyrosine kinase, also known as related adhesion focal tyrosine kinase (RAFTK) [195] or cell adhesion kinase beta (CAKβ) [196] or calcium-dependent tyrosine kinase [197]. It is a member of a family of tyrosine kinases that also

includes p125 focal-adhesion kinase (FAK). Pyk2 and FAK are structurally related and are both implicated as important integrating molecules in signal transduction cascades. Pyk2 is expressed mainly in the nervous system, while FAK is widely expressed in various tissues [198;199]. Pyk2 contains a central catalytic domain and two non-catalytic domains, the N- and the C-terminal domains. The N-terminal domain contains a tyrosine autophosphorylation site at Tyr^{402}, which serves as a docking site for SFKs to bind via their SH2 domains (reviewed in [199]). Studies have shown that Pyk2 plays a key role in cell signaling in many cell types, including rat pheochromocytoma PC12 cells [155;190;198], embryonic mouse fibroblasts [188], clone 9 (C9) hepatic cells [200] and VSMC [32;201-205].

1.13.1 Activation of Pyk2 and its implication in signal transduction pathways

Pyk2 is activated by phosphorylation on tyrosine residues ($Tyr^{402, 579, 580}$ and ^{881}) (reviewed in [199]) in a Ca^{2+}- and PKC-dependent fashion in PC12 [198] and VSMC [201] in response to stimulation by GPCR agonists. Moreover, Pyk2 is known to interact physically with SH2 domain containing molecules such as Src in PC12 cells [190;198;206], mouse embryonic fibroblasts [188], and VSMC [201]. This complex formation between Pyk2 and Src is important in mediating GPCR-induced activation of many downstream signaling, such as EGFR transactivation in mouse fibroblasts [188] and VSMC [207].

Several reports have shown that activated Pyk2 signals through the MAPK family, such as ERK1/2 [198], c-Jun amino-terminal kinase (JNK) [190] or p38mapk [208] in PC12 cells. In VSMC, Rocic et al. have shown that Pyk2 acts as an upstream

modulator of multiple signaling pathways involved in AngII-induced VSMC growth: AngII induced the formation of a complex between Pyk2 and the ERK1/2 regulators Shc and Grb2 [204]. A role for Pyk2 in AngII-induced activation of PI3-K and p70s6 kinase has also been reported [204] (**Fig. 1.3**). Later on, the same group demonstrated by using genetic approach the involvement of Pyk2 in AngII-induced protein synthesis in VSMC [209]. Another vasoactive peptide, ET-1, was shown to induce tyrosine phosphorylation of Pyk2 in cardiomyocytes [142;210] and rabbit carotid artery VSMC [211] as well as in embryonic VSMC of rat aorta [32]. In cardiomyocytes, Pyk2 activation by ET-1 is dependent on Src and PKC epsilon (PKCε) [212], as well as Ca^{2+} [142;210;212] and is followed by ROS generation [210]. Kodama et al. have reported that Pyk2 was involved in ET-1-induced ERK activation [142] and that Pyk2 activation as well as Pyk2/Src association was required for ET-1-induced JNK activation in cardiomyocytes [213]. Furthermore, Pyk2 activation appears to be critical in ET-1-induced cardiomyocyte hypertrophy [210].

1.13.2 Activation of Pyk2 and its implication in cardiovascular diseases

Direct stimulation with exogenous H_2O_2 was shown to enhance the tyrosine phosphorylation of Pyk2 in many cell types, such as PC12 [155] and VSMC [203]. Recent studies have suggested that Pyk2 may represent a key signaling molecule involved in vascular diseases because an upregulation in the basal phosphorylation of Pyk2 in VSMC from SHR as compared with VSMC from WKY has been documented recently. Moreover, AngII-enhanced phosphorylation of Pyk2 appeared to be more rapid and more potent in SHR in these studies [214]. More recently, an involvement of Pyk2 in

AngII-induced VSMC migration via JNK activation has also been reported, further strengthening an important role for Pyk2 in VSMC functioning [215].

1.14 General Conclusion

In summary, oxidative stress is believed to be involved in vascular abnormalities, such as hypertension and atherosclerosis. A number of studies have shown that ROS activate key components of growth promoting and proliferative signaling pathways, such as ERK1/2 and PKB in VSMC. An important role for receptor and non-receptor tyrosine kinases in triggering some of the H_2O_2-evoked responses has been demonstrated in many cell types, including VSMC. Several studies carried out in VSMC isolated from different vessel types have revealed that many tyrosine kinases act as upstream components in H_2O_2-induced ERK1/2 phosphorylation. These tyrosine kinases include both growth factor receptors and non-receptor tyrosine kinases. Most of these studies on ROS-induced signaling in VSMC have focused on MAPK pathways and the intermediary role of EGFR transactivation in this process (**Fig. 1.6**). However, the potential contribution of other growth factor receptors has not been investigated in any detail and not much is known on the mechanisms by which ROS activate PKB signaling in VSMC. Therefore, the studies presented in this thesis were undertaken to elucidate a possible role for receptor and non-receptor tyrosine kinases on PKB, Pyk2 and ERK1/2 phosphorylation by H_2O_2 in VSMC. These studies have used standard protocols of cellular and molecular biology such as cell culture, immunoprecipitation and western blotting.

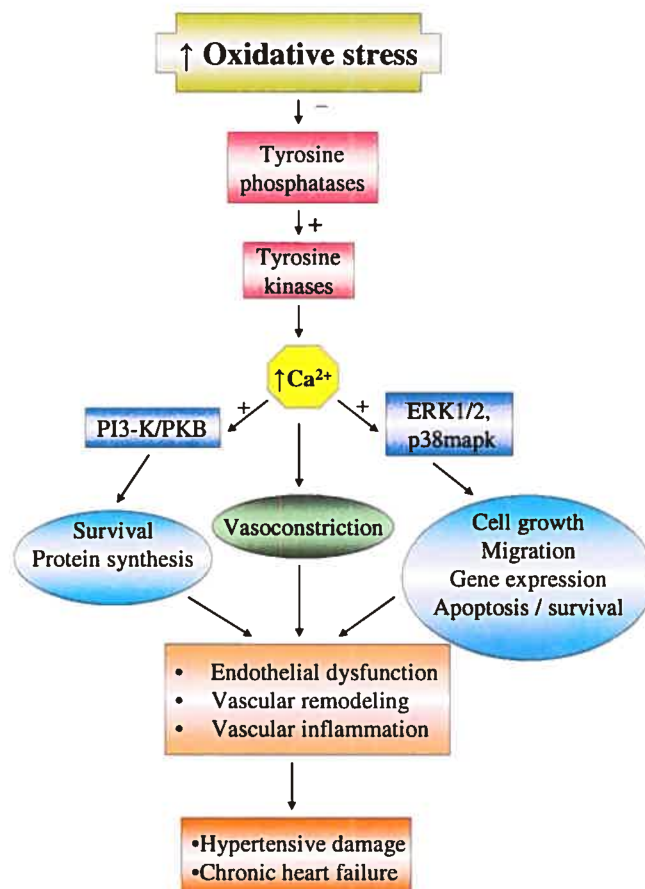


Figure 1.6: Scheme summarizing the major ROS-induced signaling pathways that are responsible of their pathological effects. High levels of ROS or oxidative stress, by inhibiting protein tyrosine phosphatases and activating thereby protein tyrosine kinases, trigger the activation of two major signaling pathways, the MAPK and the PI3-K/PKB pathway in a Ca^{2+}-dependent fashion. Aberrant activation of these pathways enhances protein synthesis, cell growth, hypertrophy, migration as well as survival responses, which are critical events in vascular remodeling, associated with cardiovascular disorders. Increased Ca^{2+} levels may also have a direct effect on vascular disorder by modifying the contractile response of the vascular smooth muscle cells.

CHAPTER 2

ARTICLE

Activation of insulin-like growth factor type-1 receptor is required for H_2O_2-induced PKB phosphorylation in vascular smooth muscle cells

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Activation of insulin-like growth factor type-1 receptor is required for H_2O_2-induced PKB phosphorylation in vascular smooth muscle cells

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Short title: IGF-1R mediates H_2O_2–induced PKB phosphorylation

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ABSTRACT

Evidence accumulated in recent years has revealed a potential role of reactive oxygen species (ROS) in the pathophysiology of cardiovascular diseases. However, the precise mechanisms by which ROS contribute to the development of these diseases are not fully identified. Previous work from our laboratory has indicated that exogenous hydrogen peroxide (H_2O_2) activates several signaling protein kinases, such as extracellular signal regulated kinase1 and 2 (ERK1/2) and protein kinase B (PKB) in A10 vascular smooth muscle cells (VSMC). However, the upstream elements responsible for this activation remain to be clarified. Although an important role of epidermal growth factor receptor (EGFR) protein tyrosine kinase (PTK) in H_2O_2-induced ERK1/2 signaling has been suggested, the contribution of this PTK or other receptor or non-receptor PTK in PKB activation is not well defined in VSMC. In the present study, we have investigated the role of receptor and Src-family-PTKs in H_2O_2-induced PKB phosphorylation using pharmacological inhibitors. AG1478, a specific inhibitor of EGFR, failed to attenuate the H_2O_2-induced increase in PKB Ser473 phosphorylation, whereas AG1024, an inhibitor of insulin-like growth factor type1 receptor (IGF-1R)-PTK, almost completely blocked this response. H_2O_2 treatment also enhanced tyrosine phosphorylation of the IGF-1Rβ subunit, which was significantly inhibited by AG1024 pretreatment of cells. Furthermore, pharmacological inhibition of Src by PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazole[3,4-d] pyrimidine) decreased PKB phosphorylation. Moreover, H_2O_2-induced PKB phosphorylation was associated with increased tyrosine phosphorylation of c-Src and Pyk2 in an AG1024 and PP2-inhibitable manner. In conclusion, these data provide evidence for the contribution of IGF-1R-PTK in initiating H_2O_2-evoked PKB phosphorylation in A10 VSMC with an intermediary role of c-Src and Pyk2 in this process.

