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Hydrogen Peroxide Enhances the Expression and Function of Giα Proteins in Aortic Vascular Smooth Muscle cells from Sprague Dawley rats: Role of Growth Factor Receptors Transactivation

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Ce mémoire intitulé :

Hydrogen peroxide enhances the expression and function of Giα proteins in aortic vascular smooth muscle cells from Sprague Dawley rats: role of growth factor receptors transactivation

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Nathan Mbong

a été évalué par un jury composé des personnes suivantes :

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Résumé

Nous avons récemment démontré que les espèces réactives oxygénées induisent une augmentation de l'expression des protéines Giα dans les cellules du muscle lisse vasculaire (CMLV) provenant d'aortes de rats spontanément hypertendus (SHR, de l'anglais *spontaneously hypertensive rats*). La présente étude a pour but d'étudier les effets du peroxyde d'hydrogène (H_2O_2), un oxydant qui induit le stress oxydatif, sur l'expression de Giα et sur l'activité de l'adénylate cyclase, et d'explorer les voies de signalisation sous-jacentes responsables de cette réponse.

Nos résultats montrent que H_2O_2 induit une augmentation de l'expression des protéines Giα-2 et Giα-3 de manière dose- et temps-dépendante avec une augmentation maximale de 40-50% à 100 μM après 1 heure, sans affecter l'expression de Gsα. L'expression des protéines Giα a été maintenue au niveau normal en presence de AG 1478, AG1295, PD98059 et la wortmannine, des inhibiteurs d'EGF-R (de l'anglais *epidermal growth factor receptor*), PDGFR-β (de l'anglais *platelet-derived growth factor receptor β)*, de la voie de signalisation ras-ERK1/2 (de l'anglais *extracellular regulated kinase1/2)*, et de la voie de la PI3Kinase-AKT (de l'anglais *phosphatidyl inositol-3 kinase*), respectivement. En outre, le traitement des CMLV avec H_2O_2 a induit une augmentation du degré de phosphorylation d'EGF-R, PDGF-R, ERK1/2 et AKT; et cette expression a été maintenue au niveau témoin par leurs inhibiteurs respectifs. Les inhibiteurs d'EGF-R et PDGF-R ont aussi induit une diminution du degré de phosphorylation de ERK1/2, et AKT/PKB. En outre, la transfection des cellules avec le siRNA (de l'anglais, *small interfering ribonucleic acid*) de EGF-R et PDGFR-β a atténué la surexpression des protéines Giα-2 et Giα-3 induite par le traitement au H_2O_2.

La surexpression des protéines Giα induite par H_2O_2 a été corrélée avec une augmentation de la fonction de la protéine Giα. L'inhibition de l'activité de l'adénylate cyclase par de faibles concentrations de GTPγS après stimulation par la forskoline a augmenté de 20% dans les cellules traitées au H_2O_2. En outre, le traitement des CMLV au H_2O_2 a aussi accru l'inhibition de l'activité de l'adénylate cyclase par les hormones inhibitrices telles que l'angiotensine II, oxotrémorine et C-ANP_{4-23}. D'autre part, la stimulation de l'adénylate cyclase induite par GTPγS, glucagon, isoprotérénol, forskoline, et le fluorure de sodium (NaF) a été atténuée de façon significative dans les cellules traitées au H_2O_2. Ces résultats suggèrent que H_2O_2 induit la

surexpression des protéines Giα-2 and Giα-3 via la transactivation des récepteurs des facteurs de croissance EGF-R, PDGFR-β et l'activation des voies de signalisation ras-ERK1/2 et PI3K-AKT

Mot-cles: Protéines Giα, peroxyde d'hydrogène, stress oxydant, récepteurs des facteurs de croissance, MAP kinases, adénylate cyclase, hypertension

Abstract

We recently have shown that reactive oxygen species contribute to the enhanced expression of $G_i\alpha$ proteins in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats (SHR). The present study was undertaken to examine if H_2O_2, an oxidant that induces oxidative stress could also enhance the expression of Giα proteins and associated adenylyl cyclase signalling in aortic VSMC and to further explore the underlying signaling pathways responsible for this response. Treatment of cells with H_2O_2 increased the expression of Giα-2 and Giα-3 proteins but not that of Gsα proteins in a concentration- and time-dependent manner. A maximal increase of 40-50% was observed at 100μM and 1h. The enhanced expression of Giα proteins was restored to control levels by AG 1478, AG1295, PD98059 and wortmannin, inhibitors of epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptor (PDGFR-β), the mitogen-activated protein kinase (MEK1/2), and PI3 kinase respectively. In addition, treatment of VSMC with H_2O_2 also increased the phosphorylation of EGF-R, PDGF-R, ERK1/2 and AKT and this increased phosphorylation was attenuated to control levels by the respective inhibitors, whereas the inhibitors of EGF-R and PDGE-R also attenuated the enhanced phosphorylation of ERK1/2 and AKT to control levels. Transfection of cells with EGF-R and PDGFR-β siRNA followed by H_2O_2 treatment restored the H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins to control levels. The increased expression of Giα proteins by H_2O_2 was reflected in the increased Gi functions. The inhibition of forskolin (FSK)-stimulated AC activity by low concentration of GTPγS (receptor- independent Gi functions) was increased by about 20% by H_2O_2 treatment. Moreover, treatment of cells with H_2O_2 also resulted in an increased Ang II-, C-ANP_{4-23}, and oxotremorine-mediated inhibition of AC (receptor-dependent functions of Gi). On the other hand, Gsα-mediated stimulation of AC by GTPγS, glucagon, isoproterenol, FSK, and NaF was significantly decreased in H_2O_2-treated cells. These results suggest that H_2O_2 increases the expression of Giα-2 and Giα-3 proteins in VSMC through the transactivation of EGF-R, PDGFR-β and associated ERK1/2 and PI3K signalling pathways.

Keywords: Giα proteins, hydrogen peroxide, adenylyl cyclase, oxidative stress, MAP kinase, growth factor receptors, hypertension.

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List of abbreviations

AC	adenylyl cyclase
ACE	angiotensin converting enzyme
Ang II	Angiotensin II
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
CO	carbon monoxide
DAG	diacyl glycerol
DNA	deoxyribonucleic acid
DPI	diphenyleneiodonium
ERK1/2	extracellular regulated kinase 1 and 2
ET-1	endothelin-1
EGF	epidermal growth factor
FGF	fibroblast growth factor
FSK	forskolin
Gs	stimulatory G protein
Gi	inhibitory G protein
Gbr2-Sos	growth factor receptor-bound protein 2 – son of sevenless
GDP	guanosine diphosphate
GPCR	G protein-coupled receptor
GSH-Px	glutathione peroxydase
GTP	guanosine triphosphate
HO^-	hydroxyl anion
H_4B	tetrahydrobiopterin
H_2O_2	hydrogen peroxide
IP_3	Inositol-(1,4,5)-triphosphate

JNK	c-jun-N-terminal kinase
kDa	kilodalton(s)
MAPK	mitogen activated protein kinase
MEK	mitogen-activated extracellular-signal kinase
mm Hg	millimetres mercury
MMP	matrix metalloprotease
NAC	N-acetyl cystein
NADPH	nicotinamide adenine dinucleotide phosphate
NaF	sodium fluoride
NO	nitric oxide
$\cdot O_2^-$	superoxide anion
$ONOO^-$	peroxynitrite
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PI3K	phosphoinositide-3 kinases
PDGF	platelet-derived growth factor
PIP_2	phosphatidylinositol-4,5-biphosphate
PLCβ	phospholipase Cβ
Ptdlns-3,4,5-P_3	phosphatidylinositol-3,4,5-triphosphate
Ptdlns-3,4-P_2	phosphatidylinositol-3,4-bisphosphate
Ptdlns-3-P	phosphatidylinositol-3-phosphate
Ptdlns-4,5-P_2	phosphatidylinositol-4,5-diphosphate
PTPs	protein tyrosine phosphatases
Ras	rat sarcoma
R-COO·	peroxyl radical
ROS	reactive oxygen species

RTK	receptor tyrosine kinase
SHR	spontaneously hypertensive rats
SOD	superoxide dismutase
VPS34	vacuolar protein sorting human
VSMC	vascular smooth muscle cells
WKY rats	Wistar Kyoto rats

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Chapter 1 – Introduction

Literature review

1.1 The Adenylyl cyclase signal transduction system

The hormone-sensitive adenylyl cyclase system is comprised of three types of plasma membrane-associated components: proteins-coupled receptors for a variety of hormones and neurotransmitters; heterotrimeric G proteins; and a catalytic entity (Schramm & Selinger, 1984). The first component of the Adenylyl cyclase system interacts with ligands to convert extracellular signals into intracellular reactions. The nature of the extracellular message may include extracellular signal molecules, such as low-molecular weight messenger proteins, or sensory signals (Krauss, 2003). Adenylyl cyclase systems in mammalian cells are activated by prostaglandins, and a variety of peptides hormones (Rodbell, Lin, & Salomon, 1974). It has been shown that adrenocorticotropic hormones, glucagon (Pohl, Krans, Kozyreff, Birnbaumer, & Rodbell, 1971), and epinephrine (Schramm, Feinstein, Naim, Lang, & Lasser, 1972) bind reversibly to specific sites in target cell plasma membranes containing adenylyl cyclase systems responding to these hormones (Schramm & Selinger, 1984). Cells can only respond to an extracellular message if they express receptors that specifically recognize and bind that particular messenger molecule. A given cell contains receptors for many different ligands, so the response to one particular ligand may depends on the level of affinity of the other ligands with the cell's signal transduction machinery (Voet, Voet, & Pratt, 2008).

1.1.1 Signalling via transmembrane receptors

Transmembrane receptors are proteins that span the phospholipid bilayer of the cell membrane. These receptors include G protein coupled receptors (GPCRs), receptor protein-tyrosine kinases (RTKs), and ligand-gated channels (Karp, 2008). The signalling molecule binds on the extracellular side of the receptor, which is thereby activated. Transmission of the signal implies specific communication with effectors proteins, and conformational change of receptors. In this process, enzymatic activities can be triggered and/or the activated receptor interacts with downstream signalling proteins. Each protein in the signalling cascade acts by altering the conformation of the downstream protein, an event that activates or inhibits that protein. An intracellular chain is set in motion, whose outcome is to trigger a defined biochemical response of the target cell (Krauss, 2003).

1.1.1.1 G protein-coupled receptors as mechanism of signal transduction

GPCRs form the largest single family of transmembrane receptors that comprise the adenylyl cyclase signal transduction system. GPCRs transduce extracellular stimuli into intracellular signal through interaction of their intracellular domains with heterotrimeric G protein (Wheatley, et al., 2007). The first GPCR to be structurally characterized at the atomic level was rhodopsin, a light-sensing protein in the retina. The mammalian genome is estimated to contain more than 1000 different GPCRs. The importance of these receptors is also evident in the fact that more than 50% of therapeutic drugs target specific GPCRs. The GPCRs include the glucagon receptor, the β-adrenergic receptor (to which epinephrine binds), and other proteins that bind peptide hormones, odorants, and biogenic amines (Voet, et al., 2008).

A characteristic structural feature of GPCRs is the presence of 7 transmembrane helices. The transmembrane helices of GPCRs are generally uniform in size: 20 to 27 residues which span the lipid bilayer. However, their N and C-terminal segments and the loops connecting their transmembrane helices vary in length (Krauss, 2003). There are three loops present on the outside of the cell, which together form a ligand biding site. There are also three loops present on the cytoplasmic side of the plasma membrane that provide biding sites for intracellular signalling proteins. GPCRs and other cell-surface receptors function much like allosteric proteins such as hemoglobin by alternating between the active and inactive conformation. The inactive conformation is stabilized by non-covalent interactions between the transmembrane α helices. Ligand biding disturbs these interactions, thus causing the receptor to assume an active conformation which is translated by a rotation or movement of the transmembrane α helices relative to each other and a change in the conformation of the cytoplasmic loops. This in turn leads to an increase in the affinity of the receptor for a G protein present on the cytoplasmic surface of the plasma membrane, and a conformational change in the α subunit of the G protein with subsequent release of guanosine diphosphate (GDP) followed by the binding of guanosine-5'-triphosphate (GTP) (Karp, 2008). One characteristic of GPCRs is that they adapt to long-term stimuli by reducing their response to them, a process named desensitization. In the case of the β-adrenergic receptor, continuous exposure to epinephrine leads to the phosphorylation of one or more of the receptor's Ser residues. The phosphorylation decreases the GPCRs affinity for epinephrine, which leads to reduced levels of epinephrine and dephosphorylation of the receptor, thereby restoring the cell's epinephrine sensitivity (Voet, et al., 2008).

1.1.2 Structure of guanine nucleotide binding proteins

Heterotrimeric G proteins were discovered, purified and characterized by Martin Rodbell, and Alfred Gilman and colleagues. The discovery confirmed the hypothesis of Rodell and his colleagues that a regulatory element is interposed between hormone receptors that control adenylyl cyclase activity and the enzyme itself. These proteins are referred to as G proteins because they bind guanine nucleotides, either GDP or GTP (Fleming, Wisler, & Watanabe, 1992). Heterotrimeric G proteins as their name implies, consist of α, β, and γ subunits. At least 20 different genes for α-subunits, 5 for β-subunits, and 12 for γ-subunits are known in mammals. The diversity of the heterotrimeric G proteins is mainly a function of their α-subunits. The α subunit confers functional specificity upon a G protein it binds, and also facilitates discrimination between different receptor and effectors molecules (Zolk, Kouchi, Schnabel, & Bohm, 2000). Most animal cells express at least 10 of the 20 α-subunit gene products. The large α-subunit of 39-46 kDa designated as Gα contains the nucleotide biding site and it forms contact with one side of the β-subunit and carries the GTPase activity. The whole assembly is anchored to the membrane by hydrophobic interactions, one at the N-terminal of the α-subunit and the other at the C-terminal of the γ-subunit. The β-subunit is tightly associated with the γ-subunit with such high affinity that they only dissociate under denaturing conditions, and together they behave as a single entity, the βγ-subunit (Gomperts, Tatham, & Kramer, 2002).

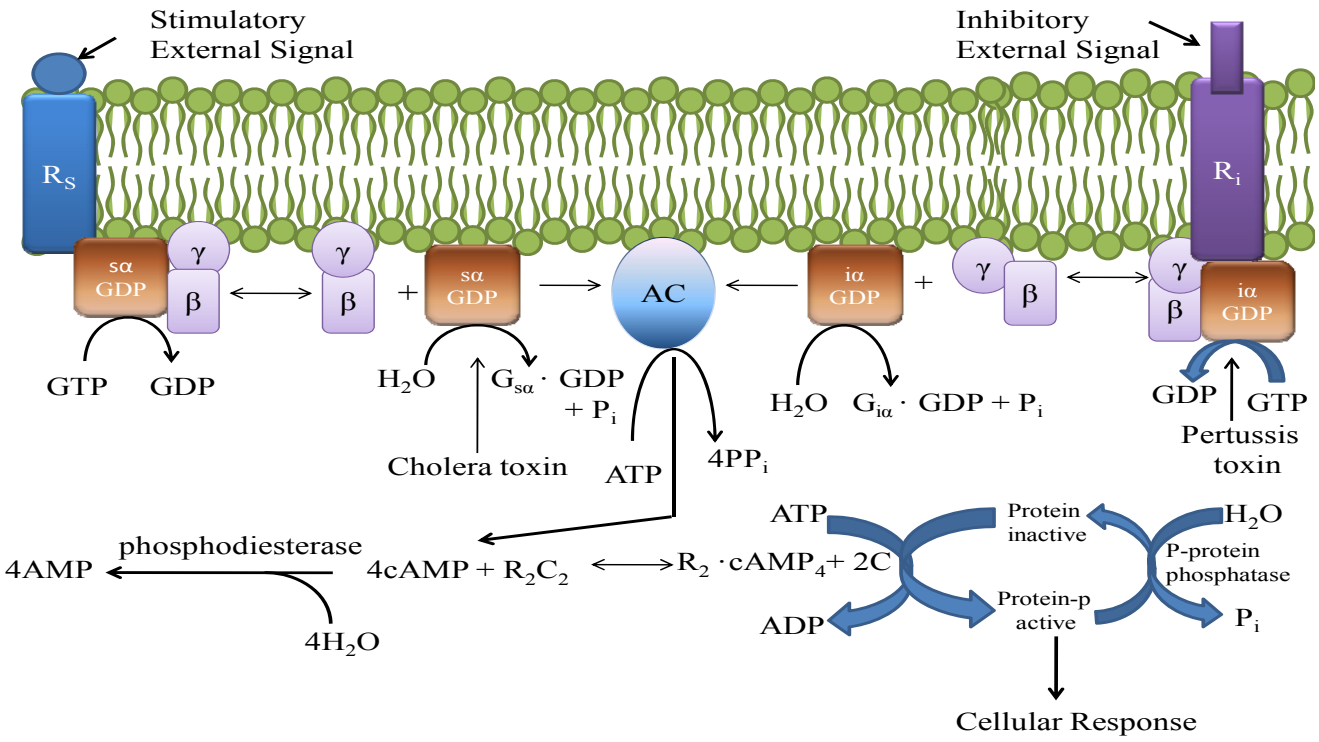

Figure 1. The adenylyl cycase signalling system.The binding of hormone to a stimulatory receptor Rs induces the binding of heterotrimeric G protein Gs, which in turn stimulates the $G_{sα}$ subunit to exchange its bound GDP for GTP. The $G_{sα}$·GTP complex then dissociates from $G_{βγ}$ and, until it catalyzes the hydrolysis of its bound GTP to GDP, stimulates adenylyl cyclase (AC) to convert ATP to cAMP. The binding of hormone to the inhibitory receptor R_i triggers the inhibition of adenylyl cyclase by $G_{iα}$·GTP. R_2C_2 represents protein kinase A (PKA) whose catalytic subunit C, when activated by the dissociation of the regulatory dimer R_2·$cAMP_4$, activates various cellular proteins by catalyzing their phosphorylation. Source: From Voet. D, Voet.j.G., and pratt, C.W.(2008). Fundamental of Biochemistry: Life at the Molecular Level. 2nd edition. Jonh wiley & Son, inc

1.1.2.1 Classification of Heterotrimeric G proteins

There are four classes of heterotrimeric G protein, including Gs, Gi, Gq and $G_{12}/_{13}$ (Table 1). This classification is based on the comparison of amino acid sequences of the Gα subunits. Molecular cloning has revealed four different forms of Gsα (Gsα-1,Gsα-2, Gsα-3, Gsα-4) resulting from the differential splicing of one gene and three distinct forms of Giα, Giα-1 Giα-2, and Giα-3 encoded by three distinct genes (Bray, et al., 1986). A characteristic of the α-subunits of Gs is that they are inhibited by cholera toxin. Giα is distinguished by the ability of *pertussis toxin* to inactivate the protein by transfer of an ADP-ribose moiety from NAD to the α-subunit of the Giα protein. Giα also has been shown to inhibit adenylyl cyclase and directly couple cell membrane receptors to ion channels (Seamon & Daly, 1982).

Table 1 : classification of heterotrimeric G protein according to subunits

Subunit	Tissue	Examples of Receptors	Effector protein, function
G_s			
α_s	ubiquitous	β-adrenergic receptors, glucagon receptors	↑ adenylyl cyclase ↑ type L Ca^{2+} channels
α_{olf}	nasal epithelium	olfactory receptor	↑ adenylyl cyclase
G_i			
α_{i-1}, α_{i-2}, α_{i-3}	Mostly ubiquitous, α_{i-1} absent in the vasculature	α_2-adrenergic receptor AT_1 receptor ET_B receptor	↓ adenylyl cyclase ↑ type T^*Ca^{2+} channels ↑ K^+ channels
G_q			
α_q α_{11}, α_{14}, α_{15}, α_{16}	ubiquitous	α_1-adrenergic receptor AT_1 receptor Endotheline receptor	↑ phospholipase Cβ
G_{12}			
α_{12}, α_{13}	ubiquitous		

Sources : Krauss, G. (2008). *Biochemistry of signal transduction and regulation*. (4e ed.). Weinheim; Chichester: Wiley-VCH .

*Lader, A. S., Xiao, Y. F., Ishikawa, Y., Cui, Y., Vatner, D. E., Vatner, S. F., et al. (1998). Cardiac Gsalpha overexpression enhances L-type calcium channels through an adenylyl cyclase independent pathway. *Proc Natl Acad Sci U S A, 95*(16), 9669-9674.

Gαq is a 42 kDa protein that exhibit slow rate of GDP-GTP exchange and GTP hydrolysis in comparison to other Gα-subunits. Gαq activates PLCβ, but is refractory to ADP-ribosylation by cholera toxin or *pertussis toxin*. Go (G "other") was first discovered as a 39 kDa *pertussis toxin* substrate in addition to Gi in the brain. Go is very similar to Gi, binds GTP, and has been shown to regulate muscarinic receptors for agonists in the brain (Fleming, et al., 1992).

1.1.2.2 Activation of guanine nucleotide proteins

In its unactivated state, a G protein maintains its heterotrimeric state and its Gα subunit binds GDP. The G protein is turned on by the interaction with an activated receptor (GPCRs) which induces the Gα subunit to exchange its bound GDP for GTP, followed by a dissociation of Gα-Gβγ complex (Fleming, et al., 1992). Subsequently, the Gα subunit dissociates from the Gβγ and binds to an effector such as adenylyl cyclase, activating the effector. The Gβγ dimer also binds to effectors such as ions channels, activate phospholipase Cβ, (PLCβ), and phospholipase A. Following hydrolysis of GTP, the Gα subunit will dissociate from the effector and reassociate with the Gβγ subunit to reform the inactive heterotrimeric G protein (Karp, 2008). The enzyme adenylyl cyclase is the catalytic unit of the hormone-sensitive adenylyl cyclase system (Voet, et al., 2008).

1.1.3 The enzyme adenylyl cyclase and its regulation

Many hormones and drugs interact with plasma membrane receptors to bring about the appropriate cellular response by generation of second messengers. The first such messenger to be identified was adenosine-3',5' cyclic monophosphate (cAMP), discovered by Sutherland and Rall in the late 1950s (Sutherland & Rall, 1958). Cellular functions are responsive to changes in concentrations of cAMP and thus to changes in the activities of adenylyl cyclases, the enzyme that catalyze the synthesis of cAMP from ATP (Voet, et al., 2008).

1.1.3.1 Structural organisation of adenylyl cyclases

Mammalian adenylyl cyclases are expressed in all tissues, but at very low levels, approximately 0.01-0.001% of membrane protein. Molecular cloning has permitted the identification of several novel isoforms of mammalian adenylyl cyclase. Ten different isoforms of adenylyl cylase have been identified, which differ in their primary sequence, tissue distribution and regulation (Taussig & Gilman, 1995). All of the isoforms of adenylyl cyclase are activated by the α-subunit of Gs, and by the diterpene forskolin. Certain isoforms (Type I, III) are also activated by Ca^{2+}-calmodulin, while some (Type I, V, VI) are inhibited by the α-subunit of the three Gi proteins, and Ca^{2+} (type V and VI). Type I and II adenylyl cyclase also appear to have independent sites for interaction with the βγ-subunits of G protein. The type I enzyme is strongly inhibited, while

type II, and IV adenylyl cyclase are activated provided Gsα is also present (Sunahara, Dessauer, & Gilman, 1996).

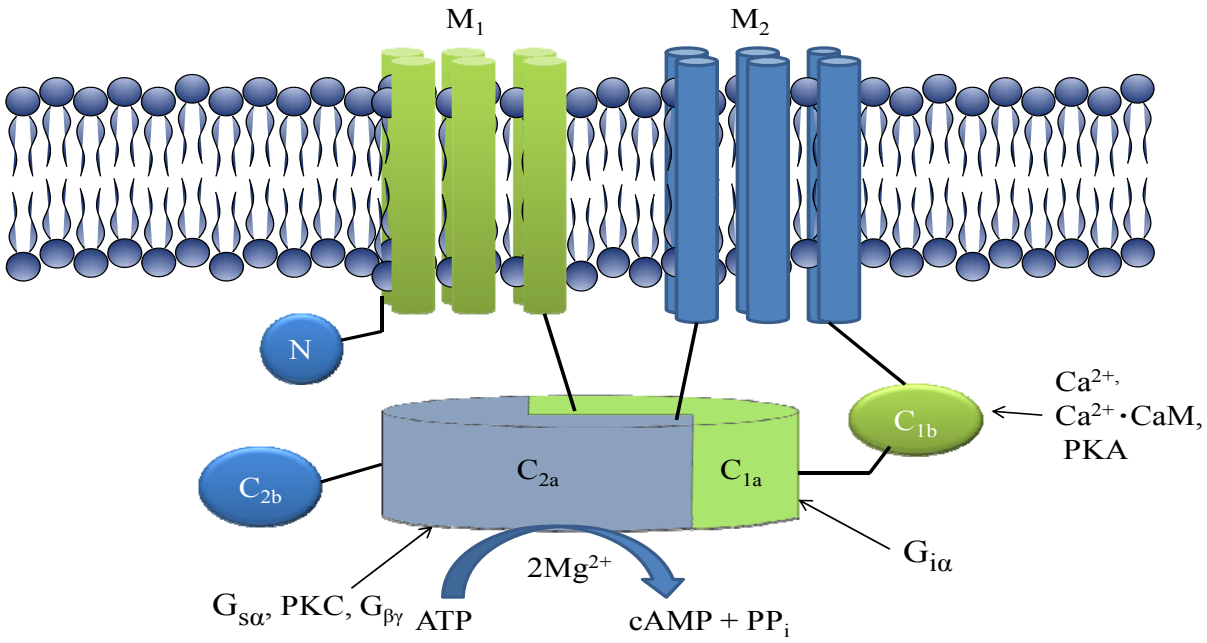

Figure 2: Diagram of a typical mammalian adenylyl cyclase.