Key words: Protein kinase B, Hydrogen peroxide, Growth factor receptor protein tyrosine kinase, Src-family protein tyrosine kinase, Proline-rich tyrosine kinase, Vascular smooth muscle cells.

Introduction

Reactive oxygen species (ROS) are believed to play a critical role in the pathophysiology of cardiovascular diseases such as hypertension, atherosclerosis and restenosis after angioplasty (Dhalla et al. 2000; Droge 2002; Fortuno et al. 2005; Pollock and Pollock 2005), as well as in vascular complications associated with diabetes (Ceriello and Motz 2004). Two key vasoactive peptides, angiotensin II (AngII) and endothelin-1 (ET-1), that are also important growth factors for vascular smooth muscle cells (VSMC) (Battistini et al. 1993; Daou and Srivastava 2004; Touyz et al. 2004), and are believed to contribute to vascular diseases (Touyz and Schiffrin 2003; Pollock and Pollock 2005; Sainani et al. 2005; Sun 2005; Tostes, and Muscara 2005) mediate their vasoactive responses through ROS generation (Zafari et al. 1998; Fei et al. 2000; Wedgwood. et al. 2001; Daou and Srivastava 2004). An important source of ROS in VSMC is NAD(P)H oxidase, which catalyzes the generation of superoxide anion (O_2^-) from molecular oxygen (O_2) (Ushio-Fukai et al. 1996; Touyz et al. 2002; Niemiec and Zak 2005). O_2^- is a relatively unstable molecule and is rapidly converted by a dismutation reaction to hydrogen peroxide (H_2O_2) (Boveris and Chance 1973).

Recent studies have demonstrated an exaggerated production of ROS such as O_2^- and H_2O_2 in many animal models of hypertension (Guo et al. 2003; Li et al. 2003; Sedeek et al. 2003). Moreover, a direct role of H_2O_2 in AngII-induced vasculature hypertrophy has been suggested recently (Zhang et al. 2005). Exogenous H_2O_2 activates several signaling protein kinases, such as mitogen-activated protein kinases (MAPK) and protein kinase B (PKB) (Wang et al. 2000; Blanc et al. 2003; Blanc et al. 2004; Mehdi et al. 2004; Zhuang and Schnellmann 2004), which have been proposed to play key roles in mediating the hypertrophic response in VSMC (Daigle et al. 2004). Although the precise mechanism

and intermediary steps by which H_2O_2 activates these signaling pathways remain poorly characterized, the involvement of receptor and non-receptor protein tyrosine kinases (PTK) as potential transducers of H_2O_2-evoked responses has been suggested (Frank et al. 2003; Mehdi et al. 2004; Zhuang and Schnellmann 2004; Purdom and Chen 2005; Tabet et al. 2005). Specifically, a prominent contribution of epidermal growth factor receptor (EGFR) transactivation in mediating H_2O_2-induced activation of extracellular signal regulated kinase 1 and 2 (ERK1/2) has been documented in many cell types (Zhuang and Schnellmann 2004; Purdom and Chen 2005). However, a precise role of growth factor receptor and Src-family PTKs in mediating H_2O_2-induced effects on PKB signaling in VSMC has not yet been elucidated.

PKB, also known as Akt, exists as 3 isoforms, α, β and γ (Akt1, Akt2 and Akt3, respectively); each isoform possesses an amino-terminal pleckstrin homology (PH) domain, a kinase domain and a carboxy-terminal regulatory domain. PKB is rapidly activated in response to insulin (Farese et al. 2005; van der Heide et al. 2005), AngII (Saward and Zahradka 1997), ET-1 (Daou and Srivastava 2004), and many other growth factors (Duan et al. 2000; Cenni et al. 2003; Dolloff et al. 2005; Markadieu et al. 2005; Zaka et al. 2005) in a phosphatidylinositol 3-kinase (PI3K)-dependent manner. PI3K is a lipid kinase that promotes the generation of 3'-phosphoinositides, such as phosphatidyl-inositol 3,4,5 triphosphate (PIP_3). PIP_3 binds to the PH domain of PKB and recruits it to the plasma membrane for phosphorylation by phosphoinositide-dependent kinases 1 (PDK-1) and 2 (PDK-2). PDK-1 phosphorylates PKB at threonine 308 in the catalytic domain whereas putative PDK-2 phosphorylates it at serine 473 in the C-terminal regulatory domain of PKB (Whiteman et al. 2002). Activated PKB phosphorylates

several downstream substrates, such as glycogen synthase kinase-3β, Forkhead transcription factor, Bcl-2-associated death, IkB kinase, mammalian target of rapamycin, Mdm2, Caspase 9 and endothelial nitric oxide synthase (Whiteman et al. 2002; Song et al. 2005). Phosphorylated forms of these substrates regulate diverse cellular functions, such as glucose transport, cell growth, gene expression, cell survival and death as well as protein synthesis (Saward and Zahradka 1997; Datta et al. 1999).

In the present study, by using a series of pharmacological and cell biological approaches, we have examined the involvement of receptor and non-receptor PTKs as potential upstream modulators of H_2O_2-induced PKB phosphorylation and activation in A10 VSMC. A10 VSMC, derived from the rat embryonic thoracic aorta, which exhibit characteristics similar to those of normal VSMC (Kimes and Brandt 1976) and have been extensively accepted as a model for the investigation of vascular cellular processes (Hashim et al. 2004, Daou and Srivastava 2004, Chen et al. 2006).

Materials and Methods

Materials

H_2O_2 was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phospho-specific antibodies to PKB (Ser473) and Pyk2 (Tyr402), total PKB antibody and anti-rabbit secondary antibody were from New England BioLabs (Beverly, MA, U.S.A.). Phospho-specific c-Src antibody (Tyr418) was from Biosource (Camarillo, CA, U.S.A.). Antiphosphotyrosine antibody (PY99), total c-Src antibody, total insulin-like growth factor receptor-1beta (IGF-1Rβ) subunit antibody and anti-mouse secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Total Pyk2 antibody was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Epidermal growth factor (EGF) and all pharmacological inhibitors were from Calbiochem (San Diego, CA, U.S.A.). Human IGF-1 was from PeproTech Inc. (Rocky Hill, NJ, U.S.A.). All cell culture materials were from Invitrogen Corp. (Grand Island, NY, U.S.A.). Protein A Sepharose beads and enhanced chemiluminescence (ECL) detection kits were from Amersham Pharmacia Biotech (Baie d'Urfé, Quebec, Canada).

Cell Culture

A10 VSMC, obtained from the American Type Culture Collection (Manassas, VA, U.S.A.), were maintained in Dulbecco Modified Eagle Medium (DMEM) containing 10% of fetal bovine serum and 1% of penicillin + streptomycin in a humidified atmosphere of 5% CO_2 exchange at 37°C as described earlier (Srivastava and Pandey 2000). They were passaged twice a week by harvesting with Trypsin/EDTA.

Stimulation of VSMC and Immunoblotting

VSMC were grown to 80-90% confluence in 60-mm culture plates and incubated in serum-free DMEM for 5 hours prior to the experiments. Cells subjected to various experimental treatments were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped on ice in lysis buffer (10 mM $Na_4P_2O_7$, 25 mM NaCl, 10 mM NaF, 2 mM benzamidine, 25 mM Tris-HCl pH 7.4, 2 mM EGTA, 2 mM EDTA, 1 mM Na molybdate, 1 mM Na orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1% (vol/vol) Nonidet P-40 and 0.1% (vol/vol) sodium dodecyl sulfate (SDS)). The lysates were clarified by centrifugation at 4°C to remove insoluble materials. Clarified lysates containing equal amounts of protein, as determined by Bradford's method, were separated by 7.5% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidine difluoride (PVDF) membranes. The membranes were blocked for 1 hour with PBS-Tween 20 containing 5% non-fat dry milk at room temperature. The blots were incubated overnight with respective primary antibodies at 4°C and gentle shaking. The following day, they were incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit or anti-mouse) at room temperature. The antigen-antibody complex was visualized by the ECL method (Mehdi et al. 2004). The intensity of the protein bands was quantified by NIH Image software (Daou and Srivastava 2004).

Immunoprecipitation

Cells were grown to 80-90% confluence in 100-mm culture plates and incubated in serum-free DMEM for 5 hours prior to the experiments. Stimulation and cell lysis was

performed as described above. The clarified lysates, normalized to contain equal amounts of protein (500 μg), were immunoprecipitated overnight with 2 μg of PY99 antibody at 4°C, followed by incubation with protein A Sepharose for an additional 2 hours. Immunoprecipitated phosphotyrosine proteins were collected by centrifugation, then washed once with PBS and twice with lysis buffer. The phosphotyrosine protein immunoprecipitates were separated by 7.5% SDS-PAGE, transferred to PVDF membranes and incubated with primary antibody (for IGF-1Rβ). Proteins were detected by a horseradish peroxidase-conjugated secondary antibody and visualized with an ECL detection kit (Mehdi et al. 2004). The intensity of the protein bands was quantified by NIH Image software (Daou and Srivastava 2004).

Statistics

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

Results

AG1024 but not AG1478 inhibits H_2O_2-induced PKB phosphorylation

Several studies have reported on the role of EGFR transactivation in H_2O_2-induced phosphorylation of MAPKs, such as ERK1/2 and p38 MAPK, in various cell types, including VSMC (Purdom and Chen 2005; Tabet et al. 2005). Therefore, we first examined if EGFR-PTK played a similar role in H_2O_2-induced PKB phosphorylation in A10 VSMC. For this purpose, we used AG1478, an ATP-binding site inhibitor of EGFR-PTK (Levitzki and Gazit 1995). Treatment of A10 cells with 10 μM of AG1478 for 30 minutes failed to inhibit H_2O_2-induced PKB phosphorylation as assessed by a phospho-specific Ser473 antibody (Fig. 1A). However, under these conditions, as expected, EGF-induced PKB phosphorylation was almost completely attenuated in AG1478-pretreated cells (Fig. 1A). In contrast, IGF-1-induced PKB phosphorylation was not affected by AG1478 pretreatment.

It was reported recently that the stimulatory effect of AngII on PI3K/PKB signaling in primary cultures of smooth muscle cells from the left anterior descending coronary artery of porcine hearts is mediated through IGF-1R transactivation (Zahradka et al. 2004). Therefore, we wished to investigate if IGF-1R contributes to H_2O_2-induced PKB phosphorylation in A10 VSMC. For these experiments, we utilized AG1024, a highly selective inhibitor of IGF-1R-PTK (Parrizas et al. 1997). As seen in Figure 1B, AG1024 treatment of A10 cells almost completely blocked H_2O_2-induced PKB phosphorylation. A similar inhibitory effect of AG1024 on IGF-1-induced PKB phosphorylation was observed, whereas this compound had no effect on EGF-stimulated PKB phosphorylation

(Fig. 1B). These results suggested a role of IGF-1R-PTK in enhanced PKB phosphorylation in response to H_2O_2 in A10 cells.

H_2O_2 increases tyrosine phosphorylation of IGF-1Rβ

Since tyrosine phosphorylation of the IGF-1Rβ subunit is essential for its intrinsic PTK activity, we next evaluated if H_2O_2 treatment enhances the level of its tyrosine phosphorylation. This was achieved by stimulating A10 cells with H_2O_2 for 10 minutes or with IGF-1 for 5 minutes. Cell lysates were subjected to immunoprecipitation with phosphotyrosine-specific antibody, followed by immunoblotting with the anti-IGF-1Rβ subunit antibody. As illustrated in Figure 2, both H_2O_2 and IGF-1 induced an increase in the tyrosine phosphorylation of IGF-1Rβ, and AG1024 markedly inhibited this response (Fig. 2).

H_2O_2-induced PKB phosphorylation requires c-Src activity

Several reports have suggested a role for the Src family of non-receptor PTKs in triggering some signaling events induced by ROS (Mehdi et al. 2004; Tabet et al. 2005) and AngII (Shah and Catt 2002; Touyz et al. 2004). Therefore, we were interested to investigate the possible involvement of these kinases in H_2O_2-induced PKB phosphorylation in A10 VSMC. As illustrated in Figure 3, pretreatment of cells with PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazole[3,4-d] pyrimidine), a selective inhibitor of Src family PTK (Hanke et al. 1996), prior to stimulation with H_2O_2, markedly attenuated PKB phosphorylation in response to H_2O_2. In contrast, PP3 (4-amino-7-

phenylpyrazol [3,4-d] pyrimidine), an inactive analogue of PP2, had no effect on H_2O_2-induced PKB phosphorylation under these conditions.

H_2O_2-induced c-Src phosphorylation requires PTK activity of IGF-1R

Src-PTKs are activated subsequent to phosphorylation of a conserved tyrosine residue (Tyr418 in human Src) located in its activation loop (Thomas and Brugge 1997). Therefore, further proof for the involvement of Src-PTK in H_2O_2-induced effects was obtained by directly determining changes in the phosphorylation status of Tyr418 in H_2O_2-treated A10 cells. As seen in Figure 4, H_2O_2 increased Tyr418 phosphorylation in these cells. To further elucidate any cross-talk between IGF-1R-PTK and Src-PTK, we studied the effect of AG1024 on c-Src phosphorylation. As shown in Figure 4, pretreatment with AG1024, but not AG1478, markedly inhibited H_2O_2-induced phosphorylation of c-Src, suggesting that IGF-1R-PTK is necessary for c-Src activation by H_2O_2 in A10 VSMC.

H_2O_2-induced Pyk2 phosphorylation depends on c-Src and IGF-1R but not on EGFR activity

Recent investigations have indicated that Pyk2, a cytosolic Ca^{2+}-dependent proline-rich tyrosine kinase (Lev et al. 1995), plays a role in AngII-induced PI3K/PKB signaling in VSMC (Rocic et al. 2001). Moreover, Pyk2 is activated by H_2O_2 (Frank et al. 2000), and is known to associate with c-Src-PTK (Dikic et al. 1996; Andreev et al. 2001). Therefore, we wished to examine if H_2O_2-induced PKB phosphorylation was linked with Pyk2 phosphorylation and if Src-PTK was involved in this response in A10 cells. As depicted in Figure 5, H_2O_2 treatment enhanced the tyrosine phosphorylation of Pyk2 that was

completely suppressed by PP2, whereas PP3 had no effect on this phosphorylation (Fig. 5A). Next, we examined the possible involvement of IGF-1R-PTK in Pyk2 phosphorylation by H_2O_2. As revealed in Figure 5B, AG1024 pretreatment markedly decreased Pyk2 phosphorylation, but AG1478 had no effect. These data suggested that c-Src and IGF-1R but not EGFR-PTK are required for Pyk2 phosphorylation by H_2O_2 in A10 cells.

Discussion

In this study, we have demonstrated that PKB phosphorylation in A10 VSMC, in response to exogenous H_2O_2, is mediated primarily through the IGF-1R-PTK-dependent pathway. Although a partial contribution of IGF-1R-PTK in AngII- and H_2O_2-induced ERK1/2 phosphorylation has been documented (Touyz et al. 2003; Cruzado et al. 2005; Tabet et al. 2005), to the best of our knowledge, the data presented here are the first to identify a role for IGF-1R-PTK in H_2O_2-induced PKB phosphorylation in VSMC.

Several investigations in different cell types have revealed that EGFR-PTK serves as a mediator of H_2O_2-induced activation of MAPK pathways (Zhuang and Schnellmann 2004; Purdom and Chen 2005; Tabet et al. 2005). However, failure of AG1478, the specific inhibitor of EGFR-PTK, to block H_2O_2-enhanced PKB phosphorylation while inhibiting EGF-induced PKB phosphorylation, indicated that EGFR-PTK does not participate in PKB activation by H_2O_2 in A10 cells. This notion is further supported by recent experiments showing that H_2O_2-induced PKB phosphorylation is independent of EGFR-PTK activity in renal cells (Zhuang and Schnellmann 2004). In contrast to these studies, H_2O_2-induced activation of PKB was found to be dependent on EGFR-PTK in HeLa cells (Wang et al. 2000). The reason for these discrepancies is not clear but it may be attributed to cell-specific differences in response to H_2O_2 or the differential expression level of EGFR in various cells.

The fact that H_2O_2 treatment induced an increase in tyrosine phosphorylation of the IGF-1Rβ subunit lends further support to a role for IGF-1R-PTK activation in triggering H_2O_2 responses in A10 cells. A similar increment of IGF-1R tyrosine phosphorylation in response to AngII has been demonstrated in primary cultures of smooth muscle cells

derived from the coronary artery of porcine hearts (Zahradka et al. 2004). These authors also reported that AngII-induced phosphorylation of the p85 subunit of PI3K and of p70s6k, a downstream substrate of PI3K, was suppressed by IGF-1R-PTK inhibitors (Zahradka et al. 2004). Thus, it appears that H_2O_2 and AngII, that signals through the generation of H_2O_2 primarily utilize IGF-1R-PTK activation as a mechanism to turn on the PI3K/PKB signaling pathway.