The M_1 and M_2 domains each contain six transmembrane helices. C_{1a} and C_{2a} form the enzyme's pseudosymetric catalytic core. The domains with which various regulatory proteins are known to interact are indicated. Abreviations: PKA, protein kinase A; PKC, protein kinase C; CaM, calmodulin. Source: From Voet. D, Voet.j.G., and pratt, C.W.(2006). Fundamental of Biochemistry: Life at the Molecular Level.2nd edition. Jonh wiley & Son, inc.

Adenylyl cyclases are large transmembrane proteins that consist of two bundles of six transmembrane segments. These 120 kD membrane-bound protein each consist of a small N-terminal domain, along with two repeats of a unit consisting of a transmembrane domain (M) followed by two consecutive cytoplasmic domains (C). The interaction between the C_1 and C_2 domains is essential for catalysis. The C_{1a} and C_{2a} domains associate to form the enzyme's catalytic core, whereas C_{1b} as well as C_{1a} and C_{2a} bind regulatory molecules (Figure 2).The two catalytic domains of the enzyme and their interactions provide a binding site for ATP that activates adenylyl cyclase to transform ATP to cAMP. Forskolin and Gsα bind to C_{2a} to activate

adenylyl cyclase, and Giα binds to C_{1a} to inhibit the enzyme. Other regulators of adenylyl cyclase activity include Ca^{2+}, calmodulin, protein kinase A (PKA), and protein kinase C (PKC) (Voet, et al., 2008).

1.1.3.2 Regulation of adenylyl cyclase activity

The identification of several isoforms of adenylyl cyclase, along with the discovery of distinct, type-specific modes of regulation allow for the classification of regulatory patterns.

1.1.3.2.1 Regulation by G protein subunits

Regulation of adenylyl cyclase by Gsα was the basis for the discovery of G protein. A ligand activated GPCR triggers the exchange of Gsα-GDP for GTP. GTP-bound Gsα subsequently undergoes conformational changes that facilitate dissociation from the βγ-subunit of G protein. Such dissociation allows the α-subunit to interact with adenylyl cyclase. Hydrolysis of Gsα-bound GTP to GDP terminates activation of adenylyl cyclase by Gsα-GTP after several seconds, then Gsα-GDP reassociates with βγ-subunits (Taussig, Tang, Hepler, & Gilman, 1994). The binding of G proteins to the receptors exposes the enzyme binding sites for ATP, which then catalyzes the transformation of ATP into cAMP. G protein βγ subunits inhibit type I adenylyl cyclase, and the effect of βγ is exerted directly on the enzyme. The concentrations of βγ subunits required to inhibit adenylyl cyclase are significantly higher than the concentration of Gsα required to activate the enzyme. The source of βγ subunits is presumed to be from Gi or Go since only low concentrations of βγ subunits can be achieved by activation of Gsα, while substantially higher concentrations can be obtained by activation of Gi or Go. Furthermore, inhibition of type I adenylyl cyclase activity by Giα is absent when the Gsα-stimulated activity is examined (Taussig & Gilman, 1995). The first evidences that Giα proteins could in fact inhibit adenylyl cyclases came from studies of a cell transfected with cDNAs encoding constitutively activated (GTPase deficient) mutants of various Gα subunits. Expression of activated Gsα-1,Gsα-2, Gsα-3 impaired accumulation of cAMP stimulated by either Gsα or forskolin (Sunahara, et al., 1996).

1.1.3.2.2 Regulation by phosphorylation

Adenylyl cyclase catalyzes the synthesis of cAMP from ATP. Cyclic AMP binds to regulatory subunits of the cAMP-dependent protein kinase A (PKA), thereby activating the kinase to phosphorylate specific Ser or Thr residues on target proteins in the cytosol, and nucleus (Voet, et al., 2008). Potential regulation of adenyly cyclase by protein kinase C has been explored. Studies have shown that phosphorylation of the enzyme by protein kinase C enhances the activity of Type II, VII, and V (type VI is inhibited) adenylyl cyclase. This is a consequence of the activation of GPCRs through Gq, followed by activation of PLCβ and generation of diacyl glycerol (DAG) (Gomperts, et al., 2002).

1.1.3.2.3 Regulation by Calcium and Forskolin

Calcium is an important regulator of adenylyl cyclases. Changes in intracellular Ca^{2+} concentration also affect the concentration of cAMP. In association with calmodulin, Ca^{2+} increases the activity of type I, III and VIII adenylyl cyclases. Intracellular concentrations of cAMP rise when transfected cells expressing isoforms of the enzyme are exposed to agonists that elevate intracellular Ca^{2+} (Taussig & Gilman, 1995). Another activator of adenylyl cyclase is forskolin, a diterpene isolated from the roots of *Coleus forskohlii*. Forskolin directly activates adenylyl cyclase, bypassing all upstream influences including receptors and GTP binding proteins. Forskolin also potentiates Gsα-mediated activation of adenylyl cyclase (Gomperts, et al., 2002).

1.2 The Adenylyl cyclase system in cardiovascular diseases

The Adenylyl cyclase/cAMP signal transduction system is one of the biochemical mechanisms that regulate arterial tone and reactivity. Hypertensive hormones such as epinephrine, isoproterenol increase cAMP formation in rat aorta through their stimulatory effect on adenylyl cyclase (Asano, Masuzawa, & Matsuda, 1988). Studies have shown that changes in cardiac contractility and myocardial metabolism that occur in heart diseases involved alteration of the membrane-bound adenylyl cyclase system (Baumann, et al., 1981). In heart failure and hypertension, several signal transduction defects leading to adenylyl cyclase desensitization have been demonstrated, such as β-adrenoceptor downregulation, increase of Giα proteins expression, and uncoupling of β-adrenergic receptors by an increase of receptor kinase activity (Castellano &

Bohm, 1997). Studies have provided evidence that the increase of Giα and desensitization of adenylyl cyclase are common features of cardiac hypertrophy in polygenic, monogenic, and acquired types of hypertension (Michel, Brodde, & Insel, 1993)

1.2.1 Blood Pressure

The hydrostatic force that blood exerts against the wall of a vessel and that propels blood is called blood pressure. Blood pressure is much greater in arteries than in veins and is highest in arteries when the heart contracts during ventricular systole (Campbell & Reece, 2005). The maximum pressure exerted in the arteries when blood is ejected into them during systole averages 120 mmHg in humans. The minimum pressure within the arteries when blood is drained off into the remainders of the vessels during diastole averages 80 mm Hg. In human, blood pressure is determined partly by cardiac output and partly by peripheral resistance. Contraction of smooth muscles in the walls of the arterioles constricts the tiny vessels, increases peripheral resistance, and therefore increases blood pressure upstream in the arteries. When the smooth muscles relax, the arterioles dilate, blood flow through the arterioles increases, and the pressure in the arteries falls (Guyton & Hall, 2006). Nerve impulses and hormones control arterioles wall muscles. Stress, both physical and emotional, can raise blood pressure by triggering nervous and hormonal responses that will constrict blood vessels. Cardiac output is adjusted in concert with changes in peripheral resistance. This coordination of regulatory mechanisms maintains adequate blood flow as the demand on the circulatory system changes (Sherwood, Klandorf, & Yancey, 2005).

1.2.2. Mechanisms of blood pressure regulation

Development and maintenance of a level of arterial blood pressure adequate to perfuse the tissues is required for the survival of all mammals. Rapid alterations of arterial blood pressure are stabilized by neural reflex and hormonal mechanisms. The nervous system detects changes in arterial pressure and provides both rapid stabilization and long term control of blood pressure by adjusting level of sympathetic tone (Cowley, 1992). The short and long-term regulation of arterial blood pressure is achieved by the interaction of the baroreceptors reflexes, the renin-angiotensin system and the sympathetic nervous system (Reid, 1992).

1.2.2.1. The Renin-Angiotensin system in the regulation of blood pressure

The renin-angiotensin system plays an important role in the regulation of renal sodium and water excretion, and thus in maintaining body sodium and fluid balance. All components of the renin-angiotensin system are present in the kidney. Renin is secreted by the juxtaglomerular cells in the form of prorenin after proteolytic removal of 43-amino acid residue at the N-terminus of prorenin. The active form of renin contains 339-343 amino acid residues, with a mass of 37 kDa. Renin provides a pathway for the secretion of Angiotensin I which is rapidly converted into Angiotensin II (Ang II) by angiotensin converting enzyme (ACE). Ang II, a peptide hormone, is the primary product of the renin-angiotensin system. The main effects of Ang II are control of cardiovascular, renal and adrenal functions (Carey, 2007). The kidneys secrete the hormone rennin in response to reduced sodium chloride, extracellular fluid volume, and arterial pressure. Renin then activates angiotensinogen, a plasma protein produced by the liver, into angiotensin I, which is converted into AngII. Ang II stimulates the adrenal cortex to secrete aldosterone, which stimulates sodium reabsorption by the kidneys. The increased sodium reabsorption by the distal portion of the tubule induces water retention, which helps restore the plasma volume, thus being important in the long-term control of blood pressure (Sherwood, et al., 2005).

1.2.3 Hypertension

Hypertension is a cardiovascular condition characterized by sustained high blood pressure. A mean arterial blood pressure greater than 110 mm Hg under resting condition is considered to be hypertensive; at that level, the diastolic blood pressure is greater than 90 mm Hg and the systolic greater than 135 to 140 mm (Guyton & Hall, 2000). Hypertension is generally classified as either primary (essential), or secondary hypertension. Essential hypertension or hypertension of unknown causes, accounts for more than 90% of cases of hypertension. Many factors have been implicated in the genesis of essential hypertension: overproduction of sodium-retaining hormones and vasoconstriction, long-term high sodium intake, sedentary lifestyle, and inappropriate renin secretion.

It is estimated that 43 million people in the United States have hypertension or are taking hypertensive medication, which represents 24% of the adult population (Carretero & Oparil, 2000). According to the Canadian hypertension society, hypertension is a substantial health concern in Canada, affecting over five millions people (Canadian Hypertension Society, 2009).

The incidence of hypertension increases with age. Its prevalence is approximately 50% in the population aged 60 to 69 years, and increases to 70% in those older than 70 years (Suri & Qureshi, 2006) Several studies have reported the impact of a variety of factors including age, race, gender, and body mass index on hypertension. Hypertension is a major risk factor for myocardial infarction, stroke, congestive heart failure, and renal diseases (Mulvany, 1991). Despite major research efforts, it remains uncertain what triggers hypertension in the general population. In most cases, genetic as well as environmental factors are responsible for the pathogenesis of hypertension (Lifton, Gharavi, & Geller, 2001). Studies have shown that mutations of a family of protein kinases genes WNK1, and WNK4 (With-no-Lysine Ks) cause Gordon's syndrome, a rare Mendelian form of hypertension by increasing renal sodium retention (Huang, Kuo, & Toto, 2008). During hypertension, small arteries undergo functional and structural changes, resulting in reduced lumen size and increase peripheral resistance (Lehoux & Tedgui, 1998).

1.2.3.1 Physiology of hypertension on vascular smooth muscle cells

During hypertension, the vascular wall is constantly subjected to mechanical forces in the form of stretch or tensile stress due to blood pressure, and shear stress due to blood flow. Shear stress is principally sensed by endothelial cells located at the interface between the blood and the vessel wall. Functional changes in either shear stress or stretch result in vascular remodelling. The processes involved in vascular remodelling include cellular hypertrophy and hyperplasia, as well as enhanced protein synthesis or extracellular matrix protein reorganization (Lehoux & Tedgui, 1998). The primary hemodynamic characteristic of essential hypertension is increased peripheral vascular resistance which is associated with structural and functional alterations of the vasculature (Korner, Bobik, Angus, Adams, & Friberg, 1989). The structural changes include reduced vessel lumen diameter and media thickening. At the cellular level, there are hyperplasia, hypertrophy, and elongation of VSMC resulting in a smaller lumen and outer diameter (Korsgaard, Aalkjaer, Heagerty, Izzard, & Mulvany, 1993; Mulvany, Baandrup, & Gundersen, 1985). Many factors regulate VSMC function, including vasoactive peptides, such as Ang II and endothelin-1 (ET-1) that stimulate vasoconstriction and growth, and vasorelaxing factors, such as nitric oxide, prostacyclin, and C-type natriuretic peptide that induce vasodilation (Rubanyi, 1991). Vascular smooth muscle cells (VSMC) are central to these events. VSMCs detect

mechanicals stimuli resulting from pulsatile stretch and transduce them into intracellular signals leading to modulation of gene expression and cellular functions such as proliferation, apoptosis, migration, and remodelling (Haga, Li, & Chien, 2007).

Using neonatal rat aortic VSMCs, Wilson and colleagues have shown that cyclical mechanical strain induces the production of platelet-derived growth factor (PDGF) αα and ββ, and that the strain-induced growth of VSMC was dependent on the autocrine action of PDGF (Wilson, Mai, Sudhir, Weiss, & Ives, 1993). Stretching of VSMCs induces a rapid phosphorylation of PDGF receptor α independent of its ligand (PDGF) activation (Hu, Bock, Wick, & Xu, 1998). Stretch also induces the phosphorylation of epidermal growth factor receptor (EGFR) and its recruitment of adaptor proteins Shc and Grb2 (growth factor receptor-bound2), which in turn activate extracellular regulated kinases (ERK1/2) (Iwasaki, Eguchi, Ueno, Marumo, & Hirata, 2000). Several stretch-induced protein kinase molecules have been identified in VSMCs, including, Phosphoinositide-3 kinases (PI3Ks), protein kinase C, nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), Rho family GTPases, and mitogen activated protein kinase (MAPKs). Stretching of VSMCs induces PI3K and PKB/AKT activation which can be inhibited by pre-treatment of VSMCs with N-acetylcysteine, a scavenger of reactive oxygen species (ROS) suggesting the role of ROS in the mechanotransduction of VSMCs (Zhou, et al., 2003).

1.2.3.2 Alteration of G proteins expression in hypertension

Heterotrimeric guanine nucleotide proteins have been shown to be implicated in various pathological conditions including hypertension, diabetes, and heart failures. Alteration of Giα proteins levels and adenylyl cyclase activity have been reported in cardiovascular tissus from spontaneously hypertensive rats (SHR) and various model of hypertension including deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Marcil, de Champlain, & Anand-Srivastava, 1998). An increased expression of Giα-1, and Giα-2 protein and mRNA in hearts and aortas from SHR, as well as in hearts from DOCA-salt hypertensive rats with established hypertension has been demonstrated (Marcil, et al., 1998). The enhanced expression of Giα protein was shown to occur before the onset of hypertension in SHR and DOCA-salt suggesting the implication of increased expression of Giα protein in hypertension (Marcil, Thibault, & Anand-Srivastava, 1997). Our laboratory has shown that overexpression of Giα-2 and Giα-3 proteins precedes the development of hypertension, and that inactivation of the enhanced Giα

proteins expression by *pertussis toxin* attenuates the development of high blood pressure in SHR (Li & Anand-Srivastava, 2002). Furthermore, we have reported that volume overload cardiac hypertrophy exhibits decreased expression of Gsα and not of Giα in hearts of Sprague-Dawley rats (Di Fusco, Hashim, & Anand-Srivastava, 2000). TsuTsui et al. have reported impaired levels of endothelial Giα proteins in atherosclerotic coronary arteries (Tsutsui, et al., 1994). Reduced function of Gsα in β adrenoreceptor-adenylyl cyclase system of femoral arteries isolated from SHR has also been reported (Asano, Masuzawa, Matsuda, & Asano, 1988).

1.2.3.3 Alteration of adenylyl cyclase/cAMP activity in hypertension

The elevation of blood pressure in essential hypertension is due to an increase in the peripheral resistance of vessels. The increase of the peripheral resistance is attributed to structural changes in the vessels, abnormalities in Ca^{2+} movement, and aberration in cyclic nucleotides metabolism (Anand-Srivastava, 1996). It has been shown that the adenylyl cyclase/cAMP system is one the biochemical mechanism participating in the regulation of arterial tone (Triner, Vulliemoz, Verosky, Habif, & Nahas, 1972). Decreased cAMP level in cardiovascular tissues have been implicated in the pathogenesis of hypertension (Ramanathan & Shibata, 1974). The adenylyl cyclase/cAMP system is involved in both the control of heart contractility, and vascular smooth muscle tone (Bar, 1974). Reduced adenylyl cyclase activity in response to β-adrenergic stimulation has been demonstrated in mesenteric vasculature (Amer, Gomoll, Perhach, Ferguson, & McKinney, 1974), aorta and myocardium of spontaneously SHR (Anand-Srivastava, 1988). Bohm and colleagues have reported the positive inotropic effect of cAMP-phosphodiesterase inhibitor pimobendan in failing human myocardium, and the role played by other factors such as increased Ca^{2+} sensitivity of myofilaments in the increase in force of contraction (Bohm, et al., 1991). In addition to the effect of PKA on myosin light chain kinase (MLCK), $G_{12/13}$ proteins also play a key role in the regulation of vascular tone (Siehler, 2009). Activation of $G_{12/13}$ by ligands such as Ang II, and endothelin activates the guanine nucleotide exchange protein pp115-RhoGEF, which in turn catalyzes the exchange of bound GDP for GTP on the small GTPase RhoA. Downstream target of RhoA-GTP include ROCK, which mediates cell contraction through the inhibition of myosin light chain phosphatase (MLCP) (Sward, et al., 2003).

1.3 Oxidative stress, redox signalling and cardiovascular diseases

Oxidative stress is caused by the overproduction of reactive oxygen species (ROS). An imbalance between oxidants and antioxidants in favour of the oxidants leads to oxidative stress. Oxidants are produced as normal products of aerobic metabolism, but can also be formed at higher rates under pathological conditions. Antioxidants include enzymatic and non-enzymatic molecules capable of inhibiting the oxidation of other proteins or molecules thereby maintaining them in their reduced state (Baynes, 1991). Antioxidants include enzymes such as catalase, superoxide dismutase (SOD), glutathione peroxidase, and molecules such as tocopherols (Vitamine E), glutathione, ascorbic acid (Vitamine C), and flavonoids (Ames, Shigenaga, & Hagen, 1993). Reactive oxygen species (ROS) are highly reactive oxygen-containing molecules due to the presence of unpaired valence shell electrons. Reduction-oxidation (redox) reactions that generate ROS are important chemical processes that regulate signal transduction (Thannickal & Fanburg, 2000).

1.3.1 Sources and chemistry of reactive oxygen species

ROS include both free radicals, which have oxygen or nitrogen-based unpaired electrons, and other species such as hydrogen peroxide (H_2O_2) that act as oxidants. A primary form of reactive oxygen species (ROS) is superoxide anion (O_2^-) but biological systems produce other species including hydroxyl anion (HO^-), peroxynitrite ($ONOO^-$) and hydrogen peroxide (H_2O_2). In the vasculature, $\cdot O_2^-$ anion is produced by a one electron reduction of oxygen using Nicotinamide adenine dinucleotide phosphate (NAD(P)H) as an electron donor (Taniyama & Griendling, 2003). Superoxide has an unpaired electron, which conferts high reactivity and renders the molecule unstable. Hydrogen peroxide is mainly produced from dismutation of $\cdot O_2^-$. This reaction can be spontaneous or catalyzed by SOD, of which there are three isoforms, CuZnSOD, MnSOD, and extracellular SOD (EC-SOD) (Fridovich, 1997).

The SOD-catalyzed dismutation is favoured when the concentration of O_2^- is low and the concentration of SOD high. Unlike $\cdot O_2^-$, H_2O_2 is not a free radical and is a much more stable molecule. It is lipid soluble, crosses cell membrane, and has a longer half-life than $\cdot O_2^-$ (Han, Antunes, Canali, Rettori, & Cadenas, 2003). In biological system, H_2O_2 is scavenged by catalase and glutathione peroxidase to produce H_2O molecules (Schafer & Buettner, 2001). Hydrogen peroxide can also generate the highly reactive hydroxyl radical HO^- in the presence of metal

containing-molecules such as Fe^{2+} (Fridovich, 1997). When $\cdot O_2^-$ is produced in excess, a significant amount of $\cdot O_2^-$ reacts with oxide nitrate (NO) to produce $ONOO^-$. In the vasculature, $\cdot O_2^-$, H_2O_2, $ONOO^-$, NO, and HO^- are produced to varying degrees. Their production is regulated by anti-oxidant enzymes such as catalase, SOD, thioredoxin, glutathione (Thannickal & Fanburg, 2000).

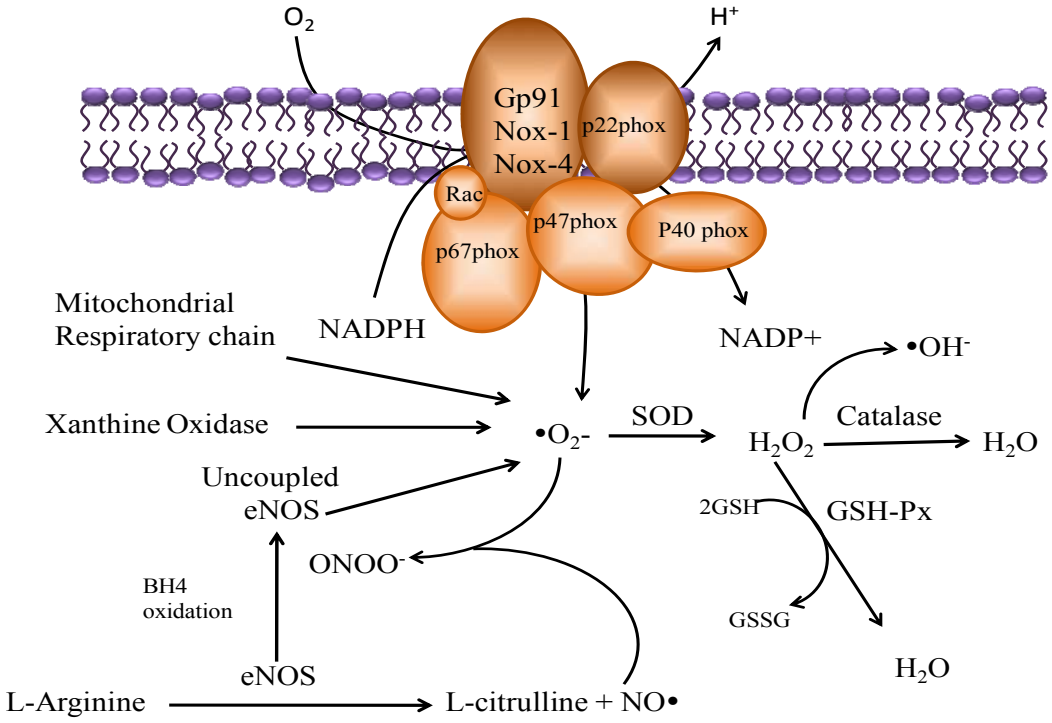

Figure 3. Generation of $\cdot O_2^-$ and H_2O_2 from NAD(P)H oxidase. Many enzyme systems, including NAD(P)H oxidase, xanthine oxidase, and uncoupled nitric oxide synthase (NOS) have the potential to generate reactive oxygen species. NAD(P)H oxidase is a multisubunit enzyme, comprising gp91phox(or its homologues, Nox1 and Nox4), p22phox, p47phox, p67phox, and p40phox. Abreviations: SOD, superoxide dismutase; GSH, glutathione ; GSH-PX, glutathione peroxydase ; GSSG, glutathione disulfide ; H_2O_2, hydrogen peroxide ; Mox-1, mitogen oxydase 1 ; $NAD(P)^+$, nicotinamide-adenine-dinucleotide ; NAD(P)H, nicotinamide-adenine-dinucleotide-phosphate ; NAD(P)H oxidase, nicotinamide-adenine-dinucleotide-phosphate oxidase ; •NO-, nitric oxide ; $\bullet O_2$-, superoxide radical ; •OH-, hydroxyl radical ; $ONOO^-$, peroxynitrite ; Rac, small G protein. Source. Tamara, M.P., Touyz, R.M. Redox signaling in hypertesion. (2006). Cardiovascular research.71 :247-258.

Under normal conditions, the rate of ROS production is balanced by the rate of elimination. Oxidative stress is the result of the imbalance between the production of ROS and the cellular

anti-oxidant capability of biological system. In biological system, ROS have enzymatic sources and non enzymatic sources multiple sources (Touyz & Schiffrin, 2004).

1.3.1.1 Enzymatic sources of reactive oxygen species

Cellular production of ROS occurs from both enzymatic and nonenzymatic sources. Any electron-transferring protein or enzymatic system can result in the formation of ROS as by-products of electron transfer reactions. The generation of ROS in the mitochondria accounts for 1-2% O_2^-, of total production under reducing conditions (Thannickal & Fanburg, 2000). In the vasculature, several enzyme systems contribute to ROS formation, including the NAD(P)H oxidases, xanthine oxidase, endothelial NO synthases, enzymes of the mitochondrial respiratory chain, lipoxygenases, cytochrome P450 monoxygenases, and cyclooxygenases (Clempus & Griendling, 2006). Mitochondria generate ROS as byproducts during ATP production via electron transfer through cytochrome c oxidases. NAD(P)H oxidase is one of the major sources of ROS in the vasculature. Xanthine oxidoreductase catalyzes the oxidation of hypoxanthine into xanthine in the process of purine metabolism. Xanthine oxidoreductase exists in two interconvertible forms, either as xanthine dehydrogenase or xanthine oxidase. The former reduces NAD+, whereas the latter reduces molecular oxygen leading to the production of $\cdot O_2^-$ and H_2O_2 (Touyz, 2004).