We also provided evidence that, along with IGF-1R-PTK, c-Src activation is required for H_2O_2-induced PKB phosphorylation in VSMC. Similar observations were reported previously in Chinese hamster ovary (CHO) cells (Mehdi et al. 2004), in rat-2 fibroblasts (Esposito et al. 2003) and in renal cells (Zhuang and Schnellmann 2004) where PP2 was found to decrease H_2O_2-induced PKB phosphorylation. Our results, showing that c-Src phosphorylation was almost completely blocked by inhibition of IGF-1R-PTK, suggested that H_2O_2 might activate c-Src through an IGF-1R-dependent pathway. Supporting these observations, recent studies have established that IGF-1 treatment increased c-Src phosphorylation in 3T3-L1 preadipocytes (Sekimoto and Boney 2003; Sekimoto et al. 2005). It thus appears that one of the consequences of IGF-1R activation is the enhancement of c-Src activity.

Pyk2, a target of H_2O_2, is known to interact physically with c-Src (Dikic et al. 1996; Sabri et al. 1998; Andreev et al. 2001; Lakkakorpi et al., 2003; Park et al. 2004), and has been implicated as an upstream regulator of PI3K/PKB signaling in response to AngII in VSMC (Rocic and Lucchesi 2001; Rocic et al. 2001; Rocic et al. 2003). It is thus possible that Pyk2 may have a similar role in H_2O_2-induced PKB phosphorylation in A10 VSMC. This notion is supported by observations showing that pharmacological inhibition of Src-

and IGF-1R-PTKs blocked not only PKB phosphorylation, but also Tyr402 phosphorylation of Pyk2. A recent report that overexpression of a dominant form of Pyk2 reduced PKB phosphorylation induced by H_2O_2 in PC12 cells lends additional support for a role of Pyk2 as a mediator of PKB activation (Banno et al. 2005).

Several laboratories have determined that the Phosphatase and Tensin homolog deleted on chromosome ten (PTEN), which catalyzes PIP_3 dephosphorylation, is a direct target of H_2O_2 and becomes inactivated by oxidation of Cys-124 in its catalytic site subsequent to H_2O_2 treatment (Lee et al. 2002). Inactivation of PTEN, which results in an increase of PIP_3 levels, has been attributed to PKB activation in response to several agents known to generate ROS (Leslie et al. 2003; Kwon et al. 2004; Seo et al. 2005). However, our earlier studies, showing that H_2O_2 treatment enhanced PI3K activity in CHO cells overexpressing human insulin receptor and that Wortmannin, an inhibitor of PI3K, suppressed H_2O_2-induced PKB phosphorylation (Mehdi et al. 2004), indicate that stimulation of PI3K by H_2O_2 also plays an important part in this response. Moreover, our observations that AG1024 blocked H_2O_2-induced PKB phosphorylation further suggested that PTEN inhibition alone cannot contribute to this effect but also requires IGF-1R-PTK activation. The precise mechanism by which H_2O_2 activates IGF-1R-PTK in A10 cells is still unclear. However, in view of the ability of H_2O_2 to inhibit protein tyrosine phosphatases (PTPases), such as PTP1B (Lee et al. 1998) and SHP-2 (Meng et al. 2002), it may be postulated that H_2O_2 treatment can shift the equilibrium of the phosphorylation-dephosphorylation cycle, culminating in a net increase of the tyrosine phosphorylation of IGF-1R and/or other proteins, which may trigger the IGF-1R-PTK signaling pathway (Fig. 6).

In summary, the data presented here demonstrate that H_2O_2 enhances PKB phosphorylation in A10 VSMC. PKB phosphorylation by H_2O_2 occurs in an IGF-1R- and Src-PTK-dependent manner with a potential intermediary role of Pyk2 (Fig. 6). Since PKB is a critical component of cell survival, hypertrophic and proliferative pathways, it may be speculated that aberrant activation of PKB and its downstream components by ROS might contribute to the pathophysiology of vascular abnormalities.

Acknowledgments

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Figure legends

Figure 1: Effect of AG1478 and AG1024 on H_2O_2-induced PKB phosphorylation in A10 VSMC. Confluent, serum-starved A10 cells were pretreated with (+) or without (-) 10 μM AG1478 **(A)** or 1 μM AG1024 **(B)** for 30 minutes, followed by stimulation in the absence (C) or presence of 500 μM H_2O_2 for 10 minutes, 10 nM EGF or 1 ng/mL IGF-1 for 5 minutes. The cells were lysed, and equal amounts of protein were separated on 7.5% SDS-PAGE. PKB phosphorylation was detected by immunoblotting with phospho-specific Ser473 PKB antibody (top panel of each section). Blots were also analyzed for total PKB (middle panel). The bottom panels represent average data quantified by densitometric scanning of the immunoblots shown in the top panels. Values are the means ± SE of at least 3 independent experiments and are expressed as percent phosphorylation where phosphorylation observed with either H_2O_2, EGF or IGF-1 alone is defined as 100%. **(A)** $^{*}P<0.001$ vs control (C), $^{\#}P<0.001$ vs control (C), $^{\S}P<0.001$ vs control (C), $^{\dagger}P<0.001$ vs EGF. **(B)** $^{*}P<0.001$ vs control (C), $^{\#}P<0.001$ vs control (C), $^{\S}P<0.001$ vs control (C), $^{\dagger}P<0.001$ vs H_2O_2, $^{\ddagger}P<0.001$ vs IGF-1.

Figure 2: Effect of H_2O_2 on IGF-1Rβ tyrosine phosphorylation in A10 VSMC. Confluent, serum-starved A10 cells were pretreated with (+) or without (-) 1 μM AG1024 for 30 minutes, followed by stimulation in the absence (C) or presence of 500 μM H_2O_2 for 10 minutes or 1 ng/mL IGF-1 for 5 minutes. The cells were lysed, and equal amounts of protein from the clarified lysates were subjected to immunoprecipitation (IP) with antiphosphotyrosine (PY99) antibody. The immunoprecipitates were immunoblotted with an antibody to the IGF-1Rβ subunit (top panel). Whole lysates were also analyzed for the total IGF-1Rβ subunit (middle panel). The bottom panel represents average data quantified by densitometric scanning of the immunoblots shown in the top panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percent phosphorylation where phosphorylation observed with either H_2O_2 or IGF-1 alone is defined as 100%. $^{*}P<0.001$ vs control (C), $^{\#}P<0.01$ vs control (C), $^{\dagger}P<0.01$ vs H_2O_2, $^{\ddagger}P<0.01$ vs IGF-1.

Figure 3: Effect of PP2 on H_2O_2-induced PKB phosphorylation in A10 VSMC. Confluent, serum-starved A10 cells were pretreated with (+) or without (-) 10 μM PP2 or its inactive analogue PP3 for 30 minutes, followed by stimulation in the absence (C) or presence of 500 μM H_2O_2 for 10 minutes. The cells were lysed, and equal amounts of protein were separated on 7.5% SDS-PAGE. PKB phosphorylation was detected by immunoblotting with phospho-specific Ser473 PKB antibody (top panel). Blots were also analyzed for total PKB (middle panel). The bottom panel represents average data quantified by densitometric scanning of the immunoblots shown in the top panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percent phosphorylation where phosphorylation observed with H_2O_2 is defined as 100%. $^{*}P<0.001$ vs control (C), $^{\dagger}P<0.001$ vs H_2O_2.

Figure 4: Effect of AG1478 and AG1024 on H_2O_2-induced c-Src phosphorylation in A10 VSMC. Confluent, serum-starved A10 cells were pretreated with (+) or without (-) 10 μM AG1478 or 1 μM AG1024 for 30 minutes, followed by stimulation in the absence (C) or presence of 500 μM H_2O_2 for 10 minutes. The cells were lysed, and equal amounts of protein were separated on 7.5% SDS-PAGE, and c-Src phosphorylation was detected by immunoblotting with phospho-specific Tyr418 c-Src antibody (top panel). Blots were also analyzed for total c-Src (middle panel). The bottom panel represents average data quantified by densitometric scanning of the immunoblots shown in the top panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percentage phosphorylation where phosphorylation observed with H_2O_2 is defined as 100%. $^{*}P<0.001$ vs control (C), $^{\dagger}P<0.001$ vs H_2O_2.

Figure 5: Effect of AG1478, AG1024 and PP2 on H_2O_2-induced Pyk2 phosphorylation. Confluent, serum-starved A10 cells were pretreated: **(A)** with (+) or without (-) 10 μM AG1478 or 1 μM AG1024 for 30 minutes, or **(B)** 10 μM PP2 or its inactive analogue PP3 for 30 minutes, followed by stimulation in the absence (C) or presence of 500 μM H_2O_2 for 10 minutes. The cells were lysed, and equal amounts of protein were separated by 7.5% SDS-PAGE. Pyk2 phosphorylation was detected by immunoblotting with phospho-specific Tyr402 Pyk2 antibody (top panel of each section).

Blots were also analyzed for total Pyk2 (middle panel). The bottom panel represents average data quantified by densitometric scanning of immunoblots shown in the top panel. Values are the means ± SE of at least 3 independent experiments and are expressed as percent phosphorylation where phosphorylation observed with H_2O_2 alone is defined as 100%. **(A)****P*<0.01 vs control(C) , †*P*<0.01 vs H_2O_2. **(B)** **P*<0.01 vs control (C), †*P*<0.05 vs H_2O_2.

Figure 6: Schematic model showing the signaling cascade induced by H_2O_2 in A10 VSMC. H_2O_2 enhances tyrosine phosphorylation of the IGF-1Rβ subunit which is blocked by AG1024. IGF-1R activation is necessary for H_2O_2-induced phosphorylation of PKB, c-Src and Pyk2 because all these events are blocked by AG1024. Src appears to be upstream of Pyk2 and PKB, since its pharmacological inhibition by PP2 decreases both Pyk2 and PKB phosphorylation. Pyk2 may be acting upstream of PKB via a yet undefined pathway. The mechanism by which H_2O_2 stimulates IGF-1Rβ phosphorylation remains obscure, but the ability of H_2O_2 to inhibit PTPases might contribute to this effect.

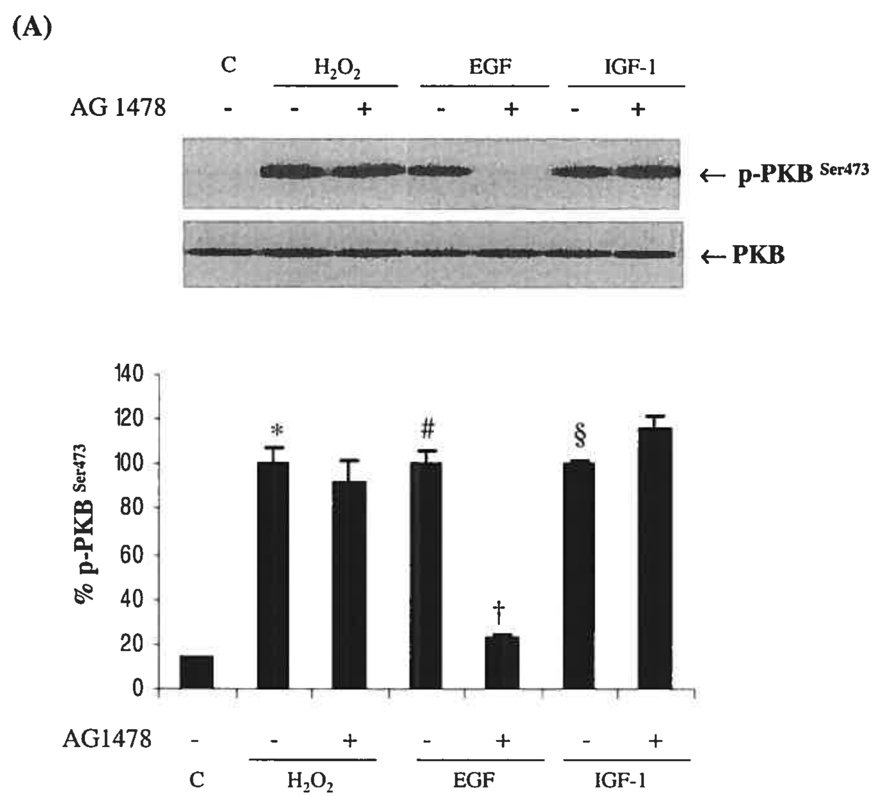


Figure 1

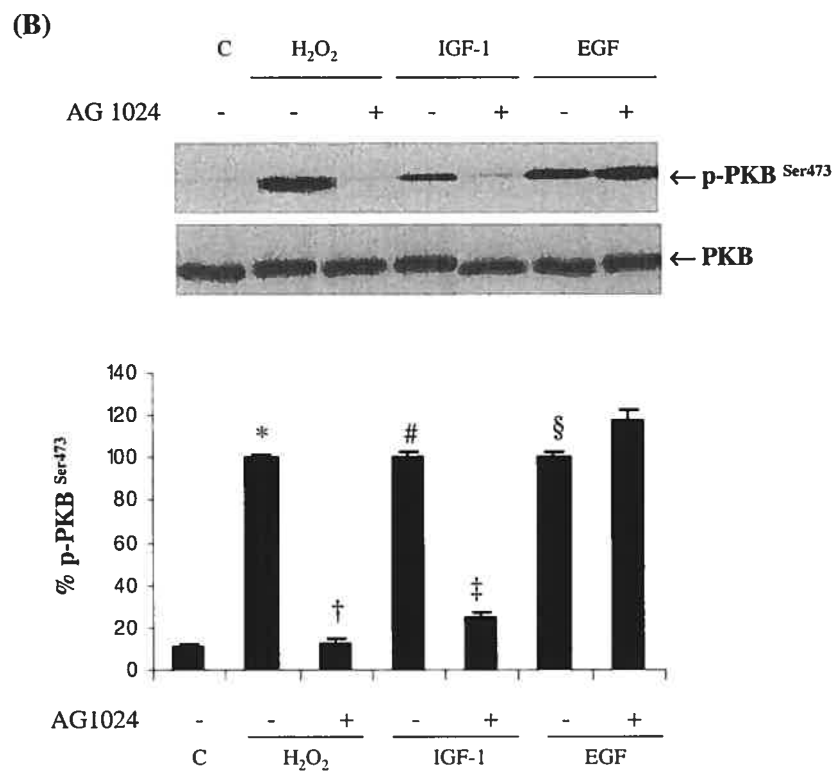


Figure 1

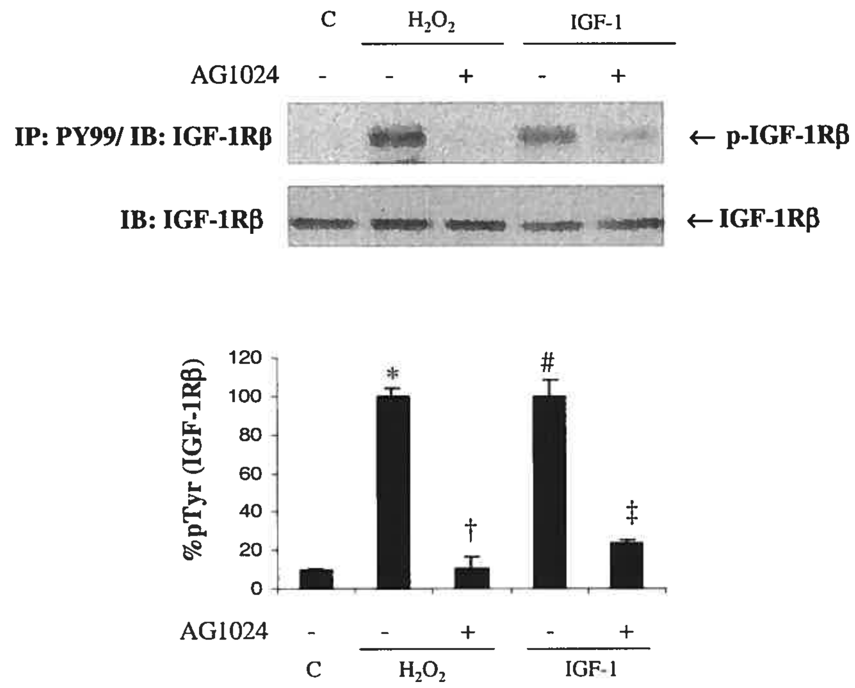


Figure 2

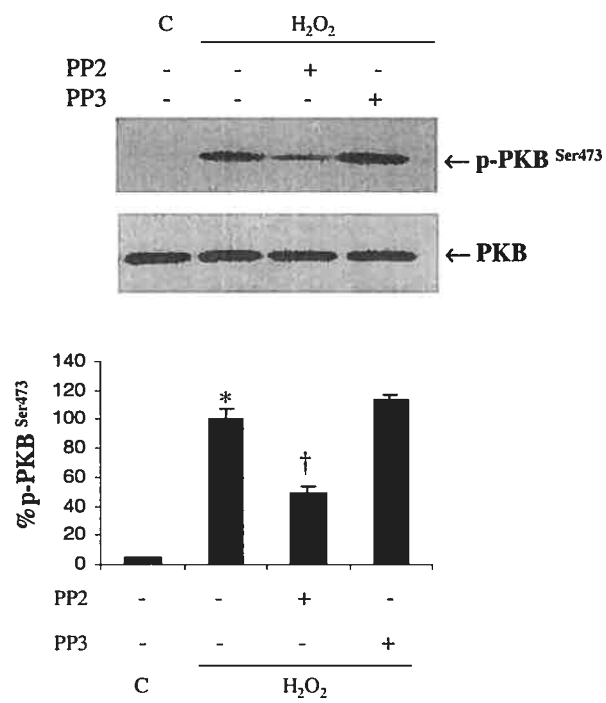


Figure 3

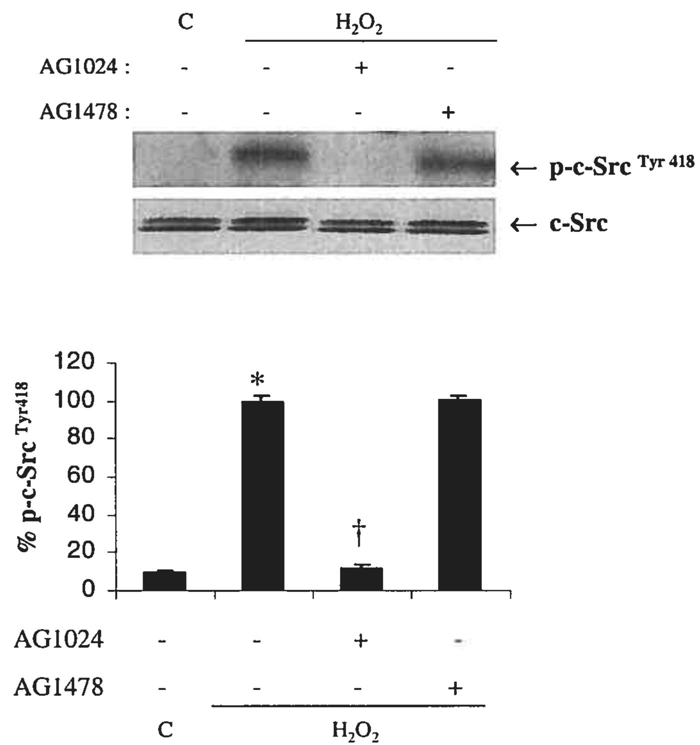
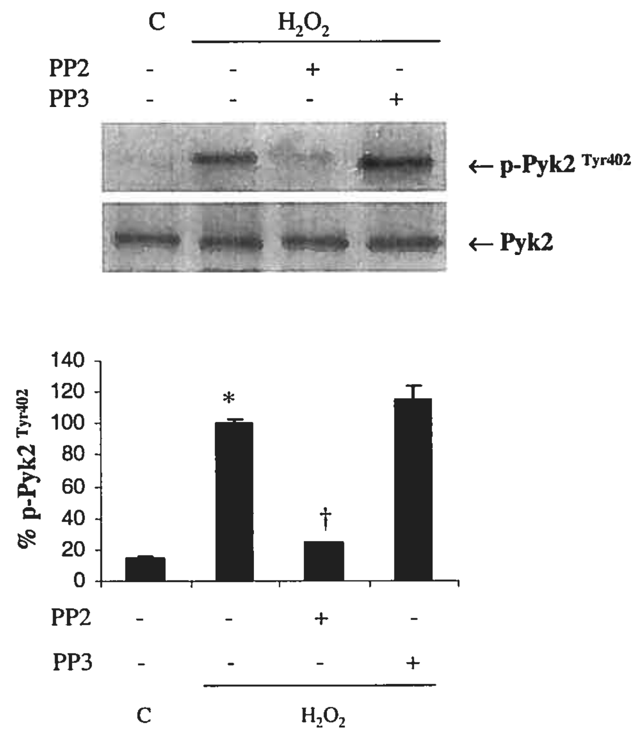


Figure 4



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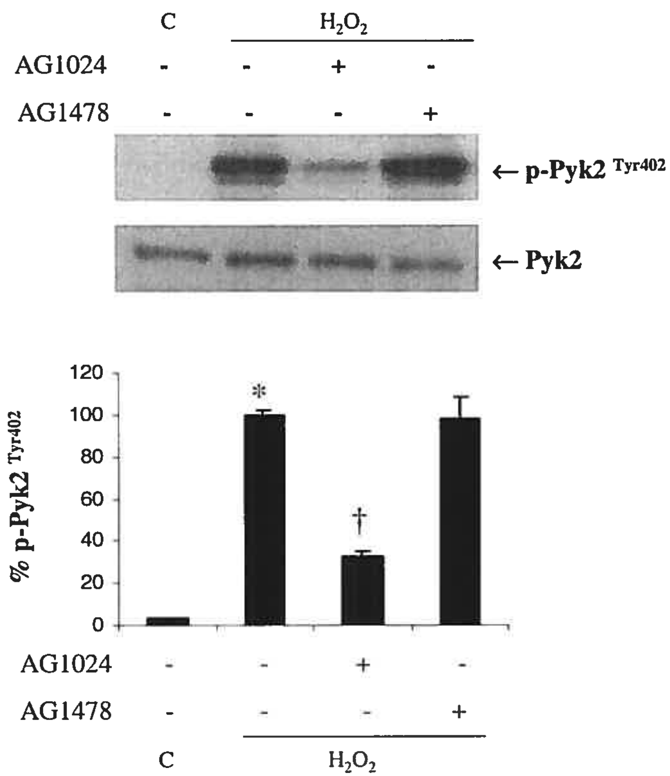

Figure 5

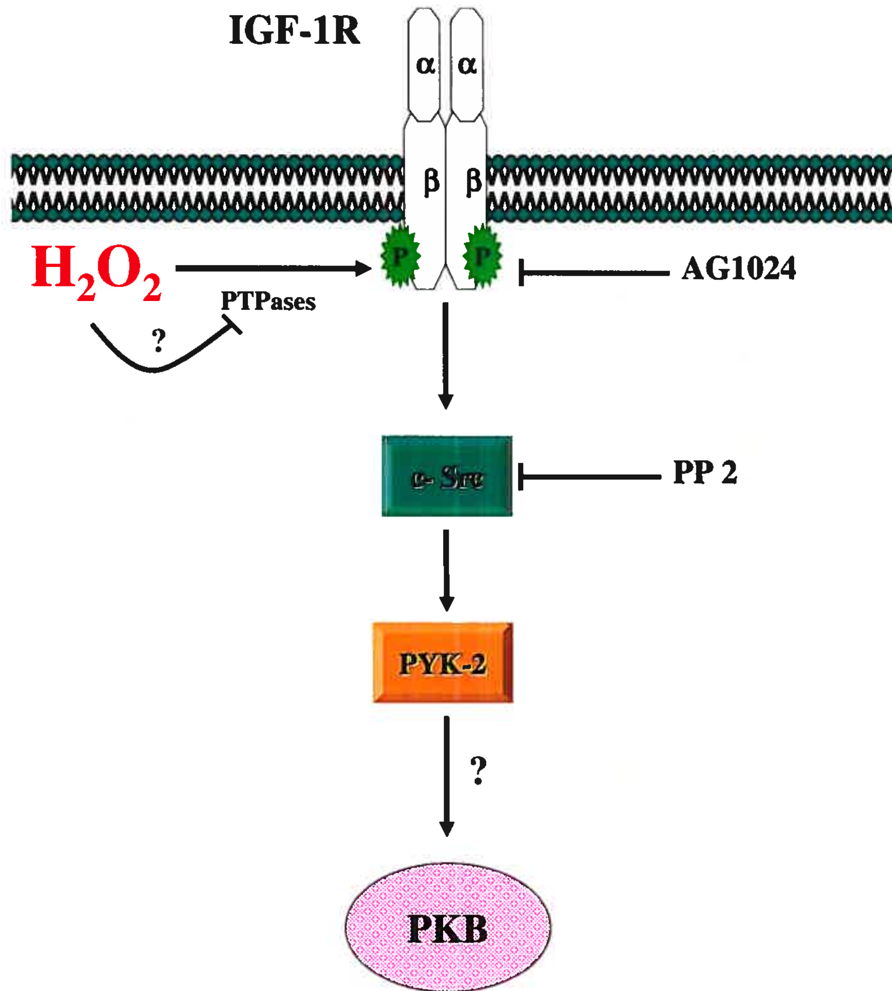

Figure 6

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CHAPTER 3

GENERAL DISCUSSION

ROS generated in the vasculature are recognized as important second messenger molecules at physiological concentrations [216]. However, a large body of evidence supports an important role for high levels of ROS (oxidative stress) in cardiovascular disorders including hypertension, atherosclerosis, vascular injury and diabetes. This is due, in part, to $^{\cdot}O_2^-$ excess and decreased bioavailability of NO and to ROS-mediated cardiovascular remodeling [17;75;217]. Both $^{\cdot}O_2^-$ and H_2O_2 were shown to increase vascular contractility, stimulate VSMC growth and induce inflammatory responses [39;218-221]. In this context, several studies have investigated the possible protective effect of antioxidants. Since ROS mediate their effects through redox-sensitive signaling pathways, a better knowledge of these pathways would be necessary for developing specific therapeutic agents directed against critical components of ROS signaling systems implicated in different pathologies.