Nitric oxide synthase (NOS) generates $\cdot O_2^-$ in addition to NO. Members of NOS are encoded by different genes. There are three isoforms including endothelia NOS (eNOSor NOS3), neuronal NOS (nNOS or NOS1) and inducible NOS (iNOS, or NOS1) (Gilkeson, et al., 1997). NOS uses L-arginine as a substrate to synthesize NO in a tetrahydrobiopterin (H_4B)-dependent manner. If the concentration of L-arginine or H_4B is low, or if H_4B is oxidized, eNOS becomes uncoupled and generates significant amounts of $\cdot O_2^-$ (Stuehr, Pou, & Rosen, 2001). eNOS uncoupling has been demonstrated in atherosclerosis, diabetes, and hypertension, all of which are associated with activation of the renin-angiotensin system and production of O_2^- from eNOS (Taniyama & Griendling, 2003).

1.3.1.2 Non enzymatic sources of reactive oxygen species

Autooxidation of small molecules such as dopamine, epinephrine, flavins and hydroquinones can be an important source of intracellular ROS production. In most cases, the direct product of such

autooxidation reactions is O_2^- (Freeman & Crapo, 1982). Peroxisomes are an important source of total cellular H_2O_2 production. They contain a number of H_2O_2-generating enzymes including glycolate oxidase, acid oxidase, urate oxidase, and fatty acyl-CoA oxidase (Thannickal & Fanburg, 2000). The cellular production of ROS may trigger the production of more ROS via a radical chain reaction. The reaction between ROS and polyunsaturated fatty acids within cell membrane may result in a fatty acid peroxyl radical ($R\text{-}COO^-$) that will react with adjacent fatty acid side chains and initiate production of more lipid radicals (Zalba, et al., 2001). Arachidonic acid metabolism involving lipoxygenase and cyclooxygenase-dependent pathway leading to leukotriene synthesis has been reported to generate ROS. Lipoxygenase activity also has been implicated in redox-regulated signalling by Ang II, and EGF (Thannickal & Fanburg, 2000).

1.3.2 The NADPH oxidases

The NADPH oxidases are enzymes that catalyze the production of superoxide from oxygen and NADPH. They are present in vascular tissue and phagocytic cells such as neutrophils, macrophages, and eosinophils (Griendling, Sorescu, & Ushio-Fukai, 2000).

1.3.2.1 Structure and expression profile of vascular NADPH oxidases

The vascular NAD(P)H oxidase is a multimeric protein complex responsible for the formation of $\cdot O_2^-$. In vascular smooth muscle cells (VSMC), O_2^- and H_2O_2 production are mainly intracellular (Griendling, Minieri, Ollerenshaw, & Alexander, 1994).The vascular NAD(P)H consists of four major subunits: a cytochrome b558, comprising of two cell membrane-associated gp91phox (or gp91phox (nox2) homologues, nox1 and nox4) and p22phox, and two cytosolic components, p47phox and p67phox. A low molecular weight G protein rac participates in subunits assembly and activation of the enzyme (Griendling, Sorescu, Lassegue, & Ushio-Fukai, 2000). The rac proteins are kept inactive by binding to a guanine nucleotide dissociation inhibitor, which prevents the exchange of guanine nucleotides from the rac proteins. An essential component of the NAD(P)H oxidase is gp91phox to which are bound the electrons carrying components of oxidase such as flavine adenine dinucleotide, and a pair of hemes molecules. P47phox is the protein that carries the cytosolic proteins to the membrane proteins to assemble the active oxidase. p67phox contains two Src homology 3(SH3) domains, one in the middle of the protein, and one near the carboxyl terminus. The SH3 domains interact with p22phox to activate the

enzyme. p22phox is located in the membrane, along with gp91phox and has a tail in the cytosol. When p22phox is phosphorylated, it binds to p47phox, an interaction that is critical in the activation of the enzyme (Brandes & Kreuzer, 2005). The distribution of catalytic subunits of NAD(P)H oxidase in VSMC is tissue and species-specific. Aortic smooth muscle cells express Nox1 and Nox4 in rodents, and also Nox5 in humans. In contrast, VSMC from human resistance arteries contain Nox2 and Nox4, but no Nox1 (Clempus & Griendling, 2006). Vascular NAD(P)H oxidase is a constitutive enzyme, but it is regulated by Ang II, platelet derived growth factors (PDGF), thrombin, and tumor growth factor-α (Zalba, et al., 2001).

1.3.3 Oxidative stress in hypertension

The impact of ROS in vascular functions and the development of hypertension has been studied extensively. It has been shown that $\cdot O_2^-$ inactivates endothelium-derived NO, one of the most important vasodilator, thereby promoting vasoconstriction (Zicha, Dobesova, & Kunes, 2001). In order to counteract the hypertensive effect of ROS, several studies have used exogenous administration of antioxidants to reduce blood pressure in animal model (Boshtam, Rafiei, Sadeghi, & Sarraf-Zadegan, 2002), and in human hypertension (Boshtam, et al., 2002; Duffy, et al., 2001). Nevertheless, the results of such studies are not conclusive and the relationship between oxidative stress and hypertension is continuously being investigated.

1.3.3.1 Oxidative stress in genetic models of hypertension

Recent work by Suzuki and associates (1995) provided evidence regarding the role of ROS in the pathophysiology of hypertension. They demonstrated that O_2^- is increased in venules and arterioles of SHR, and the administration of heparin-binding SOD, which is localized within the vessel wall normalised the blood pressure of SHR (Suzuki, Swei, Zweifach, & Schmid-Schonbein, 1995). Genetic models of hypertension, such as SHR and stroke-prone SHR exhibit enhanced NAD(P)H oxidase-mediated $\cdot O_2^-$ generation in resistance arteries, aorta, and kidneys. These processes are associated with increase expression of NAD(P)H oxidase subunits particularly p22phox, and p47phox (Touyz & Schiffrin, 2004). We have shown an increase expression of NAD(P)H oxidase subunits Nox4, and p47phox in VSMC from SHR (Saha, Li, & Anand-Srivastava, 2008). Furthermore, Fukui et al demonstrated that chronic infusion of Ang II in normotensive rats upregulates vascular p22phox mRNA and increases NADPH oxidase-

derived superoxide. Both the hypertension and the increase in p22phox mRNA were prevented by pretreatment with SOD (Fukui, Lassegue, Kai, Alexander, & Griendling, 1995). Diminished NO bioavailability as a consequence of enhanced vascular $\cdot O_2^-$ generation also contribute to oxidative stress in SHR and stroke-prone SHR. Treatment of rats with antioxidant vitamins, NADPH inhibitors, AT_1 blockers, BH4, and SOD mimetics attenuate to varying degree the development of hypertension in SHR and stroke-prone SHR (Sharma, Hodis, Mack, Sevanian, & Kramsch, 1996).Vascular oxidative stress has also been demonstrated in DOCA-salt rats. An enhanced $\cdot O_2^-$ production present in the aorta of these rats is associated with an increased NAD(P)H oxidase activity due to the increased vascular Ang II release as a consequence of nephrectomy (Zalba, et al., 2001). NO is an important endogenous antihypertensive factor that plays a role in sodium excretion and the regulation of blood pressure. A dysfunction of NOS in tissues has been observed in Dahl salt-sensitive rats. In addition, genetic deletion of eNOS has been proven to lead to hypertension in mice. NOS inhibitor L-nitro-arginine methyl ester (L-NAME) leads to a decrease in blood flow, the retention of sodium, and the development of hypertension in rats (Nakanishi, Mattson, & Cowley, 1995).

1.3.3.2 Oxidative stress in human hypertension

Clinical studies have shown the occurrence of increased ROS production in humans with essential hypertension. The level of ROS scavengers, such as vitamine E, glutathione, and SOD, have been reported to be decreased in hypertensive patients (Sagar, Kallo, Kaul, Ganguly, & Sharma, 1992). Berry et al have shown that NAD(P)H oxidase is the source of basal$\cdot O_2^-$ production in human internal arteries and saphenous veins, and that Ang II increases $\cdot O_2^-$ in human arteries. This effect is mediated by NADPH oxidase and inhibited by the AT1 receptor antagonist losartan (Berry, et al., 2000). Activation of the renin-angiotensin system has been proposed as a major mediator of NAD(P)H oxidase activation and ROS production in human hypertension. Some of the therapeutic blood pressure-lowering effects of AT1 receptors blockers and ACE inhibitors have been attributed to inhibition of NADPH oxidase activity (Touyz & Schiffrin, 2004). Several reports have shown the role of Ang II in NADPH subunits expression and activation (Touyz, et al., 2003).

1.3.4 Vasoactive peptides as inducers of oxidative stress

Vasoactive peptides such as Ang II and endothelin-1 mediate part of their responses through ROS generation (Griendling & Ushio-Fukai, 2000; Li, Watts, et al., 2003). In conditions associated with vascular damage such as hypertension, these peptides increase ROS production by activating NADPH oxidase (Touyz, Yao, Viel, Amiri, & Schiffrin, 2004).

1.3.4.1 Oxidative stress and angiotensin II

Ang II, a key component of the renin-angiotensin system regulates blood pressure, plasma volume via aldosterone-regulated sodium excretion, sympathetic nervous activity, and also plays a role in vascular remodelling in hypertension (Touyz & Schiffrin, 2000). In mammalian cells, Ang II mediates its effects via at least two plasma membrane receptors, AT_1 and AT_2. These receptors belong to the 7-transmembrane, GPCR family (Horiuchi, Akishita, & Dzau, 1999). Ang II, also regulates a variety of physiological functions including cell growth, and apoptosis (Paul & Ganten, 1992). Ang II stimulates many signalling pathways leading to cell contraction and cellular hypertrophy. In VSMCs, Ang II induces cellular hypertrophy by acting via G protein coupled AT1 receptors (Berk, Vekshtein, Gordon, & Tsuda, 1989; Geisterfer, Peach, & Owens, 1988). In endothelial and VSMc, Ang II increases the production of $\cdot O_2^-$ via the activation of membrane-associated NAD(P)H oxidase. The $\cdot O_2^-$ production upon Ang II stimulation is rapidly converted to H_2O_2 by SOD (Munzel, Hink, Heitzer, & Meinertz, 1999).

Griendling et al have demonstrated that Ang II-induced cellular hypertrophy is mediated by intracellularly produced H_2O_2. The reduction of NAD(P)H oxidase activity by transfection of antisense p22phox inhibits both H_2O_2 production and hypertrophy. In addition, infusion of Ang II in rats upregulated vascular p22phox mRNA and increased NAD(P)H oxidase-derived $\cdot O_2^-$ (Griendling, et al., 1994). It has been reported that Ang II treatment of A10 VSMC increases the production of $\cdot O_2^-$ and the expression of Nox4 and $p47^{phox}$ proteins of NADPH oxidase (Saha, Li, & Anand-Srivastava, 2008). Furthermore, the Ang II-induced phosphorylation of ERK1/2 is due to enhanced oxidative stress, since treatment of A10 VSMC with Diphenyleneiodonium (DPI) an inhibitor of NAD(P)H oxidase attenuates the Ang II-induced phosphorylation of ERK1/2 (Li, Lappas, & Anand-Srivastava, 2007).

1.3.4.2 Oxidative stress and endothelin-1

Endothelin-1 (ET-1) is a 21-amino-acid polypeptide produced by vascular endothelial cells. ET-1 has been implicated in the pathophysiology of many cardiovascular diseases including hypertension, atherosclerosis and hypercholesterolemia (Barton, et al., 1998). In addition to its vasoactive effects, ET-1 also induce cell proliferation, tissue remodelling and cell survival in VSMCs and endothelials cells (Salani, et al., 2000; Ziche, Morbidelli, Donnini, & Ledda, 1995). ET-1 has been shown to exert its biological effects through binding to specific G protein-coupled membrane receptors ET_A, and ET_B subtypes (Pollock, 2005). It has been shown that ET-1 activates NAD(P)H oxidase and induces $\cdot O_2^-$ production in cultured endothelial and smooth muscle cells (Callera, Tostes, Yogi, Montezano, & Touyz, 2006). Although Ang II is a major stimulus of vascular production of O_2^- in high-angiotensin hypertension, studies have demonstrated that ET-1 plays a major role in increasing vascular $\cdot O_2^-$ in low-renin hypertension, such as DOCA-salt model, an effect that is partially mediated by the ET_A receptor/NAD(P)H oxidase pathway (Li, Fink, et al., 2003).

1.4 Oxidative stress and signalling

Reactive oxygen species can trigger the activation of several signalling pathways that influence cytotoxicity, cell proliferation, and apoptosis. ROS are responsible for the phosphorylation of a variety of proteins kinases and transcription factors (Wang, Martindale, Liu, & Holbrook, 1998).

1.4.1 Receptor tyrosine kinase as mechanism of signal transduction

Protein-tyrosine phosphorylation is a mechanism of signal transduction that appeared with the evolution of multicellular organisms. Over 90 differents protein-tyrosine kinases are encoded by the human genome. These kinases are involved in the regulation of growth, cell migration and differentiation, inflammation, and apoptosis. Protein-tyrosine kinases are divided in two groups: Receptor protein-tyrosine kinases (RTKs) which are integral membrane proteins with an extracellular ligand binding domain, and non-receptor or cytoplasmic protein tyrosine kinases (Karp, 2008). Insulin and many other growth factors such as EGF and PDGF do not act via GPCRs and cAMP dependent pathways. Instead, these hormones and growth factors bind to RTKs, whose C-terminal domains have tyrosine kinase activity. RTKs contain a single transmembrane segment and are monomers in the unliganded state. Ligand binding causes two

monomeric receptors to form a dimer, a process called dimerization. The insulin receptor is unusual in that it is a dimer in the unliganded state; therefore ligand biding does not induce conformational change in the receptor. When a RTK dimerizes, the cytoplasmic tyrosine kinase cross-phosphorylate each other on specific tyrosine residues. This autophosphorylation activates the tyrosine kinase so it can phosphorylate other substrates (Voet, et al., 2008).

1.4.2 Signalling pathways activated by Reactive oxygen species

ROS are involved in the regulation of many signal transduction pathways. They influence cellular processes associated with growth and inflammation. Exogenous H_2O_2 affects the function of various proteins, including protein kinases, protein phosphatases, transcription factors, phospholipases, ion channels, and G proteins (Rhee, Bae, Lee, & Kwon, 2000). ROS activates mitogen-activated protein (MAP) kinases, ERK1/2, p38MAP kinase, c-jun N-terminal kinase (cJNK) (Touyz & Schiffrin, 2004). Non-receptor tyrosine kinases such as c-Src, Pyk2, janus kinase (JAK2) are regulated by ROS. Reactive oxygen species modulate intracellular free Ca^{2+} concentration, an important determinant of vascular contraction and dilation. H_2O_2 increases Ca^{2+} in VSMC and endothelial cells. These effects are attributed to redox-dependent inositol triphosphate-induced Ca^{2+} mobilization which increases Ca^{2+} influx and decreases Ca^{2+}-ATPase activation (Lounsbury, Hu, & Ziegelstein, 2000). Exogenous H_2O_2 induces tyrosine phosphorylation and activation of PDGFR and EGFR, probably due to ROS-mediated inhibition of tyrosine phosphatases. Protein tyrosine phosphatases (PTP) are susceptible to oxidation and inactivation by ROS (figure 4) (Touyz & Schiffrin, 2004). Sue Goo Rhee et al, have shown that Growth factors-induced H_2O_2 production requires the activation of phosphatidyl inositide 3-kinase (PtdIns 3-kinase) which subsequently provide phosphatidylinositol-3,4,5-triphosphate that recruits and activates a guanine nucleotide exchange factor of Rac, which is required for the activation of NADPH oxidase (Rhee, Chang, Bae, Lee, & Kang, 2003).

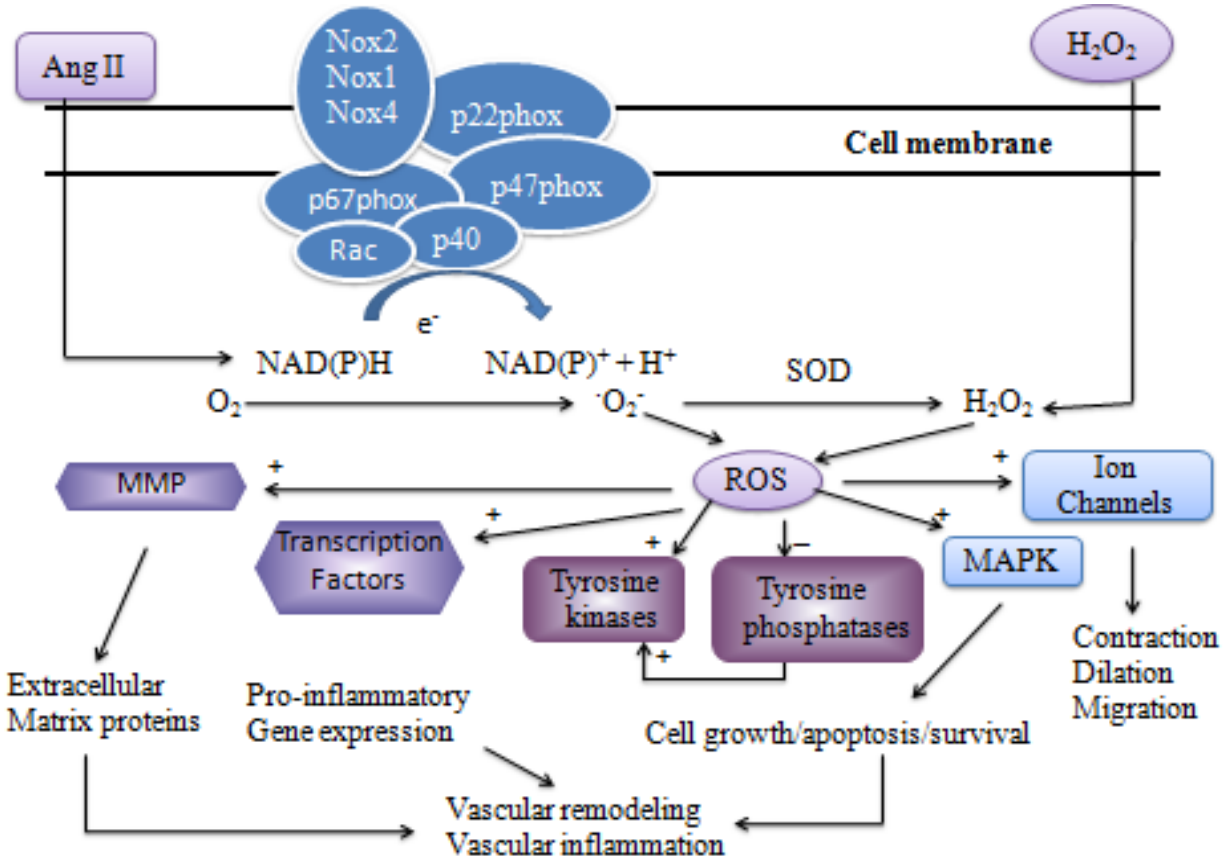

Figure 4. Redox-dependent signaling pathways by ROS in VSMC. Intracellular ROS activates redox-sensitive MAP kinases, tyrosine kinases, ions channels, and MMPs. Tyrosine phosphatases are negatively regulated by ROS, which further increases the activity of tyrosine kinase and MAP kinase. ROS also influence gene expression by activating transcription factors. Activation of these redox-sensitive pathways results in cellular responses including altered vascular tone, increased VSMC growth, inflammation, and increase deposition of extracellular matrix protein, leading to vascular remodelling, and hypertension. Abreviations: ROS, reactive oxygen species; AngII, angiotensin II; SOD, superoxide dismutase; MMP, matrix metalloproteinase; MAPK, mitogen activated protein kinase; +, stimulatory effect; -, inhibitory effect. Sources. Touyz M.R., (2005). Intracellular mechanisms involved in vascular remodelling of resistance arteries in hypertension: role of angiotensin II. Experimental Physiology.90:449-455. Touyz, R.M., Schiffrin, E.L. Reactive oxygen species in vascular biology: implications in hypertension.(2004).122:339-352.

1.4.3 Reactive oxygen species and Mitogen activated proteins kinases

Mitogen-activated protein kinases (MAPK) are serine/threonine kinases whose phosphorylation mediates nuclear transduction of extracellular signals leading to activation of transcription factors, and enhance gene expression. The mammalian MAPKs are grouped into six major

subfamilies, among which three are best characterized: the extracellular-regulated kinase1/2 (ERK1/2) also known as p44-kDa MAPK and p42-kDa MAPK respectively, the c-jun N-terminal protein kinase (c-JNK), and the p38 MAPK. This classification is based on the presence of different amino acids in their phosphorylation motif, Glu for ERK1/2, Pro for c-JNK, and Gly for p38 MAPK (Touyz & Berry, 2002).

1.4.3.1 The extracellular-regulated kinase (ERK) pathway

The extracellular-regulated kinase (ERK) signaling pathway was the first MAP kinase cascade to be characterized. The ERK pathway is involved in cell growth, proliferation and survival. There are several ERKs, the best characterized are: the 44-kDa MAPK (ERK1), the 42-kDa MAPK (ERK2), and the 63k-Da MAPK (ERK3).The 42-kDa MAPK was the first mammalian MAPK to be identified as a 42-kDa protein that increases its phosphorylation upon stimulation by mitogens, hence the name. It was later found that other stimuli such as growth factors, cytokines, and ligands for G protein linked receptors also activate p42MAPK (Torres, 2003). In the heart, ERK1 is the most highly expressed ERK. Induction of MAPK activation involves phosphorylation by a MAPK also known as MEK. MEKs (MEK1/2) are regulated by other MEK kinases, including Raf-1, and Mos. MEK1 and MEK2 function as upstream MAPKK and the Raf proteins as MAPKKK (figure4). MEKs activate MAPKs by dual phosphorylation on a tyrosine and threonine residue lying within the phosphorylation motif (TyrXThr) in the activation loop (Ruwhof & van der Laarse, 2000).

The ERK pathway can be stimulated upon G protein coupled receptor activation by hormones such as ET-1, Ang II, and through receptors protein-tyrosine kinases activation by growth factors. The ERK pathway can also be stimulated by non receptor protein tyrosine kinase such as c-Src (Chang & Karin, 2001). The ERK phosphorylation cascade is initiated by the binding of a hormone such as Ang II to AT-1 receptors, which induces Shc-Grb2-Sos formation (tyrosine phosphoryaltion of Src homology domain), and activation of Raf. The activation of Raf may involve PLC and PKC, which is independent of tyrosine kinase and Ras (Kolch, et al., 1993).

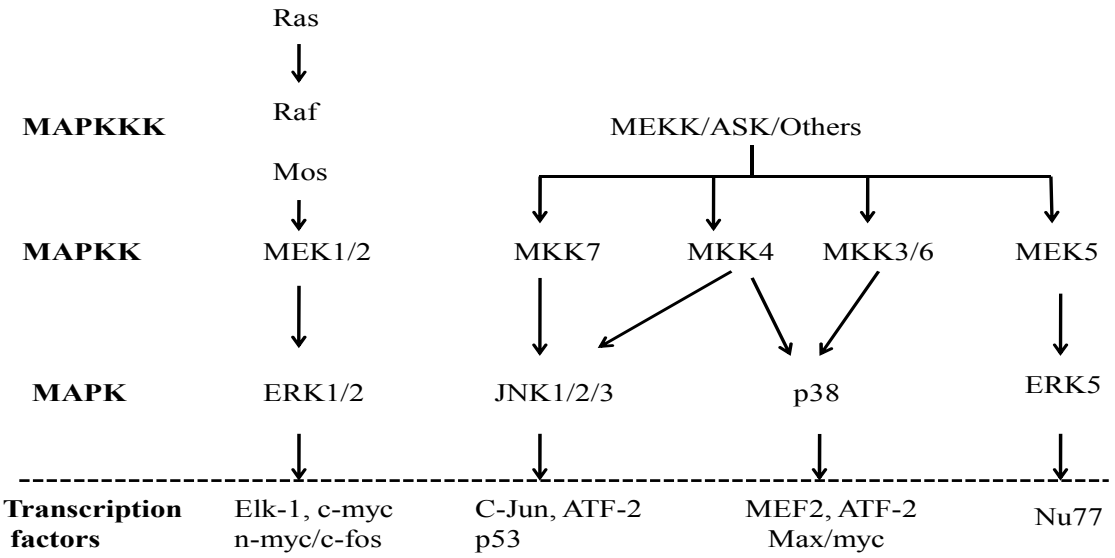

Figure 5. MAP kinase signal transduction pathway. The phosphorylation of various mitogen activated protein (MAP) kinase, ERK, JNK, and p38 leads to activation of specific transcriptions factors. Abreviations: ERK1/2, extracellular regulated kinase 1/2: ERK1/2; JNK1/2/3, c-jun-N-terminal kinase 1/2/ 3 ; MAP3K5, mitogen-activated protein kinase kinase kinase 5 ; MAPK, mitogen-activated protein kinase ; MAPKK, mitogen-activated protein kinase kinase ; MAPKKK, mitogen-ativated protein kinase kinase kinase ; MEK1/2, MAP/extracellular-signal regulated kinase 1/2 ; MEKK, MAP/extracellular-signal regulated kinase kinases; ASK, apoptosis signal regulated kinase;Ras, rat sarcoma; MEF, myocyte enhance factor; ATF-2, activating transcription factor 2.Sources. Pandya. N., Santani. D, and Jain,N. (2005). Role of mitogen-activated protein (MAP) kinases in cardiovascular diseases. Cardiovasc Drug Rev, 23(3), 247-254. Hommes, D.W.,Peppenlenbosch,M. P.,and Deventer., S.J.H.(2003). Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. Signal transduction,52 :144-151.

Once phosphorylated, ERKs translocate to the nucleus to phosphorylate transcriptions factors, and thereby regulate gene expression of cell-cycle related protein. In VSMC, another downstream target of ERK is the serine/threonine kinase $pp90^{rsk}$, which phosphorylates the S6 ribosomal protein and induces protein synthesis (Touyz & Berry, 2002). Other downstream targets of ERKs include cyclooxygenase-2, microtubule associated protein, Ca^{2+} channels, and the Na^{+}/H^{+} exchanger (Robinson & Cobb, 1997).

1.4.3.2 The p38MAP kinase pathway

There are four p38MAPKs encoded by four different genes: p38MAPKα, p38MAPKβ, p38MAPKγ, and p38MAPKδ. The p38MAPKs were first defined in a screen for drugs inhibiting tumor necrosis factor α-mediated inflammatory responses (Lee, et al., 1994). The p38MAPKs play an important role in inflammatory responses, apoptosis, and inhibition of cell growth. The p38MAPK are activated by dual specific kinases, MKK3, MKK4, and MKK6 (figure 5), which selectively phosphorylate particular isoforms, with MKK3 acting on the alpha and gamma isoforms. P38MAPKs are activated by other stimuli such as hormones, ligands for GPCR, and stresses such as heat shock and osmotic shock (Johnson & Lapadat, 2002). Downregulation of p38 activation is achieved by specific phosphatases capable to dephosphorylate activated p38 MAP kinases. The downstream targets of p38 are either other kinases or transcription factors such as ATF-2 and MEF2 (Hommes, Peppelenbosch, & van Deventer, 2003). The p38MAPK pathway has been implicated in many pathological conditions including cardiac ischemia, cardiac hypertrophy, ischemia/reperfusion injury, and arterial remodelling in hypertension. Furthermore, p38MAPK is an essential component of the redox-sensitive signaling pathways in Ang II-activated VSMC (Touyz & Berry, 2002).

1.4.3.3 The c-Jun N-terminal protein kinase (JNK) pathway

The JNKs and p38MAPK pathways were initially identified as stress-activated protein kinases (SAPKs), since they were activated by environmental stress. It is now clear that they belong to two different pathways due to differences in their phosphorylation motif, in their upstream activators, and their downstream targets (Sugden & Clerk, 1998). The c-jun N terminal kinases were named after c-jun, a DNA binding protein which is an important regulator of gene expression. The JNKs bind and phosphorylate c-jun, thus increasing its transcriptional activity. The JNKs are encoded by three genes yielding three isoforms: JNK1α, JNK2β, and JNK3γ. All isoforms have a molecular weight of approximately 46 or 54 kDa. The upstream activators of JNKs are not well-defined in heart tissues. MEK4 and MEK7 appear to activate JNKs, and MEKK5 is likely an upstream activator of MEK4 in the JNK pathway at least in vitro (Wang, et al., 1997). Initiation of the JNK pathway may be triggered by Rac and cdc42, members of the Rho family of small G proteins (Minden, Lin, Claret, Abo, & Karin, 1995). The JNK pathway

plays a role in mechanical stress-induced hypertrophy via phosphorylation of transcription factors c-jun, and ATF2. JNKs also play a role in apoptosis (Ruwhof & van der Laarse, 2000)

1.4.4 The phosphoinositide 3-kinase signalling pathways

The Phosphoinositide-3 kinases (PI3Ks) are lipid kinases involved in the regulation of cell growth, proliferation, apoptosis, and cytoskeletal remodeling. The PI3Ks are characterized by their ability to phosphorylate the 3'-OH position of the inositol ring of inositol phospholipids producing phosphatidylinositol-3-phosphate (PtdIns(3)P), phosphatidylinositol-3,4-bisphosphate (PtdIns(3,4)P_2), and phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3). There are several isoforms of PI3Ks in mammalian cells with distinct substrate and forms of regulation. The class I PI3Ks are heterodimers comprised of a p110 catalytic subunit and a regulatory/adaptor subunit (p85) (Gomperts, et al., 2002). This class is further divided into subclass IA, which is activated by receptors with protein tyrosine kinase activity, and the subclass IB, which is activated by receptors coupled with G proteins. Class I PI3Ks have been the focus of PI3K studies since they are generally coupled to extracellular stimuli. The predominant substrate for Class I PI3K is PtdIns(4,5)P_2, and hence the primary product of their action is PtdIns (3,4,5)P_3.

Class II PI3Ks are large (170-210 kDa) proteins that have a catalytic domain that is 45-50% similar to that of class I PI3Ks, however, they lack a regulatory subunit to function, and comprise three different isoforms (α, β, and γ) that diverge in the N terminus and present different domains within the C terminus (Cantrell, 2001). Class II PI3K is involved in membrane trafficking and receptor internalization and can be activated in response to RTKs, and cytokine receptors (Engelman, Luo, & Cantley, 2006). Class III PI3Ks are represented by the human homologues of the yeast gene product VPS34 (vacuolar protein sorting human), they only phosphoryalte PtdIns to produce PtdIns(3)P, and are thought to regulate vesicle transport (Cantrell, 2001).

1.4.4.1 Activation mechanisms of the phosphoinositide 3-kinase

Receptor protein tyrosine kinases and non-receptor tyrosine kinases such as c-Src activate the Class IA PI3K through interaction with the SH2 domain of the p85 regulatory subunits, which leads to allosteric activation of the catalytic subunit. PI3K activation leads to PtdIns (3,4,5)P_3 production, which regulates the activity of series of protein kinases including the Ser/Thr-specific kinase AKT/PKB, PLCγ, and protein kinase C (Gomperts, et al., 2002).

PtdIns(3,4,5)P_3 ,and PtdIns(3,4)P_2 bind to the PH domain of PKB, recruiting the kinase to the plasma membrane. PKB/AKT is phosphoryalted on the Thr308 in the kinase activation loop and on the Ser473 in a hydrophobic region of the C-terminus (Cantrell, 2001). Once phophorylated, PKB mediates the activation and inhibition of several targets resulting in cell survival, growth, and proliferation. The PI3K pathway is activated by insulin thereby mediates glucose transport, lipid metabolism, and glycogen synthesis. Furthermore, PKB has an inhibitory effect on apoptosis and programmed cell death. PI3K can be activated by guanine-nucleotide-binding proteins. Signals originating from transmembrane receptors can be transmitted from Ras protein to PI3K. Gβγ dimmers directly activate the PI3Kβ and γ subtypes (Gomperts, et al., 2002).

1.4.5 MAPK and PI3K activation by reactive oxygen species

Viedt and colleagues demonstrated that intracellular ROS are critical for AngII-induced activation of p38MAPK, JNK and ERK5 in VSMC, whereas phosphorylation of ERK1/2 appears to be redox insensitive. Furthermore, treatment of VSMC with the free radical scavenger NAC or the inhibitor of NADPH oxidase DPI (diphenyl iodonium chloride) antagonized the stimulatory effects of Ang II on JNK and p38 MAPK but not ERK1/2 activity (Viedt, et al., 2000). Phosphorylation of growth factor receptors is an important step in MAPK activation by Ang II. Activation of growth factors also triggers ROS generation (Bae, et al., 1997). Touyz et al. have shown that Ang II stimulates production of ROS in part through transactivation of IGF-R and EGF-R. Inhibition of RTKs and reduced ROS bioavailability decreased Ang II-induced phosphorylation of p38MAPK and ERK5, but not of ERK1/2 (Touyz, et al., 2003). The role of oxidative stress in ERK1/2 phosphorylation in A10 VSMC has also been reported. The enhanced phosphorylation of ERK1/2 was attenuated to control level by antioxidant suggesting the role of ROS in the enhanced ERK1/2 phosphorylation (Li, et al., 2007). Exogenous H_2O_2 stimulates AKT/PKB phosphorylation in VSMC suggesting that AKT/PKB phosphorylation is redox-sensitive (Crossthwaite, Hasan, & Williams, 2002). Ushio-Fukai and colleagues demonstrated that Ang II-induced PKB phosphorylation is significantly inhibited by both the NAD(P)H oxidase inhibitor DPI and by overexpression of catalase, suggesting the role of ROS as potential signal transducers linking the AT1 receptor to the PKB pathway in VSMC (Ushio-Fukai, et al., 1999).

1.4.6 Mechanism of activation of growth factor receptors

Cell surface receptors integrate extracellular signals such as hormones, neuropeptides, growth factors, thus regulating signal transduction pathways and cellular responses.

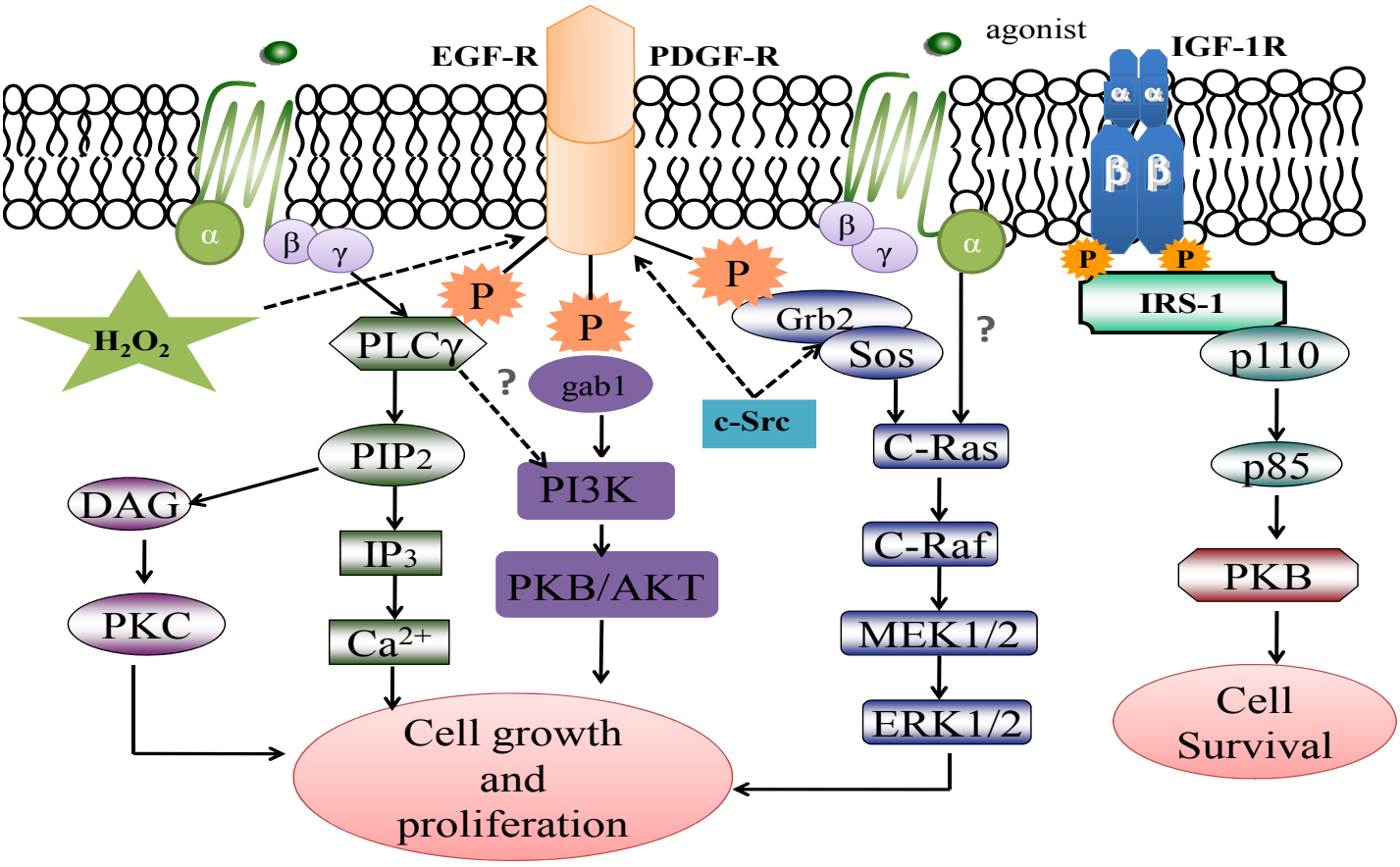

Figure 6. Signal transduction of GPCR and receptor tyrosine kinase. Ligand binding to growth factor receptors (EGF-R, PDGF-R) stimulates autophosphorylation of the receptors which then bind SH2 domains of the adaptor protein Grb2. Grb2 is complexed to the guanine nucleotide factor Son-of-sevenless(Sos), which then stimulates the exchange of RasGDP into RasGTP. Ras then activates the ERK cascade through Raf and MEK. Autophosphorylation of growth factor receptors activates PI3K which phosphorylates PKB/AKT. Extracellular ligand binding to GPCRs leads to activation of trimeric G-protein and subunits dissociation which. Gα, and Gβγ subunits elicit intracellular signals trough protein-protein interactions. The binding of insulin to the extracellular αα subunits triggers the phosphorylation of insulin receptor substrate 1, which activates PKB through p110, and p85. Abbreviations: Sos, son-of-sevenless;c-Src, cellular Sarcoma; Grb2, growth factor receptor-bound 2; DAG, diacyl glycerol; PIP2, phosphatidylinositol-3,4-bisphosphate; PLCγ, phospholipase Cγ; IRS-1, insulin receptor substrate 1; PKC, protein kinase C; PKB, protein kinase B. Sources. Wetzker. R., Bohmer, F.D. Transactivation joins multiple tracks to the ERK/MAPK cascade (2003). Molecular Cell Biology,4:651-657. Hunyady.L., Catt.K.J. Pleiotropic AT1 Receptor Signaling pathways Mediating physiological and Pathogenic Actions of Angiotensin II. Molecular Endocrinology (2006). 20(5):953-970.

Many growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) bind to receptors with tyrosine kinase activity in order to regulate cellular growth (Karp, 2008).

1.4.6.1 Activation of epidermal growth factor receptors

The EGF-Rs are members of the RTKs superfamily of receptors that possess intrinsic protein tyrosine kinase activity. The EGF-Rs are 170 kD glycoproteins that span the membrane via one α-helical segment of 23 amino acids connecting a large glycosylated extracellular ligand binding domain and an intracellular tyrosine kinase domain (Goldkorn, et al., 1998). The EGF-Rs were the first RTK to be characterized by molecular cloning, and the first proteins tyrosine kinase receptors to be shown to dimerize after ligand binding. The EGF-R family consists of four RTKs, EGFR(ErbB1), HER2(ErbB2), HER3(ErbB3), and HER4(ErbB3) (Prenzel, Fischer, Streit, Hart, & Ullrich, 2001). While EGFR has many ligands such as EGF, and TGFα, a ligand for ErbB3 and ErbB4 are the various isoforms of the neurogulin. All EGFR family are characterized by a structure consisting of an extracellular binding domain, a single hydrophobic transmembrane region, and the intracellular part containing the tyrosine kinase domain (Ullrich & Schlessinger, 1990).

Ligand-induced receptor dimerization and subsequent autophosphorylation of tyrosine residues creates docking site for adaptor proteins or enzymes with SH2 (Src homology 2 domain) or PTB (phosphotyrosine binding) domains. Adaptors such as Shc, Grb2-Sos (growth factor receptor-bound protein 2 – son of sevenless) bind to activated EGFR in order to induce the activation of the MAP kinases (Jones & Dumont, 1999). Activation of EGFR also triggers the phosphorylation of PLC which hydrolyzes phosphatidyl inositol-4,5-biphosphate (PIP_2) into inositol-(1,4,5)-triphosphate(IP_3) and diacyl glycerol (DAG) with subsequent generation of calcium (Prenzel, et al., 2001). EGFR-induced activation of PI3K is relatively weak compared to other RTKs since EGFR has no binding site for the SH2-domain of PI3K (Soltoff, Carraway, Prigent, Gullick, & Cantley, 1994). The adaptor protein Gab1 mediates the activation of PI3K by the EGFR and a report by Rodrigues and colleagues demonstrate a positive feedback loop in EGFR signaling through Gab1 and PI3K (Rodrigues, Falasca, Zhang, Ong, & Schlessinger, 2000).

1.4.6.2 Activation of platelet-derived growth factor receptor

Platelet-derived growth factor receptors (PDGFR) are membrane protein-tyrosine kinases that play an important role in the regulation of cell growth and differentiation. PDGFRs consist of two polypeptide subunits, α and β both of which have tyrosine kinase activity (Bioukar, Marricco, Zuo, & Larose, 1999). Cultured VSMC from SHR express both α and β isoforms of PDGFR, however, in VSMC from normotensive rats (WKY), PDGFRαα is almost completely suppressed (Inui, Kitami, Tani, Kondo, & Inagami, 1994). Millete et al. have shown that in rat VSMC, PDGFββ could transactivate EGF receptor to form a heterodimer between PDGFR and EGFR, which contribute to ERK1/2 activation (Millette, et al., 2005). PDGF binds to PDGFR which results in the autophosphorylation of specific tyrosine residues, creating docking sites for SH2 domains-containing proteins. These include c-Src, PI3K, PLCγ, small GTP binding protein Ras, and adaptor protein such as Shc, and Grb2. The specific binding of these proteins initiate signaling pathway leading to cell growth, and proliferation (Chen, Zhou, Zhang, & Lou, 2007).

1.4.7 Activation of growth factor receptors by reactive oxygen species

Reactive oxygen species are involved in the phosphorylation of tyrosine residues of the EGFR. Ushio-Fukai and colleagues have shown that in aortic VSMC, EGFR phosphorylation by Ang II at specific tyrosine residues requires ROS and that c-Src contributes to the redox sensitivity of this response. Thus, EGFR transactivation is an important ROS-mediated biochemical pathway activated by Ang II in VSMC (Ushio-Fukai, et al., 2001). Furthermore, redox sensitivity of EGFR phosphorylation is confirmed by the finding that exogenous H_2O_2 increases EGFR phosphorylation in VSMCs (Ushio-Fukai, et al., 1999). Bae Soo Yun and colleagues also found that addition of catalase to A431 human epidermoid carcinoma cells inhibited EGF-induced autophosphorylation of EGFR (Bae, et al., 1997). H_2O_2-induced tyrosine phosphoryaltion of the EGFR receptors involves inhibition of tyrosine phosphatases. Knebel et al demonstrated that H_2O_2 could inhibit the dephosphorylation of the EGF receptor through the inhibition of tyrosine phosphatases (Knebel, Rahmsdorf, Ullrich, & Herrlich, 1996). The interaction of growth factors with their receptors also induces a transient increase in the intracellular concentration of H_2O_2 in human heptocellular liver carcinoma cells (HepG2cells). Bae Soo Yun and colleagues reported that the binding of PI3K to PDGFRβ is necessary for the PDGF-induced H_2O_2 production (Bae, et al., 2000). Saito et al reported that H_2O_2 stimulates Tyr^{1021} phosphorylation of the PDGFRβ

receptor in VSMCs and that this phosphorylation requires the activation of two protein kinases, PKCδ and c-Src, but not the intrinsic kinase activity of the PDGFRβ receptor, which suggests the implication of both c-Src and PKC in H_2O_2-induced phosphorylation of PDGFRβ (Saito, et al., 2002). Previous work from Eguchi and colleagues has shown the implication of metalloproteases in PDGFRβ or EGF receptor transactivation by H_2O_2 in VSMC. A matrix metalloprotease (MMP)-2/9 inhibitor inhibits both Ang II-induced EGFR transactivation and H_2O_2-induced EGFR with subsequent blockage of VSMC growth and migration (Eguchi, Dempsey, Frank, Motley, & Inagami, 2001).

1.5 Transactivation of growth factor receptors

Transactivation of RTKs is a general process of GPCRs signalling. Depending on a specific receptor and cell type, GPCRs use several strategies for the activation of downstreams signalling pathways including PI3K and MAPK. Most of these involved crosstalk between GPCRs and RTKs. In some cases The GPCR itself forms part of the signalling complex, for instance the formation of complexes between the AT_1 receptor and Jak2, and also between β2 adrenergic receptor, β-arrestin and c-Src (Luttrell, Daaka, & Lefkowitz, 1999). Studies have demonstrated that Ang II-stimulated transactivation of EGF-R in VSMC requires c-Src (Ushio-Fukai, et al., 2001). Linseman and colleagues have reported that Ang II-induced transactivation of PDGFR-β involved complex formation with Shc, Grb2, as well as c-Src in cultured VSMC (Linseman, Benjamin, & Jones, 1995). In fact recent works with various GPCRs including AT_1 suggest that GPCRs-induced MAPK activation requires Shc-Grb2·Sos complex formation and subsequent activation of tyrosine kinases such as proline-rich tyrosine kinase 2 (PYK2) (Lev, et al., 1995), EGF-R (Daub, Weiss, Wallasch, & Ullrich, 1996), PDGFR-β (Linseman, et al., 1995), and c-Src (Wan, Kurosaki, & Huang, 1996).

1.6 Objectives

The interrelation between oxidative stress and hypertension has received much attention in the past years. Several experimental and clinical studies have linked an enhanced production of ROS to diseases of the cardiovascular system such as hypertension and atherosclerosis (Kojda & Harrison, 1999; Taniyama & Griendling, 2003). Enhanced oxidative stress occurs in human hypertension (Sagar, et al., 1992), and animal models including SHR, renovascular hypertension (Lerman, et al., 2001), and DOCA salt model (Callera, et al., 2006). Treatments that decrease ROS production and/or enhance antioxidant defence mechanisms also prevent vascular injury and reduce blood pressure in hypertensive patients.

Our laboratory has established the link between hypertension and the enhanced expression of Giα proteins and associated adenylyl cyclase (AC) signalling (Marcil, et al., 1998). We also have reported the role of oxidative stress in Ang II-mediated enhanced expression of Giα proteins in A10 VSMC (Li, et al., 2007). However, the signal transduction pathways responsible for ROS-induced Giα proteins expression have never been clearly elucidated. Since treatment of VSMC with Ang II enhances intracellular H_2O_2 generation via NADPH oxidase activation (Zafari, et al., 1998), H_2O_2 was used to induce oxidative stress in aortic VSMC. We examined the effect of H_2O_2, an experimental mimicker of oxidative stress on Giα protein expression and adenylyl cyclase signalling in aortic VSMC. Cells were treated with pharmacological inhibitors of MAPK and PI3K pathways. To further confirm the effect of pharmacological inhibitors of EGFR and PDGFR-β on the expression of these receptors, the EGFR and PDGFR-β were silenced with siRNA targeting the intracellular tyrosine kinase domain of these growth factor receptors. The effect of both the inhibitor treatment, and gene knockout on Giα proteins expression was assessed with Western blotting using specific antibodies. We hypothesized that H_2O_2 enhances Giα proteins expression through the activation of growth factors receptors EGFR and PDGFR-β with subsequent phosphorylation of downstream signalling pathways including ERK1/2, and PI3K.

Chapter 2 – Results

Scientific Article

Hydrogen Peroxide Enhances the Expression of Giα proteins in Aortic Vascular Smooth Muscle Cells: Role of Growth Factor Receptors Transactivation*

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Running Head: Growth Factor Receptor Activation and Giα protein expression

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Abstract

We have recently shown that the enhanced expression of $G_i\alpha$ proteins in vascular smooth muscle cells (VSMC) from spontaneously hypertensive rats was attributed to the enhanced oxidative stress. We undertook the present study to examine if hydrogen peroxide (H_2O_2), that induces oxidative stress could also enhance the expression of Gi proteins in aortic VSMC and to further explore the underlying mechanisms responsible for this response. Treatment of VSMC with H_2O_2 increased the expression of Giα proteins and not of Gs protein in a concentration- and time-dependent manner. The maximal increase (~ 40-50%) was observed at 100 μM and 1h and was restored to control levels by AG1295, AG1478, PD98059 and wortmannin, inhibitors of epidermal and platelet-derived growth factor receptors (EGFR, PDGFR), the mitogen activated protein kinase (MEK1/2), and phosphatidylinositol-3 kinase (PI3K) respectively. In addition, H_2O_2 also increased the phosphorylation of EGFR, PDGFR, ERK1/2 and AKT which was also attenuated to control levels by the respective inhibitors, whereas the inhibitors of EGFR and PDGFR also attenuated the enhanced phosphorylation of ERK1/2 and AKT to control levels. Furthermore, transfection of cells with siRNA of EGFR and PDGFR attenuated the H_2O_2-induced enhanced expression of Giα proteins to control levels. The increased expression of Giα proteins by H_2O_2 was reflected in enhanced inhibition of AC activity and decreased Gsα-mediated stimulations of AC. These results suggest that H_2O_2 increases the expression and functions of Giα proteins in VSMC through the transactivation of EGFR, PDGFR and ERK1/2 and PI3K signaling pathways.