Over the last few years, studies have identified many redox-sensitive pathways, including MAPK and PI3-K/PKB signaling systems in many cell types particularly in VSMC isolated from different artery beds [11;102;104;128;222]. Activation of these pathways has been linked to cell growth, proliferation, hypertrophy and survival, which are critical events for vascular remodeling. Since vascular diseases are often associated with excessive cell growth and proliferation, there is a lot of interest to understand the precise mechanisms by which ROS induce the activation of these pathways.

Using H_2O_2 as a mimicker of ROS, several studies have suggested a role for receptor and non-receptor PTK as well as Ca^{2+} and PKC in H_2O_2-induced activation of MAPK and PI3-K/PKB pathways. Majority of these *in vitro* studies have used high micromolar concentrations of H_2O_2. These concentrations can be considered as

"pathological" [223-226] because "normal" physiological concentration of H_2O_2 in the plasma of normotensive humans have been reported to be in low micromolar (3 μM) [227]. However, the effect of these low H_2O_2 concentrations on signaling pathways has not been examined in detail in cardiovascular system. This may be due to the possibility that acute treatment with low H_2O_2 concentration (3-5 μM) does not show detectable change in the activation/phosphorylation of the signaling components. For example, Blanc et al. have shown that both Ca^{2+} chelators and the calmodulin inhibitor Fluphenazine markedly decreased H_2O_2-induced ERK1/2, p38mapk and PKB phosphorylation in A10 VSMC cell line [102]. More recently, by using pharmacological inhibitors, Tabet el al. have reported that H_2O_2-induced ERK1/2 phosphorylation was regulated by both receptor- and non-receptor-PTK, whereas p38mapk phosphorylation was regulated exclusively by non-receptor-PTK in primary cultures of VSMC from rat mesenteric arteries [103]. Whether H_2O_2-induced PKB phosphorylation was tyrosine kinase dependent in VSMC has not yet been investigated. Therefore, these studies were undertaken to investigate a role for receptor- and non-receptor-PTK in H_2O_2-induced PKB and ERK1/2 phosphorylation.

By using pharmacological inhibitors, we have shown that H_2O_2-induced PKB and ERK1/2 phosphorylation was dependent on IGF-1R, since pretreatment of the cells with AG1024, a specific inhibitor of IGF-1R kinase, abrogated the H_2O_2-induced PKB (Chapter 2) as well as ERK1/2 phosphorylation (**Fig. 3.7**).

Further proof for the involvement of the IGF-1R in this response was provided by the results showing that H_2O_2 enhanced the tyrosine phosphorylation of the IGF-1R β-subunit in A10 VSMC (Chapter 2). A role for IGF-1R transactivation in AngII-induced

activation of PI3-K/PKB pathway has been observed in porcine coronary VSMC [168]. Thus, it appears that both exogenous H_2O_2 and AngII, which is an inducer of ROS generation, mainly utilize IGF-1R to trigger the activation of PI3-K/PKB pathway in VSMC.

Several studies have shown that EGFR-PTK plays a role in triggering the H_2O_2-induced MAPK activation in renal cells [99], cardiomyocytes and cardiac fibroblasts [101] as well as primary cultures of VSMC [103;145]. Moreover, H_2O_2 is able to enhance tyrosine phosphorylation of EGFR in VSMC [129;145;146]. On the other hand, H_2O_2-induced PKB activation has been shown to be EGFR-dependent in HeLa cells [127], but not in renal cells [99] or PC12 cells [155]. Our data suggests that EGFR transactivation does not participate in neither PKB nor ERK1/2 activation by H_2O_2 in A10 VSMC, since AG1478, the specific inhibitor of EGFR kinase, had no inhibitory effect on PKB (Chapter 2) or ERK1/2 phosphorylation (**Fig. 3.7**) in response to H_2O_2. The reason for this conflicting data is unknown, but could be related to cell-specific responses of H_2O_2.

The PDGFR has been shown to undergo tyrosine phosphorylation in response to H_2O_2 in rat aortic VSMC [146] and appears to be implicated in H_2O_2-induced ERK1/2 phosphorylation in VSMC derived from mesenteric arteries of SHR and WKY rats [103]. Therefore, we tested the possible implication of the PDGFR-PTK in H_2O_2-induced PKB and ERK1/2 phosphorylation in A10 cells by using AG1295, the specific inhibitor of PDGFR-PTK [228]. Pretreatment of cells with AG1295, failed to inhibit the H_2O_2-induced PKB (**Fig. 3.8(A)**) and ERK1/2 phosphorylation (**Fig. 3.8(B)**), whereas it blocked completely the PDGF-evoked effect. The reason for the discrepancy between our

studies and that of others is unclear. However, it could be due to the type of VSMC used, which might affect the responsiveness to the stimulant.

We also provided evidence that not only receptor-PTK could play important roles in mediating H_2O_2 responses, but also non-receptor-PTK such as Src, contributes to this effect. By using PP2 as a specific inhibitor for c-Src, previous work from our laboratory has shown that H_2O_2-induced ERK1/2 and PKB phosphorylation were Src-dependent in CHO cells [100]. Similarly, PP2 significantly inhibited H_2O_2-stimulated ERK1/2 and p38mapk phosphorylation in VSMC [103] as well as PKB activation in Rat-2 fibroblasts [98] and in renal cells [99]. Our data was the first to demonstrate a role for c-Src as an upstream regulator of PKB activation in response to H_2O_2 in VSMC (Chapter 2). Moreover, we have also shown that H_2O_2 enhanced the phosphorylation of c-Src on Tyr^{418} (Chapter 2), which is located in its activation loop. Interestingly, this effect of H_2O_2 was completely attenuated by IGF-1R but not by EGFR-PTK inhibitor, supporting the idea that IGF-1R acts upstream of c-Src in inducing the effect of H_2O_2 in these cells. c-Src has been shown to be phosphorylated in 3T3-L1 preadipocytes in response to IGF-1 [229;230]. Thus, it appears that activation of the IGF-1R is followed by Src activation in some cell types.

PKB is implicated in many cellular functions, such as glucose transport, cell growth, gene expression, cell survival and death as well as protein synthesis. PKB might be recruited under physiological conditions at a certain basal level that contributes to the normal function of the cell in equilibrium with the other signaling pathways. The work presented here has investigated PKB activation in response to severe oxidative stress. It has been shown previously that PKB phosphorylation under these conditions is transient

(peaks at 10 minutes in A10 VSMC) and becomes rapidly inactivated within 60 minutes of incubation with H_2O_2 [102]. Thus, it is possible that long time treatment with H_2O_2 might promote apoptotic pathways in the absence of an active PKB.

We have also demonstrated that, along with PKB phosphorylation, H_2O_2 induced the phosphorylation of another non-receptor PTK, Pyk2, on Tyr^{402}, which is the autophosphorylation site and is critical for its activity. This response appears to be IGF-1R- but not EGFR-dependent. Moreover, pharmacological inhibition of c-Src markedly attenuated Pyk2 phosphorylation. Observations of Rocic et al. have suggested a role for Pyk2 as an upstream regulator of PI3-K/PKB pathway in VSMC in response to AngII [204;205;209]. Thus, further investigation by using genetic approaches would be necessary to prove the involvement of Pyk2 as a possible upstream molecule in ERK1/2 and PKB activation by H_2O_2 in A10 VSMC.

Taken together, we have demonstrated that IGF-1R serves as a mediator of H_2O_2-induced activation of PKB, ERK1/2, Src and Pyk2 in A10 VSMC. Since ERK1/2, PKB and Pyk2 are believed to play crucial roles in VSMC growth, hypertrophy, death and survival, it may be suggested that targeting the IGF-1R could be a possible therapeutic approach for vascular damage and remodeling caused by severe oxidative stress.

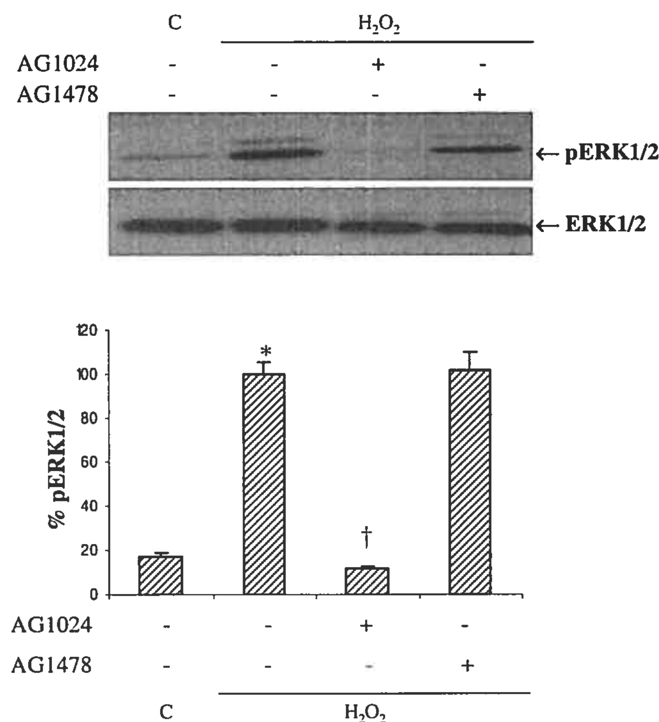


Figure 3.7: Effect of AG1478 and AG1024 on H_2O_2-induced ERK1/2 phosphorylation in A10 VSMC. Confluent, serum-starved A10 cells were pretreated with (+) or without (-) 10 μM AG1478 or 1 μM AG1024 for 30 minutes, followed by stimulation in the absence (C) or presence of 500 μM H_2O_2 for 10 minutes. The cells were lysed, and equal amounts of protein were separated on 7.5% SDS-PAGE. ERK1/2 phosphorylation was detected by immunoblotting with phospho-specific Thr202-Tyr204/Thr185-Tyr187 ERK1/2 antibody (top panel). Blots were also analyzed for total ERK1/2 (middle panel). The bottom panels represent average data quantified by densitometric scanning of the immunoblots shown in the top panels. Values are the means ± SE of at least 3 independent experiments and are expressed as percent phosphorylation where phosphorylation observed with H_2O_2 alone is defined as 100%. $^{*}P<0.001$ vs control (C), $^{\dagger}P<0.001$ vs H_2O_2. (Unpublished data)

(A)