Key Words: H_2O_2, Gi proteins, adenylyl cyclase, growth factor receptors, si RNA, ERK1/2, PI3Kinase, VSMC

INTRODUCTION

Guanine nucleotide proteins, known as G proteins are a family of GTP-binding proteins that play a critical role in the regulation of a variety of signal transduction systems, including the adenylyl cyclase/cAMP system (Tang & Gilman, 1991). The adenylyl cyclase (AC) system is composed of three components: a receptor, a catalytic subunit, and a guanine nucleotide regulatory protein that transmits the signal from the hormone-occupied receptor to the catalytic subunit (Schramm & Selinger, 1984).Well characterized members of G protein family include Gs, Gi, Gq, and Go. Gs and Gi are involved in the stimulation and inhibition of hormone-sensitive AC respectively (Itoh, et al., 1988). The G proteins are heterotrimeric, consisting of α, β, and γ subunits (Gα, Gβ, and Gγ). The α-subunit binds and hydrolyses GTP and confers specificity in the receptor and effector interactions (Gilman, 1987). Molecular cloning has revealed four different forms of Gsα, resulting from differential splicing of one gene, and three distinct forms of Giα, Giα-1, Giα-2, and Giα-3 encoded by three distinct genes (Bray, et al., 1986; Gilman, 1987).

Alterations in Giα proteins and associated AC signaling have been implicated in various pathological conditions, such as hypertension (Li & Anand-Srivastava, 2002), diabetes (Li, Descorbeth, & Anand-Srivastava, 2008) and heart failure (Di Fusco, et al., 2000). We previously have reported an increased expression of Giα-2, and Giα-3 proteins and mRNA in hearts and aortas from spontaneously hypertensive rats (SHR) (Anand-Srivastava, 1992), 1K1C (Bohm, et al., 1993), Nomega-nitro-L-arginine methyl ester (L-NAME) (Hashim & Anand-Srivastava, 2004), and deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Anand-Srivastava, de Champlain, & Thibault, 1993) with established hypertension. The enhanced expression of Giα proteins was shown to be attributed to the enhanced levels of vasoactive peptides including angiotensin II (Ang II) because the treatment of hypertensive rats with losartan, and captopril, an angiotensin converting enzyme (ACE) inhibitor or AT1 receptor antagonist, was shown to restore the enhanced expression of Gi proteins to control levels (Pandey & Anand-Srivastava, 1996). The enhanced expression of Giα proteins occured before the onset of hypertension in SHR and DOCA-salt (Hashim & Anand-Srivastava, 2004; Marcil, et al., 1997) suggesting the implication of increased levels of Giα protein in the pathogenesis of hypertension. We have earlier reported that VSMC from SHR also exhibit the increased expression of Giα proteins which was attributed to the enhanced oxidative stress (Lappas, Daou, & Anand-Srivastava,

2005). In addition, a role of oxidative stress in Ang II-induced enhanced expression of Giα proteins in A10 VSMC has also been demonstrated (Li, et al., 2007). Ang II has also been reported to increase the levels of H_2O_2, another reactive oxygen species (ROS) inducing oxidative stress (Zafari, et al., 1998).

The present study was undertaken to investigate if the increased oxidative stress induced by H_2O_2, could also increase the expression of Giα proteins and associated adenylyl cyclase signaling in aortic VSMC and to further explore the underlying signaling mechanisms responsible for this response.

We showed for the first time that treatment of aortic VSMC with H_2O_2 increased the expression of Gi proteins through the transactivation of growth factor receptors and MAP kinase/PI3Kinase signaling.

MATERIALS AND METHODS

3-Isobutyl-1-methylxanthine, glucagon, oxotremorine, isoproterenol, forskolin (FSK), guanosine 5'-[3-thio] triphosphate (GTPγS), and guanosine triphosphate (GTP) were purchased from Sigma-Aldrich Chemical (St-Louis, MO, USA). Adenosine triphosphate isotope [α-^{32}P]ATP was purchased from PerkinElmer (Boston, MA, USA). PDGFβ-R inhibitor AG1295, EGFR inhibitor AG1478, and MEK1 inhibitor PD98059, were purchased from Calbiochem (Gibbstown, NJ, USA). EGFR siRNA(r), PDGFR-β siRNA(m), siRNA transfection medium, and control siRNA were purchased from Santa Cruz Biotechnology,Inc (Santa Cruz, CA,USA). Lipofectamine 2000 was purchased from Life Technology (CA, USA). Antibodies against Giα-2 (L5), Giα-3 (C-10), ERK1/2 (C-14), p-ERK1/2 (phosphospecific-tyrosine^{204}), p-AKT (phosphospecific-serine 473), AKT (473), p-PDGFR (phosphospecific-tyrosine^{857}), PDGFR (958), p-EGFR (phosphospecific-tyrosine^{1173}), EGFR (1005) were from Santa-Cruz Biotechnologies (Santa Cruz, CA, USA). All other chemicals used in the experiments were purchased from Sigma-Aldrich.

Cell culture and incubation.

Vascular smooth muscle cells from thoracic aorta of Sprague-Dawley rats (200-225 g) (6-8 weeks old), were cultured in Dulbecco's modified Eagle's medium (DMEM) (with glucose (5.5mM), L-glutamine and sodium bicarbonate) containing antibiotics and 10% heat-inactivated fetal bovine serum (FBS), and incubated at 37˚C in 95% air and 5% CO_2 as previously described (Hashim, Li, & Anand-Srivastava, 2006b). Confluent cells were starved by incubation for 24h in DMEM without FBS at 37˚C. To study the effect of pharmacological inhibitors on Giα expression, the cells were incubated in the absence or presence of PD98059 (10μM), wortmannin (0.1μM), AG1478 (5μM), AG1295 (5μM) before treatment with H_2O_2 (100μM) for 1h. The cells were scraped into ice-cold homogenization buffer containing 10 mM Tris-HCl buffer, and 1mM EDTA (pH 7.5). The homogenate was centrifuged at 1,000 g for 10 min. The supernatant was used for immunoblotting, and the pellet was resuspended in 10 mM Tris-HCl buffer containing 1mM EDTA (pH 7.5) and used for adenylyl cyclase assay.

Adenylyl cyclase activity determination:

Adenylyl cyclase activity was determined by measuring [α-^{32}P] cAMP formation from [α-^{32}P]ATP as previously described (Li, et al., 2008). The assay medium contained 50 mmol/l of glycylglycine (pH 7.5), 0.5 mmol/l MgATP, [α-^{32}P]ATP (1.5 x 10^6 cpm), 5 mmol/l $MgCl_2$ (in excess of the ATP concentration), 100 mmol/l NaCl, 0.5 mmol/l cAMP, 1 mmol/l IBMX, 0.1 mmol/l EGTA, 10μmol/l GTPγS, and an ATP-regenerating system consisting of 2 mmol/l creatine phosphate, 0.1 mg of creatine kinase/ml and 0.1 mg of myokinase/ml in a final volume of 200μl. Incubation was initiated by the addition of the membrane preparation (20-30μg) to the reaction mixture, which had been thermally equilibrated for 2 min at 37˚C. The reactions were conducted in triplicate, and were terminated by the addition of 0.6 ml of 120 mM Zinc acetate. cAMP, was purified by coprecipitation of other nucleotides with $ZnCO_3$, an addition of 0.5 ml of 144 mM Na_2CO_3, and subsequent chromatography by the double column system as previously described (Hashim, Li, Nagakura, Takeo, & Anand-Srivastava, 2004).

Transfection of small interfering RNA.

Vascular smooth muscle cells from thoracic aorta of Sprague-Dawley rats (200-225 g) (6-8 weeks old), were cultured in DMEM containing antibiotics and 10% FBS. Forty-eight hours before transfection, cells were trypsinized and cultured in DMEM supplemented with 10% FBS without antibiotics. Confluents cells were starved for five hours in siRNA transfection medium. Cells were transfected with 90 nM siRNA and 4μg/ml of lipofectamine per 60mm cell dishes. After 48 hours of transfections, the cells were lysed for immunoblotting.

Immunoblotting.

Immunoblotting of G proteins (Gsα, Giα-2, and Giα-3), AKT, ERK1/2, PDGFβ-R, and EGFR was performed using specific antibodies as previously described (Lappas, et al., 2005). After sodium dodecyl sulphate-polyacrylamide gel electrophoresis, the separated proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Canada) with a semi-dry transblot apparatus (Bio-Rad) at 15V for 45 minutes. Upon transfer, the membranes were stained with Rouge Ponceau to confirm the presence of migrated proteins. The membranes were then blocked for 1h at room temperature in phosphate-buffer saline (PBS) containing 5% dehydrated milk and 0.2% Tween 20, and incubated with antibodies against Giα-2 (L5), Giα-3 (C-10), p-

ERK1/2 (phosphospecific-tyrosine^{204}), p-AKT (phosphospecific-serine473), p-EGFR (phosphospecific-tyrosine^{1173}), and p-PDGFR (phosphospecific-tyrosine^{857}) overnight at 4˚C. The antibody-antigen complexes were detected by incubating the membranes with secondary antibodies conjugated with horseradish peroxidase at room temperature for 1 h. The blots were visualized with enhanced-chemiluminescence (ECL) Western blotting detection reagents from Santa Cruz. Quantitative analysis of the proteins was performed by densitometric scanning of the autoradiographs using an enhanced laser densitometer (LKB Ultroscan XL, Pharmacia, Canada) and gel-scan XL evaluation software (version 2.1) from Pharmacia. The scanning was one dimensional and scanned the entire area of protein bands in the blot.

Statistical analysis. Results are expressed as means ± standard error (±SE) and were analyzed by one-way ANOVA followed by Newman-Keul test. Results were considered statistically significant at a value of $P < 0.05$

RESULTS

Effect of hydrogen peroxide on Giα-2, Giα-3, and Gsα proteins expression in aortic vascular smooth muscle cells

We previously have shown that oxidative stress contributes to the enhanced expression of Giα proteins in VSMC from SHR (Lappas, et al., 2005). To further establish the relationship between oxidative stress and Giα proteins levels, we examined the effect of various concentrations of H_2O_2 (50μM to 250μM) on the levels of Giα-2 and Giα-3 proteins. Results shown in Figure 1A&B indicate that treatment of VSMCs with H_2O_2 for 1h increased the levels of both Giα-2 and Giα-3 proteins in a concentration dependent manner. The maximal increase of about 75% in Giα-2 and 60% in Giα-3 was observed at 100 μM. In addition, the increase in the expression of Giα-2 and Giα-3 proteins by H_2O_2 (100 μM) was also time-dependent. As shown in Figure 1C&D, the levels of Giα-2 and Giα-3 were enhanced as early as 30 min then reached a maximum at about 180% of control at 1 to 2h, and levelled off after 3h, however, the levels of Gsα were not affected by H_2O_2 treatment (Figure 1E).

Effect of actinomycin D on H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins in aortic vascular smooth muscle cells

To investigate whether H_2O_2-induced enhanced levels of Giα-2 and Giα-3 proteins were due to increased RNA synthesis, the effect of actinomycin D, an inhibitor of RNA synthesis was examined on H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins. Results shown in Figure 2 indicate that actinomycin D attenuated the H_2O_2-induced enhanced level of Giα-2 and Giα-3 proteins to control levels.

Effect of H_2O_2 on Gi functions in aortic vascular smooth muscle cells

To investigate if the H_2O_2-induced enhanced expression of Giα is also reflected in Giα functions, the effect of H_2O_2 on receptor- dependent and - independent functions of Giα proteins was examined. The receptor-independent function of Giα was investigated by studying the effect of low concentrations of GTPγS (10^{-12} to 10^{-7}M) on FSK-stimulated AC activity. As illustrated in Figure3A, GTPγS inhibited FSK-stimulated adenylyl cyclase activity in a concentration dependent manner in both control and H_2O_2-treated cells; however, the inhibition was greater by about 25% in H_2O_2-treated cells.

The receptor-dependent function of Giα proteins was also examined by studying the effects of H_2O_2 treatment on angiotensin II-, C-ANP_{4-23}-, and oxotremorine-mediated inhibition of AC activity in aortic VSMC. The results in Figure 3B, indicate that Ang II, C-ANP_{4-23} and oxotremorine, inhibited AC activity by about 25 to 30% in control cells. However, these inhibitions were significantly augmented (~ 55%) by H_2O_2 treatment.

Effect of H_2O_2 on Gs-mediated stimulation of adenylyl cyclase activity in aortic vascular smooth muscle cells

The interaction of Giα and Gsα with adenylyl cyclase (AC) activity has been well established (Cerione, et al., 1985). Since H_2O_2 enhanced Giα protein expression without altering the levels of Gsα, we investigated whether increased level of Giα induced by H_2O_2 could affect Gsα-mediated stimulation of AC. To test this, the effects of isoproterenol, a β-adrenergic agonist, glucagon, sodium fluoride (NaF), and forskolin (FSK) on AC activity was examined. Figure 4B, illustrates that isoproterenol and glucagon stimulated adenylyl cyclase activity in both control cells and H_2O_2-treated cells to various degrees. However, the extent of stimulation was significantly decreased (~ 40 %) in H_2O_2-treated cell compared to control cells. In addition, FSK and NaF- stimulated AC activities were also attenuated by about 34% by H_2O_2 treatment.

Role of extracellular-regulated kinase (ERK1/2) in H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins in aortic vascular smooth muscle cells

The involvement of ERK1/2 in the enhanced expression of Giα proteins in VSMCs from SHR has been demonstrated (Lappas, et al., 2005). To investigate whether ERK1/2 is also implicated in H_2O_2-induced enhanced expression of Giα in VSMCs, the effect of an inhibitor of ERK phosphorylation, PD98050 (10μM) was examined. Results shown in Figure 5A, indicate that the increased expression of Giα-2 and Gi□-3 proteins in H_2O_2-treated cells were restored to control levels by PD98059 treatment. However, PD98059 did not have any effect on the levels of Giα proteins in control cells.

Role of phosphoinositide-3-kinase (PI3K) in H_2O_2-induced enhanced expression of Giα proteins in aortic vascular smooth muscle cells

Since the involvement of the PI3K signalling in enhanced expression of Gi proteins in VSMC from SHR has been reported (Ge & Anand-Srivastava, 1998), it was of interest to examine the contribution of PI3K in H_2O_2-induced enhanced expression of Giα proteins in aortic VSMC. For this, the effect of wortmannin (0.1μM), an inhibitor of PI3K on the expression of Gi proteins was investigated in control and H_2O_2-treated cells and the results are shown in Figure 5B. H_2O_2 increased the levels of Giα-2 and Giα-3 by about 80% and 60% respectively, which were restored to control levels by wortmannin. However, the levels of Giα proteins in control cells were unchanged by wortmannin treatment.

Effect of H_2O_2 on ERK1/2 phosphorylation in aortic vascular smooth muscle cells

Fig. 6A shows the effect of H_2O_2 on ERK1/2 phosphorylation in VSMC. Treatment of cells with H_2O_2 increased the phosphorylation of Tyr^{204} on ERK1/2 by about 90% compared to control cells which was attenuated towards control level by PD98059. In addition, PD98059 also inhibited the ERK1/2 phosphorylation in control cells by about 50%.

Effect of H_2O_2 on the phosphorylation AKT in aortic vascular smooth muscle cells

Since H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins was abolished by PI3K inhibitor wortmannin, it was of interest to examine whether treatment of aortic VSMC with H_2O_2 would increase the phosphorylation of AKT. To test this, the effect of H_2O_2 on AKT phosphorylation was investigated VSMC. As shown in Fig.6B, H_2O_2 increased the phosphorylation of phosphospecific-$serine^{473}$ on AKT by about 60% compared to control cells and this increased phosphorylation was attenuated to control levels by wortmannin.

Effect of growth factors receptor inhibitors on H_2O_2-induced enhanced expression of Giα proteins in aortic vascular smooth muscle cells

Since H_2O_2 has been shown to transactivate growth factor receptors such as EGF-R and PDGFR-β in A10 VSMC (Descorbeth & Anand-Srivastava, 2009), it may be possible that the H_2O_2-induced enhanced expression of Giα-2 and Giα-3 could also be due to the enhanced activity of both EGF-R and PDGFR-β. To investigate this, the effect of AG1478, an inhibitor of EGF-R and

AG1295, an inhibitor of PDGFR-β on H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins in aortic VSMC was examined. The results shown in Figure 7, indicate that the increased expression of Giα-2 and Giα-3 proteins (~50%) in H_2O_2-treated cells compared to control cells was restored to control levels by AG1478 (7A) and AG1295(7B) . However, these inhibitors did not have any significant effect on the levels of Giα-2 and Giα-3 proteins in control cells.

Effect of H_2O_2 on the phosphorylation of growth factor receptors in aortic vascular smooth muscle cells

To investigate whether H_2O_2 could also transactivate growth factor receptors in aortic vascular smooth muscle cells, the phosphorylation of EGF-R and PDGFR-β in response to H_2O_2 was examined and the results are shown in Figure 8. Treatment of aortic VSMCs with H_2O_2 increased the phosphorylation of Tyr^{1173} on EGF-R and Tyr857 on PDGFR-β by about 40% as compared to control cells. However, the increased phosphorylation of EGF-R and PDGFR-β induced by H_2O_2 was attenuated to control levels by AG1478 and AG1295 respectively.

Effect of siRNA of growth factor receptors (EGF-R, PDGF-R) on H_2O_2-induced enhanced expression of Giα proteins in aortic vascular smooth muscle cells

To further confirm the implication of EGF-R and PDGFR-β in H_2O_2-induced enhanced expression of Gi□ proteins, the effect of siRNA of EGF-R and PDGFR-β on the expression of Gi□ proteins was investigated in control and H_2O_2-treated cells. Results shown in Figure 9, indicate that the increased expression of Giα-2 (A, C) and Giα-3 (B, D) proteins (by about 60% and 50% respectively) in H_2O_2-treated cells compared to control cells, was attenuated to control levels by EGFR and PDGFR-β siRNA. However, cells transfected with lipofectamine alone, scrambled siRNA, siRNA of EGF-R or PDGFR-β did not affect the expression of Giα-2 and Giα-3 proteins in control cells.

Effect of siRNA of growth factor receptors (EGF-R, PDGF-R) on H_2O_2-induced increased phosphorylation of growth factor receptors in aortic vascular smooth muscle cells

To investigate if the attenuation of H_2O_2-induced enhanced expression of Gi□ proteins by siRNA of EGF-R and PDGF-R in VSMC was attributed to the decreased activation of these

growth factor receptors, the effect of siRNA on the phosphorylation of EGF-R and PDGF-R was examined and the results are shown in Figure 10. H_2O_2 increased the phosphorylation of Tyr^{1173}on EGF-R and Tyr^{857}on PDGF-R by approximately 200% (10A) and 250% (10C) respectively as compared to control cells which was significantly attenuated by EGF-R siRNA and PDGFR-β siRNA by 150 % and 125 %. In addition, the levels of total EGF-R (10B) and PDGF-R (10D) were also significantly decreased by 65% and 80% respectively in cells transfected with EGF-R siRNA and PDGFR-β siRNA. These data suggest that siRNA of EGF-R and PDGFR-β specifically inhibits the activation of EGFR and PDGFR-β induced by H_2O_2 which may contribute to the attenuation of the enhanced expression of Gi□ proteins.

Implication of growth factor receptors in H_2O_2-induced enhanced activation of ERK1/2 and AKT signaling in aortic vascular smooth muscle cells

To investigate the implication of growth factor receptors in H_2O_2-induced enhanced activation of ERK1/2 and AKT, the effect of EGF-R and PDGF-R inhibitors, AG1478 and AG1295 on the phosphorylation of ERK1/2 and AKT was examined in control H_2O_2-treated cells. As illustrated in Figure 11, H_2O_2 increased the phosphorylation of p-ERK1/2 (11A) and AKT (11B) by about 60% and 50% which was restored to control levels by AG1478 and AG1295, whereas the level of p-ERK1/2 and pAKT remained unchanged in control cells.

DISCUSSION

We earlier reported that the enhanced expression of Gi proteins in VSMCs from SHR was attributed to the increased oxidative stress and increased MAP kinase activity (Lappas, et al., 2005). However, in the present study, we demonstrate that H_2O_2, a mimicker of oxidative stress could also increase the levels of Giα proteins in aortic VSMC through the transactivation of growth factors receptors.

Although the role of oxidative stress in Ang II-mediated cell signaling has been well established (Touyz, et al., 2004), evidence for a direct role of oxidative stress and associated signaling in the increased expression of Giα proteins and associated adenylyl cyclase has been lacking. We report that H_2O_2 increases the expression of Giα proteins in a time- and concentration- dependent manner, however, a slight decrease in the expression of Giα-2, and Giα-3 proteins that was observed beyond 150 μM H_2O_2 and at 3 and 4h of treatment may not be due to apoptosis, because the total cell count determined by hemocytometer was not different between control and H_2O_2-treated cells. Furthermore, the H_2O_2-evoked enhanced expression of Giα-2, and Giα-3 in aortic VSMC may be at the transcriptional level because actinomycin D, an inhibitor of RNA synthesis, inhibited the H_2O_2-induced enhanced expression of Giα-2, and Giα-3 proteins in these cells.

The enhanced expression of Giα-2 and Giα-3 by H_2O_2 treatment was also reflected in increased Giα functions as demonstrated by the enhanced inhibition of adenylyl cyclase by inhibitory hormones and FSK-stimulated adenylyl cyclase activity by GTPγS in H_2O_2-treated cells as compared to control cells. In this regard, the relationship between increased expressions of Giα proteins and enhanced Giα function has previously been reported (Anand-Srivastava, 1992). In addition, the decreased responsiveness of AC to isoproterenol and glucagon stimulation in H_2O_2-pretreated cells may also be attributed to increased levels of Giα proteins and not to the decreased levels of Gsα proteins because H_2O_2 treatment did not alter the expression of Gsα proteins. In this regard, a relationship between Giα and Gsα proteins has been well established [24-26]. An increased expression of Gi proteins and resultant decreased stimulation of adenylyl cyclase by stimulatory hormones (Marcil & Anand-Srivastava, 2001) and decreased levels of Gi proteins and augmented stimulation of adenylyl cyclase by stimulatory hormones has been shown by several studies (Bassil & Anand-Srivastava, 2006; Bassil, Li, & Anand-Srivastava, 2008)

Our results showing that blockade of PDGFR-β and EGF-R by specific inhibitors or by silencing of PDGFR-β and EGF-R using siRNA, restored the H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins to control levels without affecting the expression of Giα proteins in control cells, suggest the implication of growth factor receptors in H_2O_2–induced enhanced expression of Giα proteins. It appears that H_2O_2 enhances the levels of Giα-2 and Giα-3 proteins through the transactivation of PDGFR and EGF-R, because H_2O_2, enhanced the phosphorylation and not the levels of PDGFR-β and EGF-R in VSMC. This notion is further supported by our results showing that siRNA of PDGFR and EGFR that decreased the levels of these receptors in control cells had no effect on the levels of Gi□ proteins. In addition, the fact that siRNA of PDGFR-β and EGF-R decreased the H_2O_2-induced enhanced phosphorylation of these receptors further suggests the role of transactivation of growth factor receptor in enhanced expression of Giα proteins induced by H_2O_2. Taken together, it may be suggested that the transactivation of growth factor receptors by H_2O_2 contributes to the enhanced expression of Giα proteins in VSMC. Furthermore, the implication of EGF-R in Ang II-induced enhanced expression of Giα proteins and proliferation in A10 VSMC has recently been shown (Gomez Sandoval, Levesque, & Anand-Srivastava, 2009).

The role of MAPK and PI3K in protein synthesis has been well established (Hashim, et al., 2006b; Wang, et al., 2008). We previously showed the implication of ERK1/2 in the enhanced expression of Giα-2 and Giα-3 protein in VSMC from SHR (Lappas, et al., 2005). In addition, Ang II has also been shown to enhance the expression of Giα proteins through ERK1/2 signaling pathways in A10 VSMC (Li, et al., 2007). However, in the present study, we demonstrate that the phosphorylation of ERK1/2 and AKT by H_2O_2 in aortic VSMC may be responsible for the H_2O_2- induced enhanced expression of Giα proteins because PD98059 and wortmannin, the inhibitors of MAP kinase and PI3kinase, respectively restored the H_2O_2-induced enhanced level of Giα-2, and Giα-3 proteins to control levels. Furthermore, our results showing that the inhibitors of PDGFR-β and EGF-R attenuated the H_2O_2-induced enhanced phosphorylation of ERK1/2 to control levels suggest a role for PDGFR-β and EGF-R in the enhanced activity of ERK1/2 induced by H_2O_2 in VSMC. In this regard, H_2O_2 has been shown to increase the tyrosine phosphorylation of tyrosine kinase receptors in the absence of growth factors in VSMC and to induce the activation of downstream pathways such as MAPK and phosphatidylinositol-3 kinase (PI3K/protein kinase B/AKT) (Mehdi, Azar, & Srivastava, 2007). Furthermore, a role of receptor

tyrosine kinase transactivation in redox-dependent MAP kinase signaling by Ang II has also been shown in VSMC (Touyz, et al., 2003). Thus taken together, it may be suggested that the phosphorylation of PDGF-R and EGF-R by H_2O_2 activates downstream signaling pathways ERK1/2 and PI3K which in turn may be responsible for the increased expression of Giα-2 and Giα-3 proteins in aortic VSMC. The precise mechanism by which H_2O_2 transactivates growth factor receptor-protein tyrosine kinases (PTKs) is still not clear. However, ROS-mediated inhibition of tyrosine phosphatases (Denu & Tanner, 1998) that can shift the equilibrium of the phosphorylation-dephosphorylation cycle resulting in a net increase of tyrosine phosphorylation of R-PTKs and non receptor PTKs (Rhee, et al., 2000), may be a possible mechanism by which H_2O_2 induces transactivation of growth factor receptors.