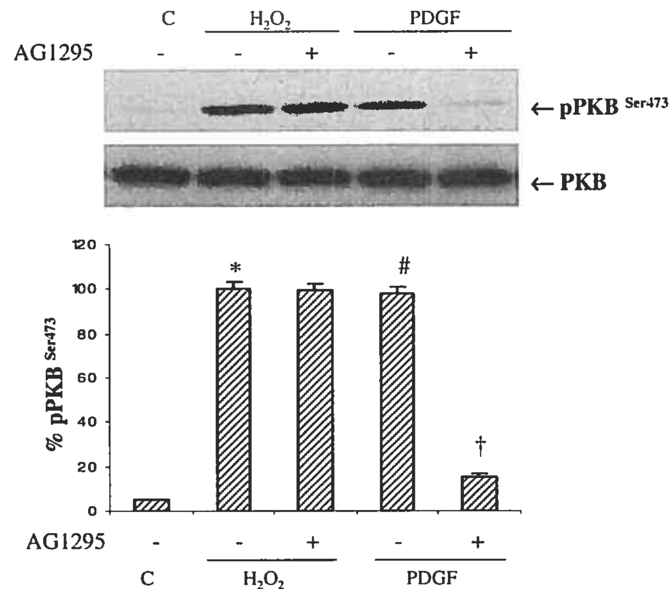
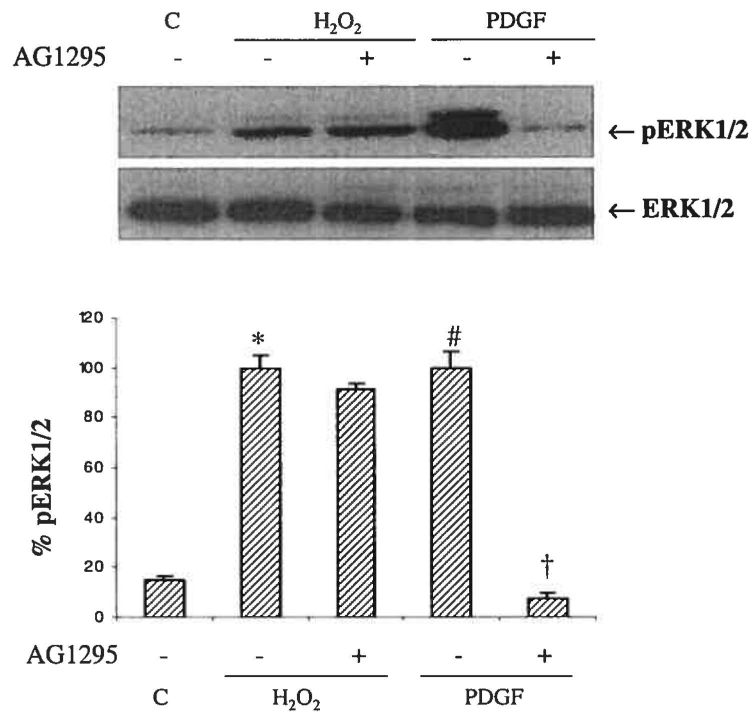


Figure 3.8: Effect of AG1295 H_2O_2-induced PKB and ERK1/2 phosphorylation in A10 VSMC. Confluent, serum-starved A10 cells were pretreated with (+) or without (-) 10 μM AG1295 for 30 minutes, followed by stimulation in the absence (C) or presence of 500 μM H_2O_2 for 10 minutes or 1ng/mL PDGF for 5 minutes. The cells were lysed, and equal amounts of protein were separated on 7.5% SDS-PAGE. PKB **(A)** and ERK1/2 **(B)** phosphorylation were detected by immunoblotting with phospho-specific antibodies (top panel of each section). Blots were also analyzed for total PKB and ERK1/2 (middle panel). The bottom panels represent average data quantified by densitometric scanning of the immunoblots shown in the top panels. Values are the means ± SE of at least 3 independent experiments and are expressed as percent phosphorylation where phosphorylation observed with either H_2O_2 or PDGF alone is defined as 100%. **(A)** $^*P<0.001$ vs control (C), $^\#P<0.001$ vs control (C), $^\dagger P<0.001$ vs PDGF. **(B)** $^*P<0.001$ vs control (C), $^\#P<0.001$ vs control (C), $^\dagger P<0.001$ vs PDGF. (Unpublished data)

(B)



CHAPTER 4

CONCLUSION

The results presented here demonstrate for the first time the involvement of the IGF-1R in mediating H_2O_2-induced phosphorylation of PKB, Src and Pyk2 in VSMC. These results were obtained by using AG1024, the specific pharmacological inhibitor of IGF-1R-PTK. We provided further evidence for the implication of the IGF-1R in H_2O_2 signaling by showing that H_2O_2 enhanced the tyrosine phosphorylation of the β–subunit of this receptor, which was inhibited by AG1024. Moreover, we observed that AG1024 abrogated H_2O_2-induced ERK1/2 phosphorylation, which is in accord with recent studies reporting a role for IGF-1R as an upstream modulator for ERK1/2 phosphorylation by H_2O_2 in primary cultures of VSMC from SHR. On the other hand, H_2O_2-induced PKB and ERK1/2 phosphorylation appeared to be EGFR- and PDGFR-independent in A10 VSMC, since neither AG1478 nor AG1295 had any inhibitory effect on these H_2O_2-induced responses. The precise mechanism by which H_2O_2 activates IGF-1R-PTK in A10 cells remains unclear. However, considering the ability of H_2O_2 to inhibit protein tyrosine phosphatases (PTPases), it may be suggested that H_2O_2 treatment can shift the equilibrium of the phosphorylation-dephosphorylation cycle, resulting thereby in a net increase of the tyrosine phosphorylation of IGF-1R and/or other proteins, which may trigger the IGF-1R-PTK signaling pathway (**Fig. 4.9**).

Since ERK1/2 and PKB are critical mediators of cell growth, hypertrophy, proliferation, protein synthesis and survival, it may be speculated that an aberrant activation of these pathways by ROS could contribute to vascular remodeling (**Fig. 4.9**). Moreover, targeting of IGF-1R could serve as a possible therapeutic approach in treating cardiovascular diseases.

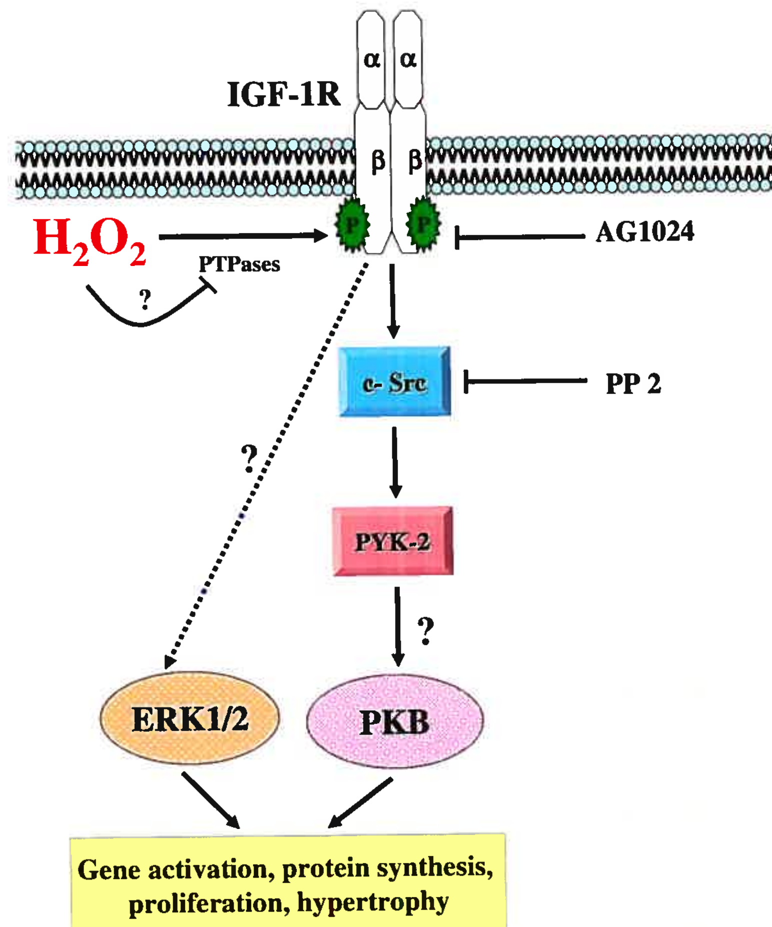


Figure 4.9: Schematic, hypothetical model showing key steps in H_2O_2-evoked responses in VSMC. IGF-1R activation appears to be necessary for H_2O_2-induced phosphorylation of PKB, ERK1/2, c-Src and Pyk2 since all of these events were blocked by AG1024. Src appears to act upstream of Pyk2 and PKB, since its pharmacological inhibition by PP2 decreased both Pyk2 and PKB phosphorylation. Whether Pyk2 is acting upstream of PKB and/or ERK1/2 or not remains to be clarified. The mechanism by which H_2O_2 stimulates IGF-1Rβ phosphorylation is still unknown, but the ability of H_2O_2 to inhibit protein tyrosine phosphatases (PTPases) might contribute to this effect. (Adapted from [231]).

CHAPTER 5

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