In summary, we provide the first direct evidence that H_2O_2 that causes oxidative stress transactivates PDGFR-β and EGFR which through the activation of downstream signaling pathways including ERK1/2, and PI3K contribute to the enhanced expression of Giα-2 and Giα-3 proteins and inhibition of AC activity in VSMC. It may be suggested that the increased expression of Gi□ proteins and resultant decreased levels of cAMP induced by oxidative stress may be one of the factors responsible for the vascular remodelling and thereby vascular complications observed in various pathological states including hypertension, and atherosclerosis

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ABBREVIATIONS: H_2O_2, Hydrogen peroxide; Mitogen activated protein kinases (MAPK), Extracellular regulated kinase (ERK), Phosphatidyl inositide 3 kinase (PI3K), Epidermal growth factor receptors (EGFR), platelet-derived growth factor receptors (PDGFR); AC, Adenylyl

cyclase; VSMC, vascular smooth muscle cells; GTP, Guanosine triphosphate; Giα, inhibitory G protein; FSK, forskolin

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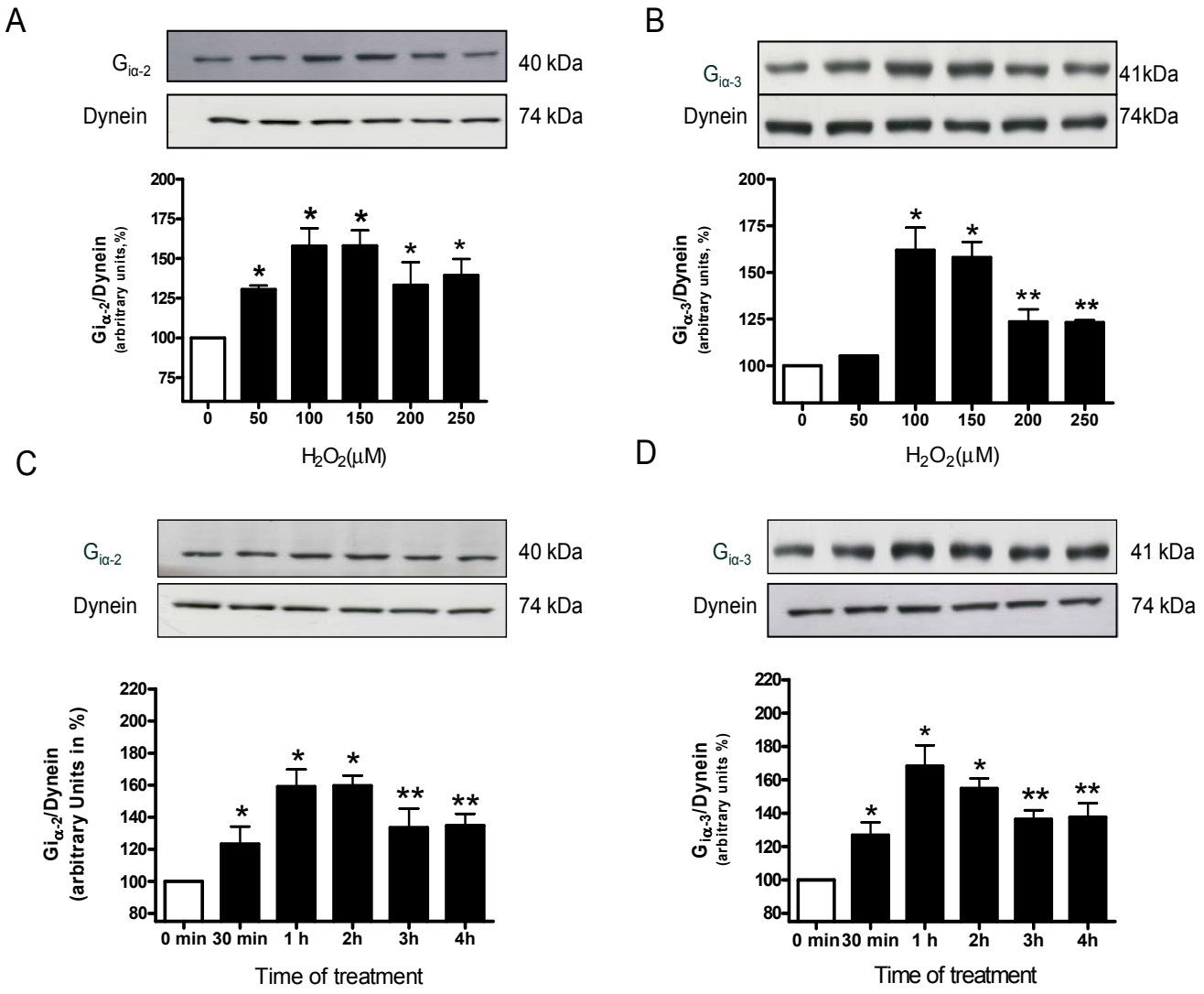
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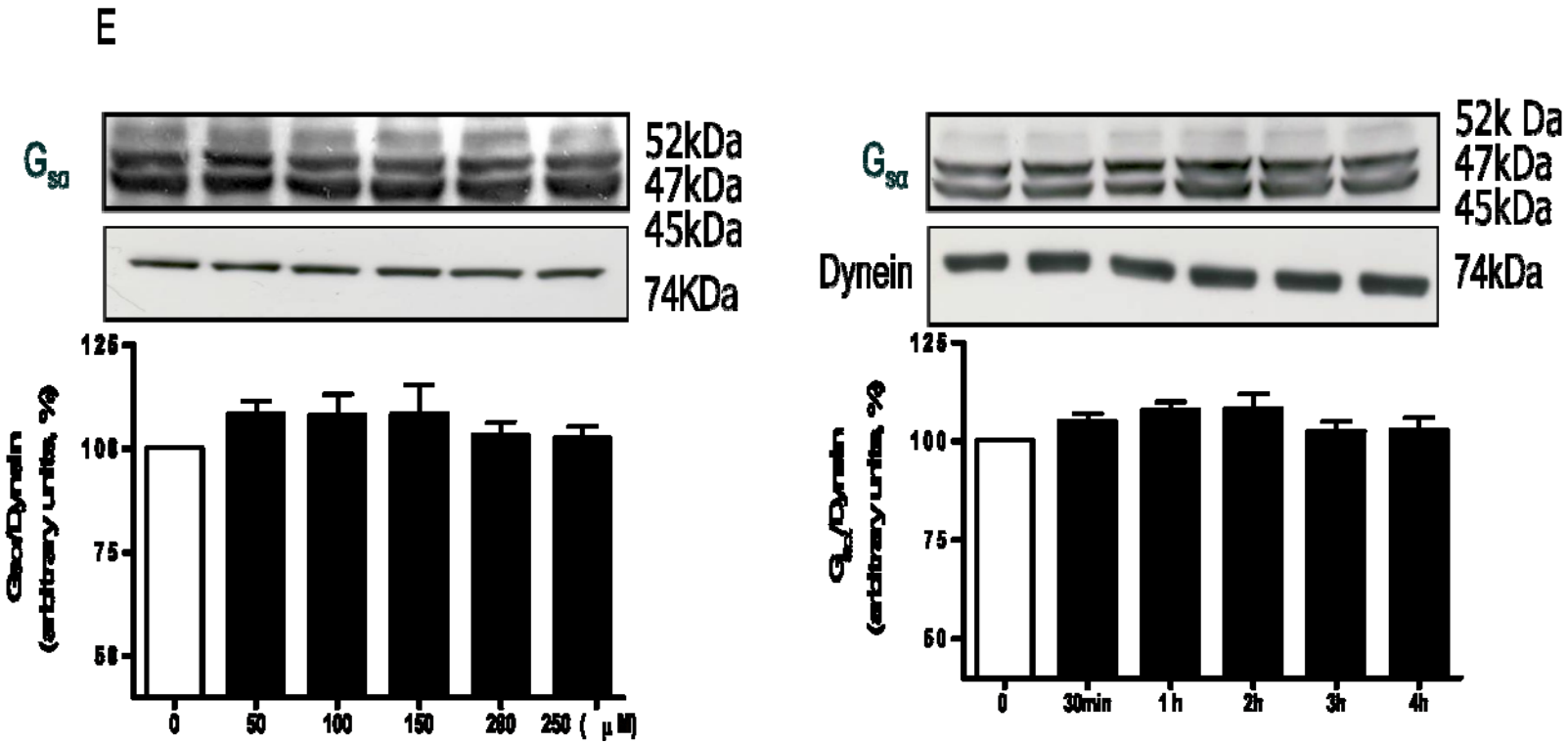

Figure 1. **Effect of various concentrations of H_2O_2 on the levels of Giα-2 and Giα-3 proteins in aortic vascular smooth muscle cells (VSMCs) from Sprague Dawley rats.** VSMCs were incubated in the absence (0μM) or presence of H_2O_2 (50 to 250μM) (A, B) for different time periods (30 min to 4h) (C, D) as described in Materials and Methods. Cell lysates were prepared and subjected to Western blotting using specific antibodies against Giα-2, Giα-3, and Gsα. Dynein was used as a loading control. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Data are means ± SE of 3 separate experiments. $^{*}P < 0.01$, vs 0 μM, $^{*}P < 0.01$ vs 0 min, $^{**}P < 0.05$ vs 100μM, $^{**}P < 0.05$ vs 1h

Figure 2

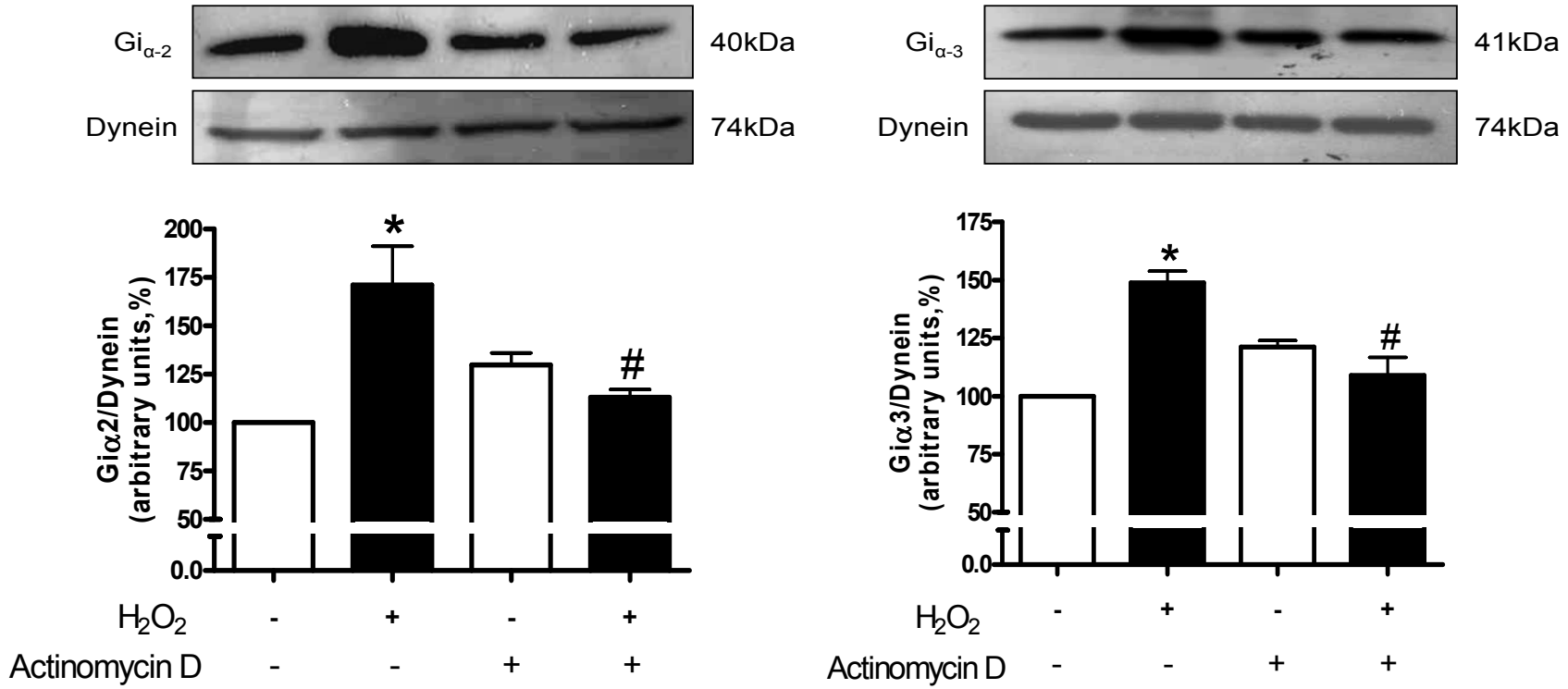

Figure 2. **Effect of actinomycin D on H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins in aortic VSMC.** Aortic VSMCs were pretreated without or with actinomycin D (5μM) for 24 hours, and were further incubated in the absence (white bars) or presence (black bars) of 100μM of H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using specific antibodies against Giα-2 and Giα-3 as described in Materials and Methods. Dynein was used as a loading control. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Values are means SE of 3 separate experiments. $^{*}P < 0.05$ vs CTL, $^{\#}P < 0.01$ vs H_2O_2

Figure 3

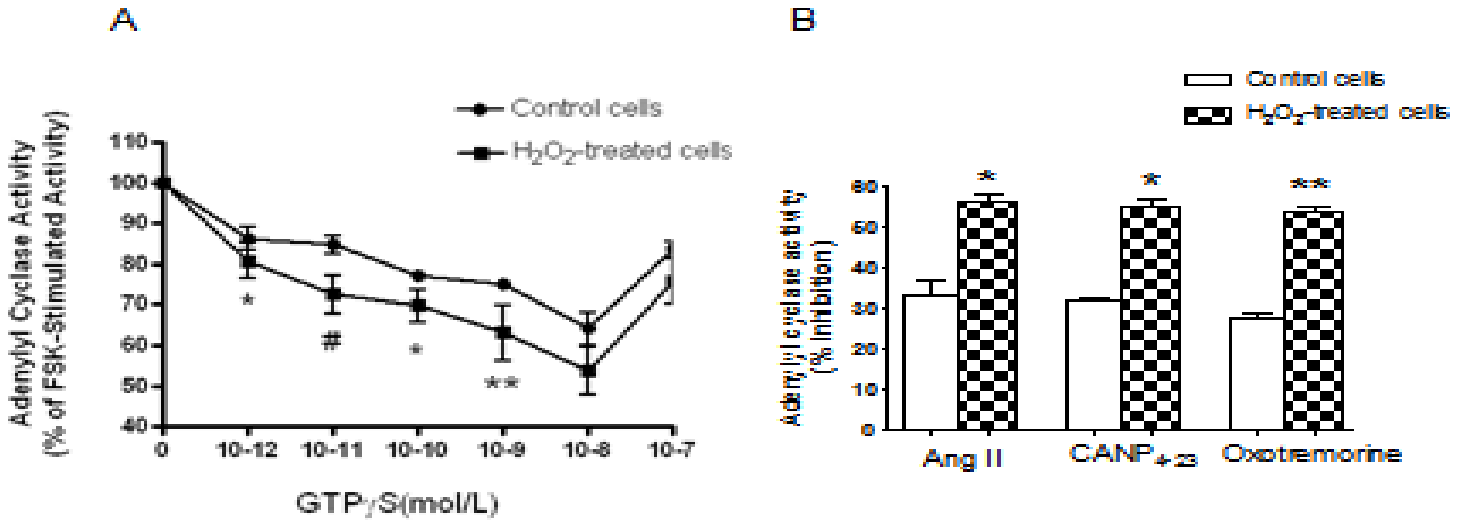

Figure 3. **(A) Effect of H_2O_2 on GTPγS-mediated inhibition of forskolin (FSK)-stimulated adenylyl cyclase activity in aortic vascular smooth muscle cells**. Aortic VSMC were incubated in the absence or presence of 100µM H_2O_2 for 1h and half. Membranes were prepared as described in Materials and Methods. Adenylyl cyclase activity was determined in these membranes in the presence of 100µM FSK alone, taken as 100%, and in the presence of various concentrations of GTPγS (10^{-12} to 10^{-7}M). Basal enzyme activity values in the absence of GTPγS in control or H_2O_2-treated cells were 102 ±2.1 pmol cAMP/mg protein min^{-1} and 68 ±1.5 pmol cAMP/mg protein min^{-1}.**(B) Effect of hydrogen peroxide (H_2O_2) on hormonal inhibition of adenylyl cyclase activity in aortic vascular smooth muscle cells (VSMC).** Aortic VSMC were treated without (white bars) or with H_2O_2 (100µM) for 1h and half. Adenylyl cyclase activity was determined in the presence of 10µMGTPγS alone, taken as 100% or in combination with 10µM Angiotensin II (Ang II), 0.1µM C-ANP_{4-23}, or 50µM oxotremorine. Basal enzyme activity values in the absence of GTPγS in control or H_2O_2-treated cells were 21 ±0.45 pmol cAMP/mg protein min^{-1} and 10 ±0.25 pmol cAMP/mg protein min^{-1} respectively. Values are means ± SE of 3 separate experiments. $^{*}P < 0.05$, $^{\#}P < 0.001$, $^{**}P < 0.05$ vs CTL

Figure 4

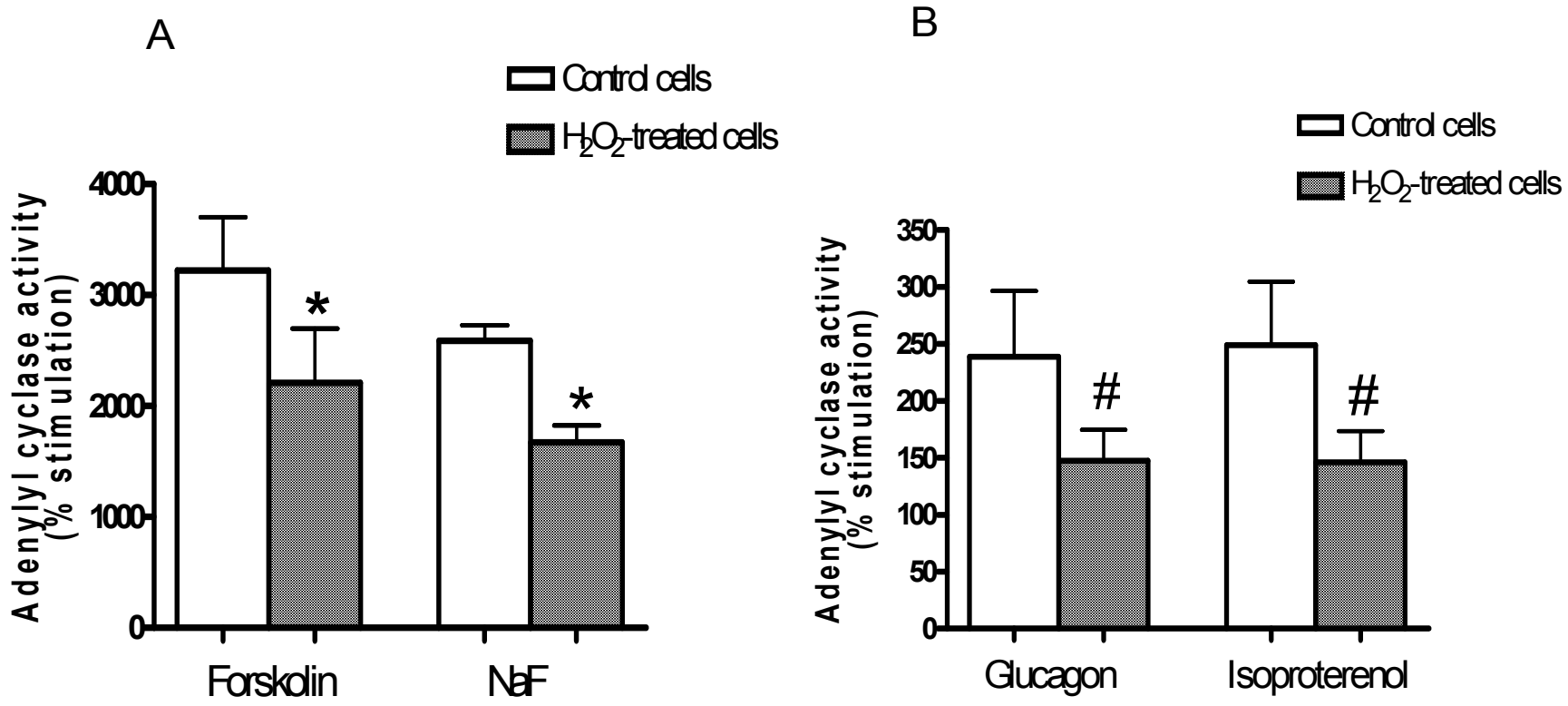

Figure 4. Effect of hydrogen peroxide on agonist-mediated stimulation of adenylyl cyclase in aortic vascular smooth muscle cells (VSMC). Aortic VSMC were treated without (white bars) or with H_2O_2 (100µM) for 1h and half. Membranes were prepared as described in Materials and Methods. Adenylyl cyclase activity was determined in the presence of 10µMGTP alone, taken as 100% or in combination with 50µM isoproterenol (Iso) or 1µM glucagon, in the absence or presence of 10mM sodium fluoride (NaF) or 50µM Forskolin (FSK). Basal adenylyl cyclase activity values in the absence of GTP in control or H_2O_2-treated cells were 24.6 ±0.18 pmol cAMP/mg protein min^{-1} and 16 ±0.71 pmol cAMP/mg protein min^{-1} respectivey.Values are means ± SE of 3 separate experiments. $^{*}P < 0.05$, $^{\#}P < 0.01$ vs CTL

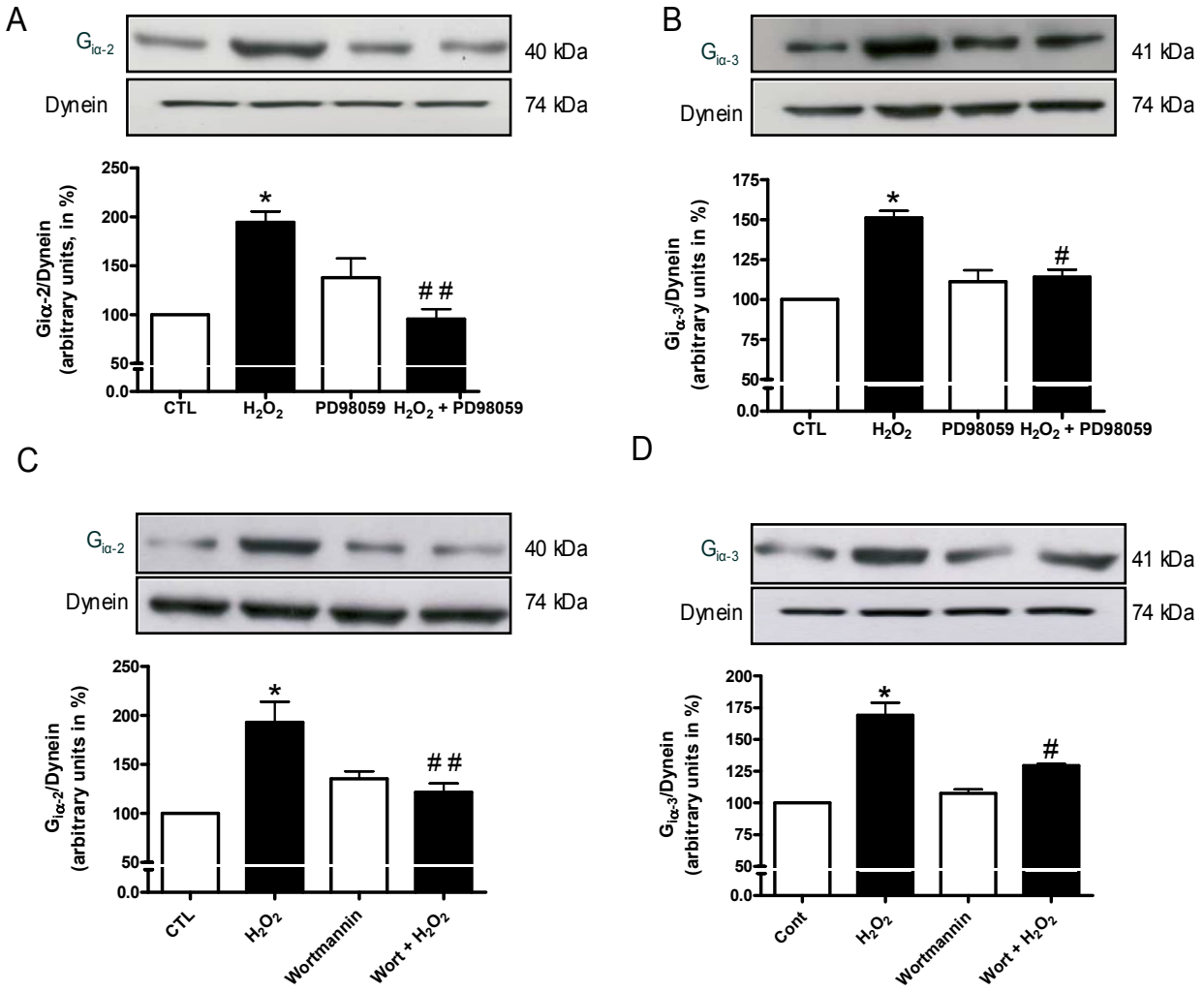

Figure 5. **Effect of ERK1/2 (5A, 5B) and AKT (PKB) (5C, 5D) inhibitors on H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins in aortic vascular smooth muscle cells**. Cells were pretreated without or with PD98050 (10μM) or wortmannin (0.1μM) for 1h, then stimulated with 100μM of H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using specific antibodies against Giα-2 and Giα-3 as described in Materials and Methods. Dynein was used as a loading control. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Values are means ± SE of 8 separate experiments. $^{*}P < 0.01$, vs CTL, $^{\#\#}P < 0.001$, $^{\#}P < 0.05$ vs H_2O_2

Figure 6

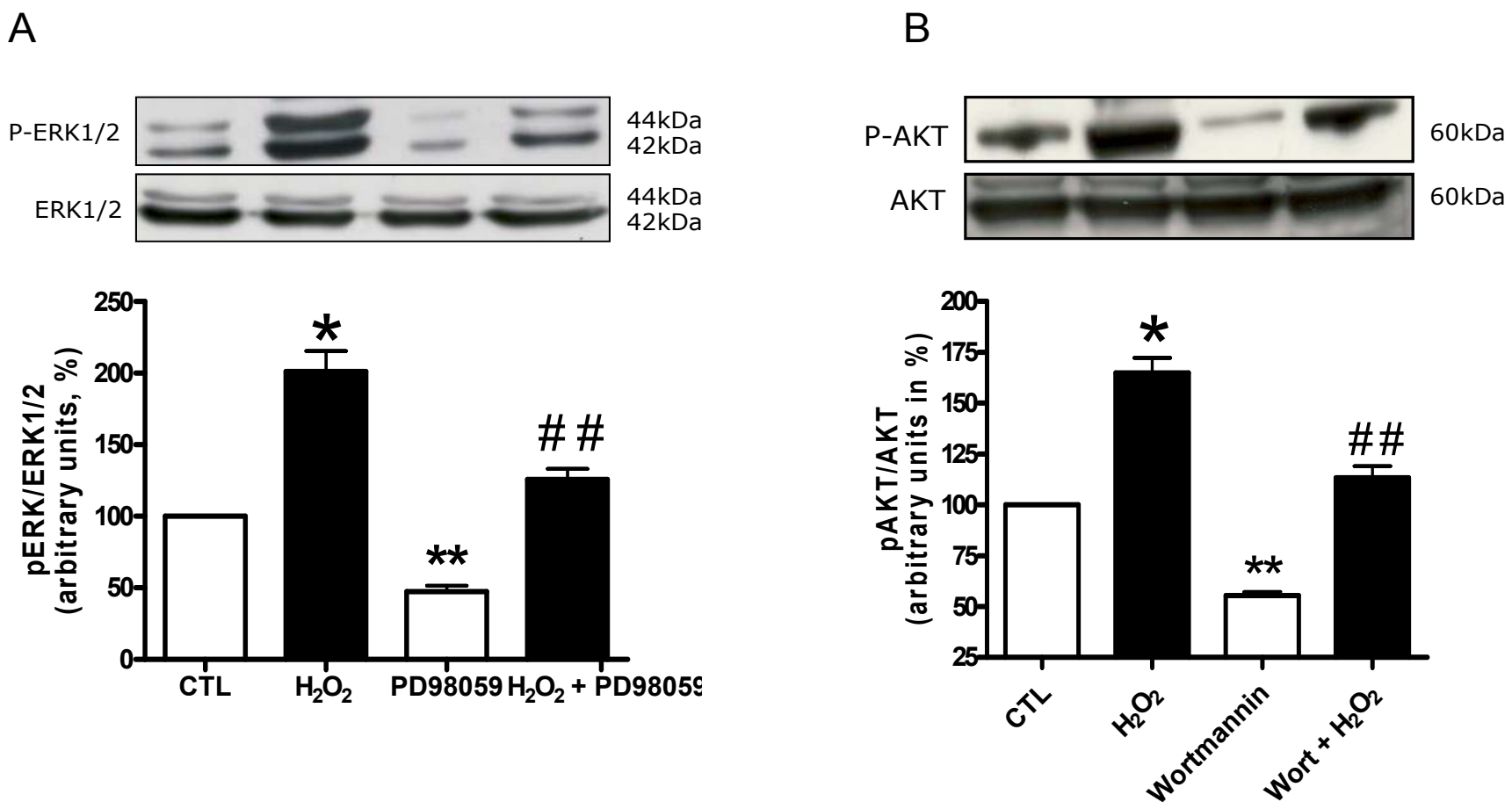

Figure 6. Effect of H_2O_2 on ERK1/2 and AKT phosphorylation in aortic vascular smooth muscle cells (VSMCs). Confluent cells were incubated in the absence or presence of PD98059 (10µM) or wortmannin (0.1µM) for 1h, then challenged with 100µM of H_2O_2 for 1h then challenged with 100µM of H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using phospho-specific-^{Tyr204} ERK or phosphospecific-serine^{473} AKT antibodies (top), and also analyzed for total ERK and total AKT (bottom) as described in Material and Methods. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Values are means ± SE of 3 to 6 separate experiments. $^{*}P < 0.01$, $^{**}P < 0.05$ vs CTL, $^{\#\#}P < 0.01$ vs H_2O_2

Figure 7

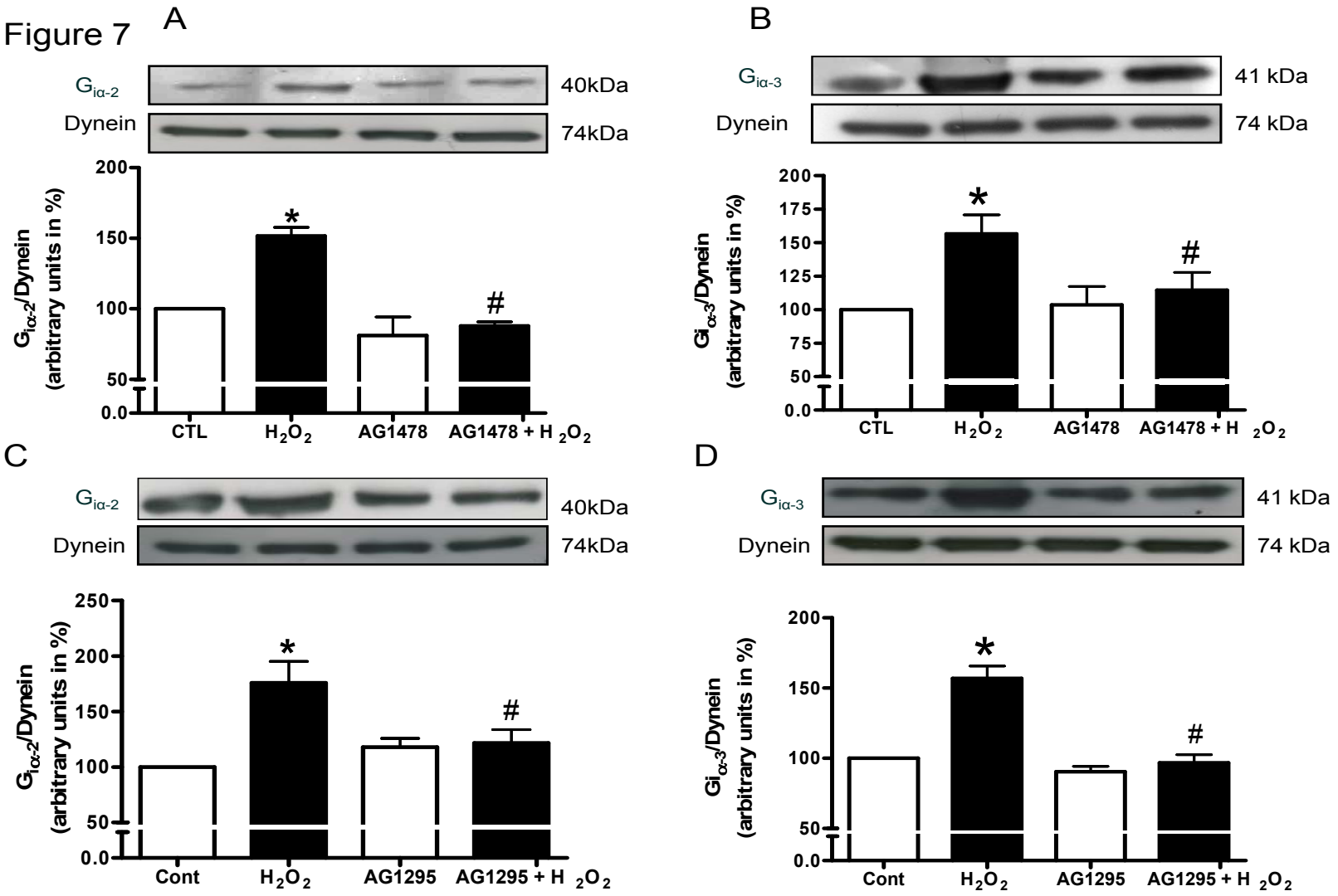

Figure 7. **Implication of EGFR, and PDGFR-β inhibitors on the expression of Giα-2 and Giα-3 proteins.** Aortic vascular smooth muscle cells (VSMC) were treated with either AG1478 (5μM) or AG1295 (5μM) for 1h, and then treated with 100μM H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using specific antibodies against Gi Giα-2 and Giα-3 as described in Materials and Methods. Dynein was used as a loading control. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%.Values are means ± SE of 4 separate experiments. $^{*}P < 0.05$ vs CTL, $^{\#}P < 0.01$ vs H_2O_2. $^{*}P < 0.05$ vs CTL, $^{\#}P < 0.01$ vs H_2O_2

Figure 8

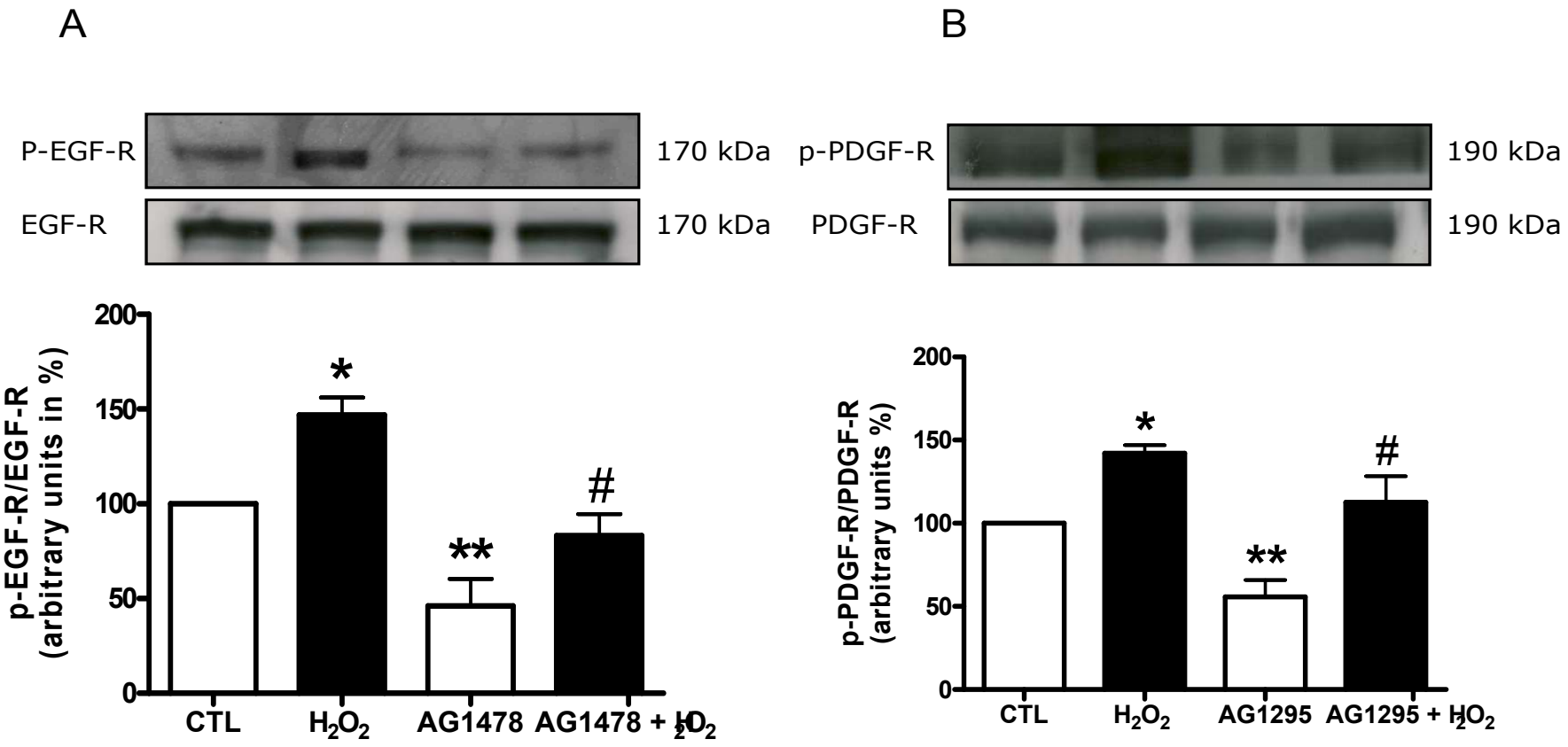

Figure 8. Effect of H_2O_2 on the phosphorylation of EGF-R and PDGFR-β in aortic vascular smooth muscle cells (VSMCs). Confluents aortic VSMCs were incubated in the absence or presence of AG1478 (5μM), or AG1295 (5μM) for 1h, then challenged with 100μM of H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using phosphospecific-tyrosine^{1173} EGF-R (8A) and phosphospecific tyrosine^{857} PDGFR-β (8B), and also analysed for total EGF-R, and PDGFR-β. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Values are means SE of 4 separate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs CTL, $^{\#}P < 0.01$ vs H_2O_2

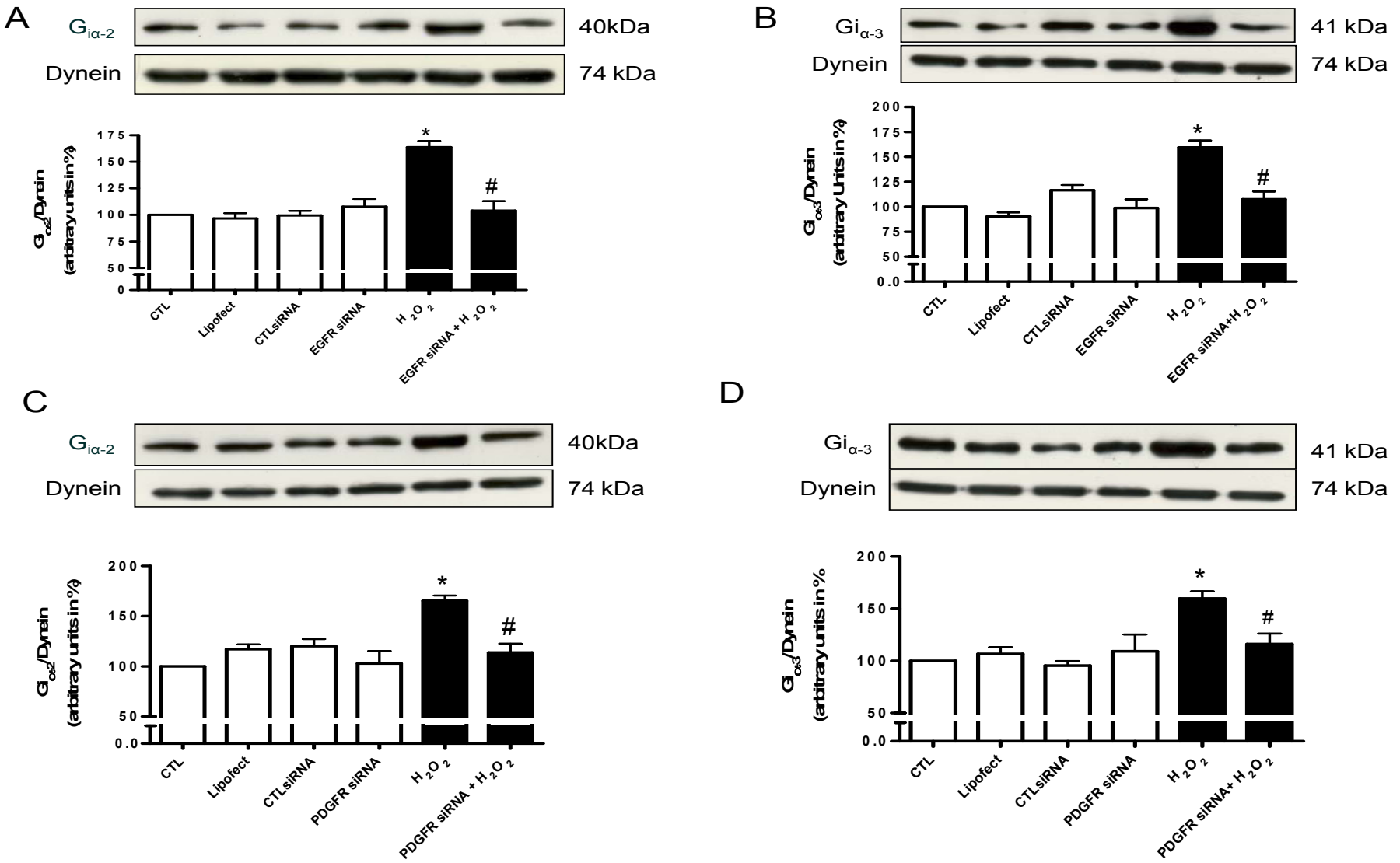

Figure 9. **Effect of EGFR, and PDGFR-β silencing on the expression of Giα-2, and Giα-3 proteins.** Aortic vascular smooth muscle cells (VSMC) were silenced with either EGFR (A, B) or PDGFR-β (C, D) siRNA for 48h, and then treated with 100μM H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using specific antibodies against Giα-2 and Giα-3 as described in Materials and Methods. Dynein was used as a loading control. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Values are means ± SE of 4 separate experiments. $^{*}P < 0.05$ vs CTL, $^{\#}P < 0.01$ vs H_2O_2

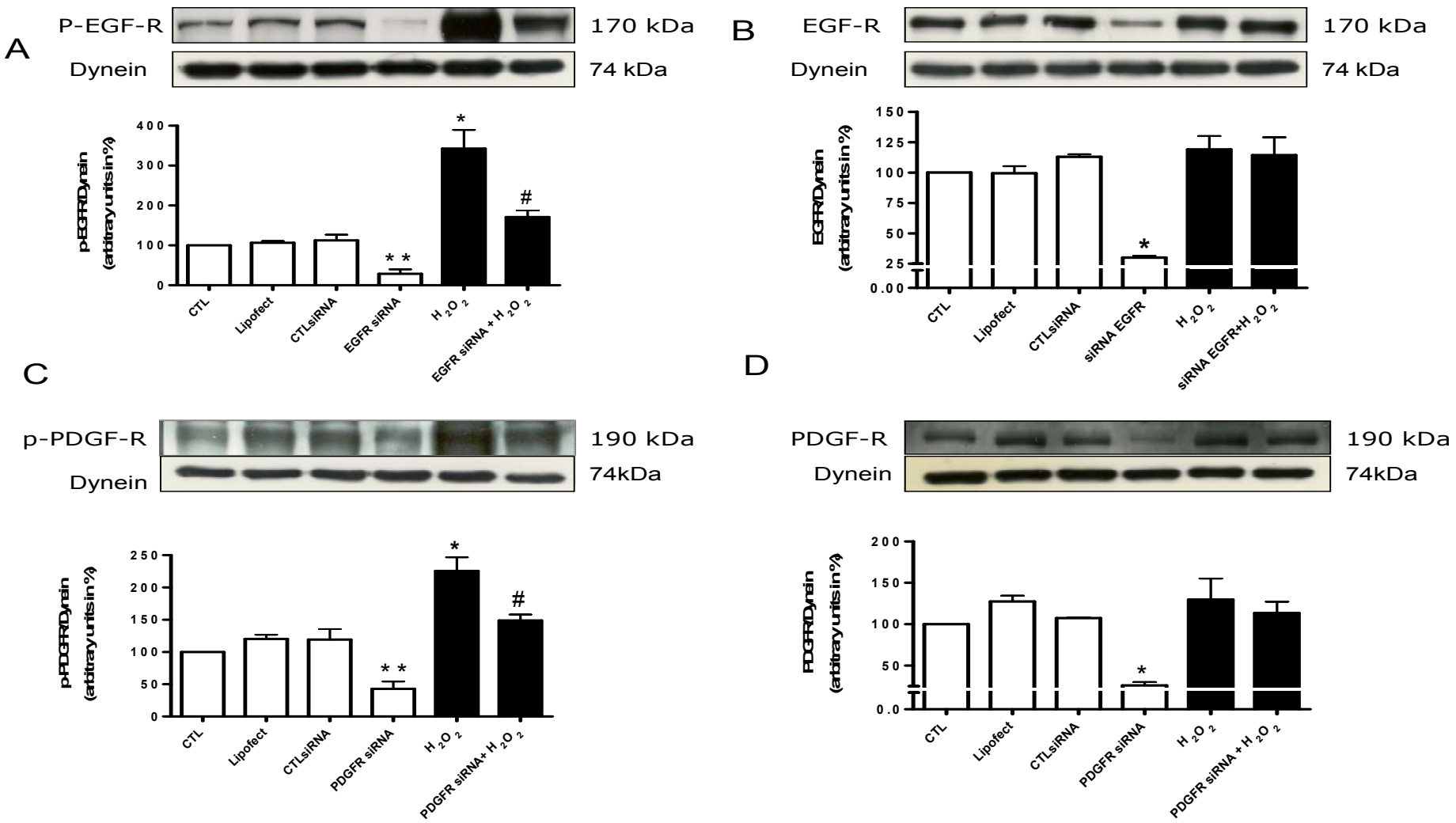

Figure 10. **Effect of hydrogen peroxide on the phosphorylation of EGF-R and PDGFR-β in aortic vascular smooth muscle cells (VSMC)**. Confluent VSMCs were treated with control siRNA (scrambled), or silenced with EGFR siRNA or PDGFR-β siRNA for 48h, and then stimulated with 100µM H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using phosphospecific-tyrosine^{1173} EGF-R (A) or phosphospecific tyrosine^{857} PDGFR-β (C) and also analyzed for total EGF-R (B) and total PDGFR-β (D) as described in Material and Methods. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Values are means ± SE of 4 separate experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ vs CTL $^{\#}P < 0.05$ vs H_2O_2

Figure 11

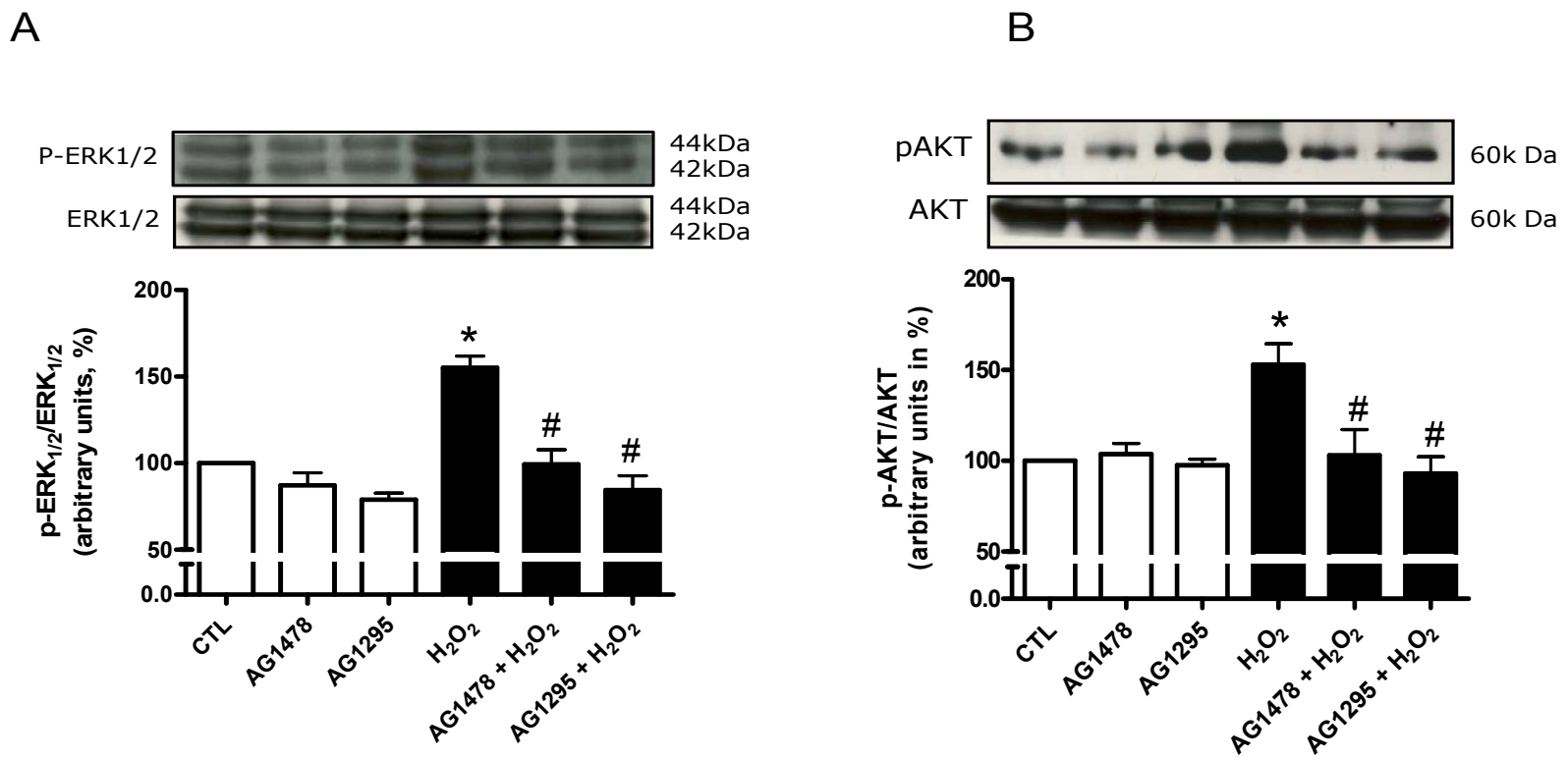

Figure 11. **Effect of growth factor receptors inhibitors on the phosphorylation of ERK1/2 and AKT in aortic VSMC treated with H_2O_2**. Confluent aortic VSMC were incubated in the presence of 5µM of AG1478 or 5µM of AG1295 for 1h, and then stimulated with 100µM H_2O_2 for 1h. Cell lysates were prepared and subjected to Western blotting using phospho-specific-Tyrosine^{204} ERK1/2 or phosphospecific-serine^{473} AKT antibodies (top) and also analyzed for total ERK1/2 and AKT (bottom) as described in Material and Methods. Proteins were quantified by densitometric scanning and plotted as a percentage of CTL taken as 100%. Values are means SE of 3 separate experiments.$^{*}P < 0.05$ vs CTL, $^{\#}P < 0.001$ vs H_2O_2

Chapter 3

Discussion, Conclusion and Perspectives

General discussion

Heterotrimeric guanine nucleotide proteins play a critical role in the regulation of a variety of signal transduction systems. Several studies have reported the impact of G proteins overexpression, mutations, and defects in the pathophysiology of many diseases (Farfel, Bourne, & Iiri, 1999). An enhanced Giα protein expression coupled with decreased adenylyl cyclase activity in myocardial membranes from hearts with dilated cardiomyopathy has been reported (Bohm, et al., 1990). Mutation of the gene encoding the β3 subunit of heterotrimeric Gs protein has been shown in patients with essential hypertension (Siffert, et al., 1998). Eschenhagen et al have reported an increase of the levels of Giα-2 mRNA in patients with congestive heart failure (Eschenhagen, et al., 1992). Our laboratory has reported the implication of Giα proteins and associated adenylyl cyclase signalling in various pathological conditions, such as hypertension (Li & Anand-Srivastava, 2002), heart failure, and diabetes (Hashim, Li, & Anand-Srivastava, 2006a). We demonstrated that the inactivation of the enhanced expression of Giα proteins by *pertussis toxin* attenuates the development of high blood pressure in SHR (Li & Anand-Srivastava, 2002). The increased expression of Giα-2, and Giα-3 proteins and mRNA in hearts and aortas from SHR (Anand-Srivastava, 1992), 1K1C (Bohm, et al., 1993), L-NAME (Hashim & Anand-Srivastava, 2004), and DOCA-salt hypertensive rats with established hypertension has also been reported (Anand-Srivastava, et al., 1993). Furthermore, we have reported an overexpression of Giα proteins before the onset of DOCA-salt-induced hypertension (Marcil, et al., 1998).

The Adenylyl cyclase/cAMP system is implicated in the control of heart contractility and vascular smooth muscle tone (Katz, Tada, & Kirchberger, 1975). Levels of cAMP are regulated by Giα and Gsα proteins. The Giα proteins are involved in the regulation of adenylyl cyclase inhibition (Itoh, et al., 1988). Alteration in Giα proteins expression, Adenylyl cyclase activity and cAMP levels have been reported in cardiovascular tissues from genetic models of SHR and experimentally induced models of hypertensive rats (Anand-Srivastava, Picard, & Thibault, 1991; Di Fusco & Anand-Srivastava, 2000; Marcil, et al., 1997). The Adenylyl cyclase/cAMP pathway is thought to be important in the initiation of cardiac hypertrophy because several vasoactive peptides such as Ang II and Endothelin that increase Giα protein can also trigger hypertrophic responses in cultured myocytes (Zolk, et al., 2000). In various model of hypertension including the genetic SHR (Saha, Li, Lappas, & Anand-Srivastava, 2008), the

glucose-induced model as well as the angiotensin II-induced model of hypertension, we have demonstrated an increase in the production of superoxide anion in VSMC (Li, et al., 2008). The implication of oxidative stress in the enhanced expression of Giα-2 and Giα-3 proteins in VSMC from SHR also has been reported (Lappas, et al., 2005). We also have demonstrated the link between enhanced production of reactive oxygen species and diseases of the cardiovascular system including hypertension and diabetes (Li, et al., 2008).

Although the role of oxidative stress in Ang II-mediated enhanced expression of Giα proteins and the indirect implication of signal transduction pathway including the MAPK have been reported, the specific signalling mechanisms by which reactive oxygen species enhance Giα proteins has not been elucidated. We have studied the implication of several signalling pathways including the ERK1/2, p38MAPK, C-Jun-N terminal protein kinase, PI3K and growth factors receptors in the H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins and associated Adenylyl cyclase (AC) in VSMC. To correlate the Giα protein expression with Giα functions, we examined the stimulatory effects of isoproterenol, FSK, NaF, and glucagon as well as the inhibitory effects of Ang II, oxotremorine, and C-ANP_{4-23} on AC activity. Aortic VSMC were treated with H_2O_2, in order to induce oxidative stress. Our first results showing that H_2O_2 induces the overexpression of Giα-2 and Giα-3 proteins not that of Gsα, in a concentration and time dependent-manner are in agreement with previously reported findings illustrating the role of oxidative stress in Ang II-induced enhanced expression of Giα-2 and Giα-3 proteins in A10 VSMC (Li, et al., 2007). To investigate whether H_2O_2 exerts its effects on Giα proteins at a transcriptional level, we studied the effect of Actinomycin D, an inhibitor of RNA synthesis. The H_2O_2-induced increased expression of Giα-2 and Giα-3 proteins was decreased to control level by Actinomycin D.

To examine whether the augmentation of Giα-2 and Giα-3 levels by H_2O_2 treatment is also reflected in Giα functions, we studied the effect of H_2O_2 pretreatment on AC activity. The AC activity is dually regulated by stimulatory and inhibitory receptors as wells as G proteins including Gsα and Giα (Bohm, et al., 1993). The relationship between increased expressions of Giα proteins and enhanced Giα functions has previously been reported (Anand-Srivastava, 1992). In SHR, a reduction of cardiac AC activity has been reported to occur (Robberecht, et al., 1981). In aortic VSMC pretreated with H_2O_2, the stimulatory response of glucacon and isoproterenol was significantly decreased by roughly 90% compared to control cells.

Furthermore, FSK, and NaF stimulated AC activity by a receptor independent mechanism by about 100 % (fold) in control cells compared to H_2O_2-treated cells. Since Giα-2 and Giα-3 levels were significantly increased in H_2O_2-treated smooth muscle cells than in control cells, the decreased responsiveness of AC to isoproterenol and glucagon in H_2O_2-pretreated cells can also be attributed to increased levels of Giα proteins. The decreased stimulation of AC by FSK and NaF in H_2O_2-pretreated VSMC is in agreement with earlier studies on A10 VSMC (Li, Hashim, & Anand-Srivastava, 2005) and SHR (Ge, Garcia, & Anand-Srivastava, 2006) and may be due to overexpression of Giα proteins or defective AC subunit or both. Since the levels of Gsα were not altered by H_2O_2 treatment of aortic VSMC, the decreased stimulation of AC by isoproterenol and glucagon is not due to Gsα, rather to the upregulation of Giα proteins. The overexpression of Giα-2 and Giα-3 proteins in H_2O_2-pretreated cells was further demonstrated by the greater inhibition of FSK-stimulated AC with low concentrations of GTPγS (an index of Giα function). GTPγS inhibited FSK-stimulated AC activity in a concentration dependent manner with a greater inhibition of about 25% in H_2O_2-treated cells. Moreover, the fact that the Ang II, C-$ANP_{4-23,}$ and oxotremorine-mediated inhibition of AC was significantly increased in H_2O_2-treated cells, compared with untreated control cells, may be explained by the increased expression of Giα-2 and Giα-3 proteins. We previously have established a correlation between the phosphorylation of MAPK and Gq/G11, and Giα proteins expression in aortic VSMC (Descorbeth & Anand-Srivastava, 2010; Li, et al., 2007). The identification of signaling pathways responsible for the H_2O_2-induced expression of Giα proteins is critical for understanding mechanisms that regulate vascular smooth muscle cell function, hence vascular pathological processes involved in hypertension.

Mitogen-activated protein kinases (MAPK) act as transducer of extracellular signalling via tyrosine-kinases growth factors and G-protein-linked receptors to elements regulating transcription (Sivaraman, Wang, Nuovo, & Malbon, 1997). The role of MAPK in cell growth, proliferation, and apoptosis has been demonstrated by several investigators (Zhang & Liu, 2002). However, we demonstrate for the first time a direct correlation between oxidative stress, ERK1/2 signalling pathway and Giα proteins expression. We report that the inhibition of ERK1/2 phosphorylation by PD98059 restores the H_2O_2-induced enhanced level of Giα-2, and Giα-3 proteins to control levels suggesting a role of ERK1/2 signalling pathway in the increased expression of Giα proteins. Previous studies from our lab have shown the implication of ERK1/2

signaling pathway in the enhanced expression of Giα proteins in VSMC from SHR (Lappas, et al., 2005). Moreover, the current study also reports the role of oxidative stress in the phosphorylation of ERK1/2 in aortic VSMC. The H_2O_2-induced enhanced phosphorylation of ERK1/2 was attenuated to control level by PD98059. However, Viedt and colleagues have reported that intracellular ROS are critical for AngII-induced activation of p38MAPK, JNK and ERK5 in VSMC, whereas phosphorylation of ERK1/2 appears to be redox insensitive (Viedt, et al., 2000). Touyz and collaborators also have shown that inhibition of ROS with antioxidants decreased Ang II-induced phosphorylation of p38MAPK, JNK and ERK5, but not that of ERK1/2 in VSMC (Touyz, et al., 2003). In contrast to these findings, Frank et al. have demonstrated that antioxidants diphenyleneiodonium (DPI) and N-acetyl cysteine (NAC) inhibit ERK1/2 activation produced by Ang II in VSMC and that exogenous H_2O_2 stimulate protein tyrosine phosphorylation of ERK1/2 (Frank, et al., 2000). Other investigators have reported the involvement of ROS in ERK1/2 phosphorylation in cardiac fibroblasts (Sano, et al., 2001) and cardiac myocytes (Nishida, et al., 2000). We also have reported that the enhanced expression of ERK1/2 phosphorylation in both A10 VSMC and VSMC from SHR is attenuated to control levels by antioxidant DPI, and NAC, suggesting the role of ROS in enhanced ERK1/2 phosphorylation (Lappas, et al., 2005; Li, et al., 2007). The reasons for the discrepancies between published results are still unclear. It is however, important to note that, the discrepancies lie in the activation of ERK1/2 pathway by ROS generated by Ang II, rather than exogenous ROS. In fact there is a consensus among published results that exogenous $O_2^{-\cdot}$ and H_2O_2 are potent activator of ERK1/2, p38 MAPK, JNK, and ERK5 (Droge, 2002; Thannickal & Fanburg, 2000).There are multiple pathways leading to ERK1/2 phosphorylation by Ang II, which differ between cell types. Previous reports have revealed that Ang II activates ERK via either c-Src or EGFR in cardiac fibroblasts (Murasawa, et al., 1998). Other reports suggest that Ang II-mediated ROS activation of ERK1/2 phosphorylation involves a tyrosine kinase-dependent, PKC-dependent (Zou, et al., 1996), or PI3K and MEK-dependent pathways in VSMC (Aikawa, et al., 1997). The MAP kinases effects of endogenous ROS seem to be ligand or cell-type specific. However, on the basis of the present results and previous findings, it is most likely that ROS induce ERK activation by a tyrosine kinase, MEK-dependent pathway in VSMC.

We also examined the implication of p38 MAPK and c-Jun N-terminal protein kinase on Giα proteins expression in VSMC. Although exogenous H_2O_2 induced the phosphorylation of both

p38 MAPK and JNK, the activation of these pathways does not seem to be involved in Giα-2 and Giα-3 proteins expression. Pretreatment of aortic VSMC with SB 203580 and dicoumarol, which are pharmacological inhibitors of p38 MAPK and JNK respectively, did not alter the expression of Giα-2 and Giα-3 proteins. One possible explanation for the differences in the stimulation of Giα proteins expression between ERK1/2 and p38 MAPK, JNK pathways lie in the physiological processes they induce. ERK1/2 is a major growth-signaling kinase, whereas JNK and p38 MAPK influence cell survival, apoptosis, inflammation, and differentiation (Pearson, et al., 2001). Activation of p38 MAPK and JNK would result in the phosphorylation of specific transcriptions factors responsible for cell death, and inflammation such as c-jun and ATF-2. On the other hand, activation of ERK1/2 signalling pathway leads to the phosphorylation of transcription factors involve in cell growth, and differentiation such as c-myc, c-fos and Elk1 (Zhang & Liu, 2002).

The PI3Ks are activated in response to growth factors and other agents, including vasoactive peptides, and have been reported to regulate cell adhesion and motility, cell differentiation, cell growth and apoptosis (Gomperts, et al., 2002). We have reported the implication of PI3K signalling in high glucose-induced enhanced expression of Gq/G11α and PLCβ in aortic VSMC (Descorbeth & Anand-Srivastava, 2010). Pretreatment of Aortic VSMC with wortmannin attenuated the H_2O_2-induced enhanced level of Giα-2, and Giα-3 proteins to control levels, which suggest the implication of PI3-K signaling in the enhanced expression of Giα proteins. PKB/AKT is considered to be a marker for PI3K activation since PKB/AKT activation is thought to occur via the binding of PtdIns $(3,4,5)P_3$ to the pH domain of PKB/AKT, thereby causing a proportion of AKT to translocate to the plasma membrane where it undergoes conformational changes and is subsequently phosphorylated on Ser^{473} by PtdIns$(3,4,5)P_3$-dependent kinase (Qin & Chock, 2003). In our studies, exogenous H_2O_2 was shown to increase the phosphorylation of Ser^{473} on PKB/AKT. The phosphorylation was blocked by wortmannin, implicating PI3K as an upstream mediator of H_2O_2 response. Consistent with our findings, a direct activation of PI3Ks by H_2O_2 in VSMC has been reported (Ushio-Fukai, et al., 1999). The underlying mechanisms responsible for PKB/AKT activation in response to H_2O_2 in VSMC have not been fully elucidated. One possible explanation is that H_2O_2 might simply triggers membrane translocation of PI3K and enables it to access it substrate (PtdIns$(3,4)P_2$, thereby enhancing it catalytic efficiency to PtdIns $(3,4,5)P_3$ production. Other reports have suggested the role of EGF-R,

PDGF-Rβ, as well as src family of protein tyrosine kinases such as c-Src and fyn in the activation of PI3K signalling pathway (Abe, Okuda, Huang, Yoshizumi, & Berk, 2000; Jin, et al., 2000). Phosphorylation of growth factors receptors is an important step in both MAPK, and PI3K activation. We tested the hypothesis that H_2O_2-induced activation of ERK1/2 and PI3K is mediated via growth factors receptors transactivation in VSMCs. The H_2O_2-induced phosphorylation of p-ERK1/2 and p-AKT were restored to control levels by EGF-R and PDGF-Rβ inhibitors AG1478 and AG1295 respectively. These results are in accordance with our previous findings illustrating that high glucose-induced enhanced phosphorylation of ERK1/2 and AKT is restored to control levels by growth factors inhibitors, AG1478 and AG1295 (Descorbeth & Anand-Srivastava, 2009). Our findings suggest the implication of EGF-R and PDGFR-β transactivation in H_2O_2-induced enhanced activation of ERK1/2 and AKT. Kim et al. have shown that EGF induces vasoconstriction through the PI3K mediated MAPK pathway in DOCA-salt hypertensive rats (Kim, et al., 2006). Other investigators have demonstrated the role of PDGFR-β in the activation of ERK1/2 and AKT. The inhibition of PDGF-Rβ tyrosine phosphorylation by melittin a bioactive component of bee venom toxin also inhibits downstream intracellular signal transduction pathways such as ERK1/2 and AKT/PKB in rat aortic VSMC further confirming the involvement of growth factors receptors in ERK1/2 and AKT phosphorylation (Son, et al., 2007).

Migration and proliferation of VSMCs is critical in the pathogenesis of vascular diseases, and the activation of growth factor receptors including EGF-R and PDGF-R play a major role in vascular remodelling (Touyz, 2005). We have reported the contribution of EGF receptor transactivation in Ang II-induced enhanced expression of Giα proteins and proliferation in A10 VSMC (Gomez Sandoval, et al., 2009). Vasoactive peptides such as Ang II and endothelin mediate their physiological effects through the activation of growth factors receptors EGF-R, PDGF-R and IGF-R in a variety of cell types (Itoh, Mukoyama, Pratt, Gibbons, & Dzau, 1993). Aortic VSMC exposed to H_2O_2 exhibit an enhanced phosphorylation level of EGF-R and PDGFR-β and the inhibition of these receptors by AG1478 and AG1295 respectively, attenuated the enhanced expression of Giα-2 and Giα-3 proteins to control levels. Furthermore, the suppression of EGF-R and PDGFR-β expression with siRNA decreased the H_2O_2-induced enhanced phosphosphorylation of EGF-R and PDGFR-β with subsequent decrease of Giα proteins expression. In summary, hydrogen peroxide increases the expression of Giα-2 and Giα-3 proteins

via the phosphorylation of growth factor receptors EGF-R, and PDGFR-β, which in turn activate downstream signalling pathways including ERK1/2, and PI3K. The induction of the above pathways may trigger transcription of genes responsible for Giα proteins synthesis. Several reports have established the link between the activation of growth factors receptors and hypertension. Sarzani et al. showed that hypertension induces high expression of PDGF-Rα and β in the aorta of DOCA-salt hypertensive rats (Sarzani, Arnaldi, & Chobanian, 1991). Other evidence shows that in stroke-prone SHR, treatment with ACE inhibitor reduced aortic PDGF-R-β phosphorylation and ERK1/2 activity (Kim, Zhan, et al., 2000). Kagiyama et al. also have demonstrated that the inhibition of EGF-R with specific antisense oligonucleotides decreases blood pressure and prevents cardiac hypertrophy in Ang II-induced hypertension (Kagiyama, et al., 2002). These findings show the implication of growth factors receptors in vascular diseases. Moreover, the role of hydrogen peroxide in the activation of growth factor receptors has been reported. Exogenous H_2O_2 induces tyrosine phosphorylation and activation of PDGFR-β and EGF-R in VSMC (Droge, 2002). The precise mechanisms by which H_2O_2 induces the phosphorylation of growth factors receptors have been studied extensively.

Hydrogen peroxide targets proteins containing reactive cysteine (Cys) residues. The pK_a of the sulfhydryl group (Cys-SH) of most Cys residues is approximately 8.5, because unaltered Cys-SH groups are crucial for the catalytic and structural functions of many proteins (Kim, Yoon, Kwon, Lee, & Rhee, 2000). Since the Cys-SH group is less readily oxidized by H_2O_2 than the cysteine thiolate anion ($Cys\text{-}S^-$), few proteins are vulnerable to oxidation by H_2O_2 in cells. However, some protein cysteine residues have low pK_a values and exist as $Cys\text{-}S^-$ at neutral pH due to nearby positively charged amino acid residues that are available for interaction with the negatively charged $Cys\text{-}S^-$ (Rhee, et al., 2003). Proteins with low pKa cysteine residues include protein tyrosine phosphatases (PTP). All PTP contain cysteine residues (pKa, 4.7 to 5.4) in the catalytic domain (Denu & Tanner, 1998). Exogenous H_2O_2 induces tyrosine phosphorylation and activation of PDGFR-β and EGFR, probably due to ROS-mediated inhibition of tyrosine phosphatases. Protein tyrosine phosphatases (PTP) are susceptible to oxidation and inactivation by H_2O_2 (Rhee, et al., 2003). PTPs exist in two forms, an active state with a reduced cysteine or an inactive state with an oxidized cysteine. The reversible oxidation of the cysteine residues to sulfenic acid by H_2O_2 renders PTPs inactive (Brigelius-Flohe, Banning, Kny, & Bol, 2004). The inhibition of PTPs by H_2O_2 results in increase tyrosine phosphorylation of protein tyrosine

kinase and non-receptor tyrosine kinase which promote the activation of downstream signalling cascade including MAPK and PI3K. Recent reports have indicated that other mechanisms may be involved in the activation of EGF-R and PDGF-R by H_2O_2. Eguchi et al. have shown that metalloprotease-dependent HB-EGF cleavage is required for EGF-R activation by H_2O_2 in VSMC (Eguchi, et al., 2001), and Chen et al. demonstrated the role of c- Src in H_2O_2-stimulated EGF-R activation in endothelial cells (Chen, Vita, Berk, & Keaney, 2001). Our laboratory also has reported the implication of c-Src in high glucose-induced EGF-R and PDGF-R transactivation that contributes to the increased expression of Gq/11α and PLCβ proteins in VSMC (Descorbeth & Anand-Srivastava, 2009). The mechanisms involved in the activation of c-Src by H_2O_2 are still unclear. Furthermore, it remains unclear whether the transactivation of growth factors receptors by H_2O_2 alone is sufficient to induce the enhanced expression of Giα-2 and Giα-3 proteins in aortic VSMC. Growth factor receptors have been proposed to interact with GPCR during growth factor stimulation, and subsequently activating c-Src family kinases. It has been reported that GPCR can associate with PDGF-R to form a functional signalling complex in human embryo kidney cells (Pyne, Waters, Moughal, Sambi, & Pyne, 2003). In addition, the inhibition of Giα protein by *pertussis toxin* partially suppress both the PDGFR-β-induced ROS production, and the downstream ERK1/2, JNK, and AKT activation in the lens epithelial cells (Chen, et al., 2007). Taken together, it may be possible that the H_2O_2-induced enhanced expression of Giα-2 and Giα-3 proteins requires the concerted activation of upstream membrane-associated components of growth factors receptors including c-Src, GPCR as well as PI3K and ERK1/2 signalling.

General conclusion

We have provided the first evidence demonstrating that H_2O_2 increases the levels of Giα proteins without affecting the levels of Gsα in VSMC. Our studies demonstrate the role of oxidative stress in the enhanced expression of Giα-2 and Giα-3 proteins and associated adenylyl cyclase signalling. The increase expression of Giα proteins was reflected in enhanced Giα and decreased Gsα-mediated functions. The activation of PDGFR-β and EGFR by H_2O_2 triggers the activation of downstream signalling pathways including ERK1/2, and PI3K which may be responsible for enhanced expression of Giα-2 and Giα-3 proteins. The increased expression of Giα proteins results in the inhibition of adenylyl cyclase activity.

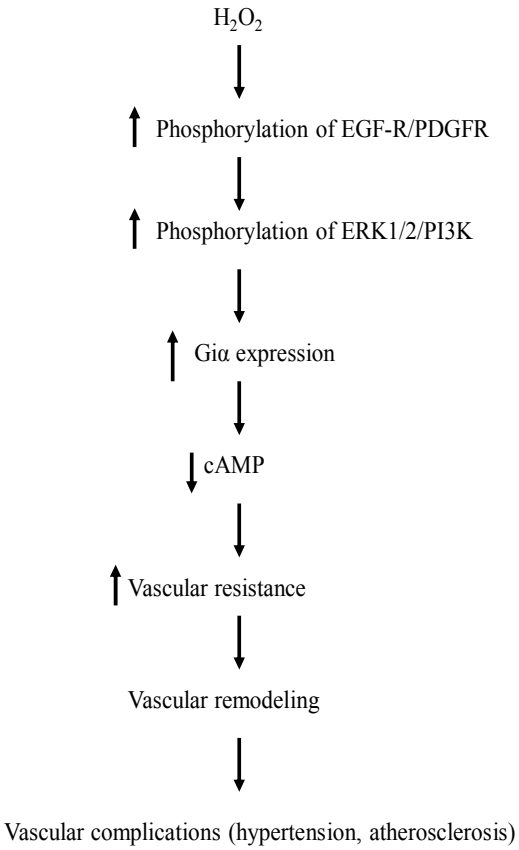

Figure 7. **Proposed mechanism**: Mechanism responsible for the enhanced expression of Giα proteins by H_2O_2 in aortic vascular smooth muscle cells.

Future work

Our study elucidates some of the signalling mechanisms involved in the overexpression of Giα proteins by hydrogen peroxide. We demonstrate that the phosphorylation of growth factors receptors EGFR and PDGFR-β is critical to the H_2O_2-induced overexpression of Giα proteins. It will be of interest to also investigate the involvement of other kinases or signalling molecules such as c-Src, and metalloproteases in the activation of growth factors receptors by H_2O_2. The implication of both c-Src and metalloproteases in EGF-R activation has been reported (Chen, et al., 2001; Eguchi, et al., 2001). Vascular remodelling during hypertension includes cellular

hypertrophy and hyperplasia, as well as enhanced protein synthesis (Lehoux & Tedgui, 1998). A direct role of oxidative stress in hypertension could be explored by studying the effect of H_2O_2 on the proliferation and hypertrophy of aortic VSMC by detection of tritiated thymidine H^3 uptake. In vivo studies could further establish the correlation between increased Giα proteins expression, oxidative stress, and hypertension. Studies have shown that H_2O_2 increases Ca^{2+} in VSMC and endothelial cells (Lounsbury, et al., 2000). It has also been shown that oxidative stress increases intracellular Ca^{2+} which can regulate the phosphorylation and dephosphorylation of proteins and modulate signal transduction pathways (Ermak & Davies, 2002). It will be of interest to examine the role of Ca^{2+} in the H_2O_2-induce enhanced expression of Giα proteins

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