Université de Montréal

Modulation of Endothelin-1 and Insulin-like Growth Factor Type 1-induced Signaling by Curcumin in A-10 Vascular Smooth Muscle Cells

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Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de Maîtrise en Sciences (M.Sc)
en Sciences Biomédicales
option Générale

Août 2011

Université de Montréal
Faculté des études supérieures et postdoctorales

Ce mémoire intitulé :

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Résumé

Les maladies cardio-vasculaires (MCV), telles que l'hypertension et l'athérosclérose, s'accompagnent de modifications structurales et fonctionnelles au niveau vasculaire. Un fonctionnement aberrant de la migration, l'hypertrophie et la prolifération des cellules musculaires lisses vasculaires (CMLV) sont des évènements cellulaires à l'origine de ces changements. L'endothéline-1 (ET-1) contribue à la pathogénèse des anomalies vasculaires, notamment via l'activation des protéines MAPK et PI3-K/PKB, des composantes clés impliquées dans les voies prolifératives et de croissance cellulaires. Il a été suggéré que le stress oxydant jouerait un rôle intermédiaire dans les effets pathophysiologiques vasculaires de l'ET-1. En conséquence, une modulation de la signalisation induite par l'ET-1 peut servir comme éventuelle stratégie thérapeutique contre le développement des MCV. Il apparaît de nos jours un regain d'intérêt dans l'utilisation des agents phyto-chimiques pour traiter plusieurs maladies. La curcumine, constituant essentiel de l'épice curcuma, est dotée de plusieurs propriétés biologiques parmi lesquelles des propriétés anti-oxydantes, anti-prolifératrices et cardio-protectrices. Cependant, les mécanismes moléculaires de son effet cardio-protecteur demeurent obscurs. Dans cette optique, l'objectif de cette étude a été d'examiner l'efficacité de la curcumine à inhiber la signalisation induite par l'ET-1 dans les CMLV. La curcumine a inhibé la phosphorylation des protéines IGF-1R, PKB, c-Raf et ERK1/2, induite par l'ET-1 et l'IGF-1. De plus, la curcumine a inhibé l'expression du facteur de transcription Egr-1 induite par l'ET-1 et l'IGF-1, dans les CMLV. Ces résultats suggèrent que la capacité de la curcumine à atténuer ces voies de signalisation serait un mécanisme d'action potentiel de ses effets protecteurs au niveau cardiovasculaire.

Mots-clés : CMLV, Curcumine, Egr-1, ERK1/2, ET-1, IGF-1, IGF-1R, PKB.

Abstract

Cardiovascular diseases (CVDs), including hypertension and atherosclerosis, are associated with vascular functional and structural changes. Some of the cellular events underlying these processes include aberrant vascular smooth muscle cell (VSMC) proliferation, hypertrophy and migration. Endothelin-1 (ET-1) has been implicated in the pathogenesis of vascular abnormalities through the hyperactivation of key components of growth promoting and proliferative signaling pathways, including MAPKs and PI3-K/PKB. Vascular oxidative stress has also been suggested to play an intermediary role in mediating ET-1-induced pathophysiological effects. Interference with ET-1-induced signaling may therefore serve as a potential therapeutic strategy against the progression of cardiovascular disorders. There is presently a surge of interest in the use of plant-derived phytochemicals for the treatment of various diseases. Curcumin, the main constituent of the spice turmeric, exhibits multiple biological properties, amongst them, antioxidant, anti-proliferative and cardioprotective properties. However, the molecular mechanisms of its cardiovascular protective action remain obscure. Therefore, in the present studies, we investigated the effectiveness of curcumin to inhibit ET-1-induced signaling events in VSMC. Curcumin inhibited ET-1-induced as well as IGF-1-induced phosphorylation of IGF-1R, PKB, c-Raf and ERK1/2, in VSMC. Furthermore, curcumin inhibited the expression of transcription factor early growth response-1 (Egr-1) induced by ET-1 and IGF-1, in VSMC. In summary, these results demonstrate that curcumin is a potent inhibitor of ET-1 and IGF-1-induced mitogenic and proliferative signaling events in VSMC, suggesting that the ability of curcumin to attenuate these effects may contribute as potential mechanism for its cardiovascular protective response.

Keywords: Curcumin, Egr-1, ERK1/2, ET-1, IGF-1, IGF-1R, PKB, VSMC

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Liste des abréviations, sigles et acronymes

ANF	atrial natriuretic factor
Ang II	Angiotensin II
ANP	atrial natriuretic peptide
AP-1	activator protein 1
ApoE	apolipoprotein E
AT1R	Angiotensin II type 1 receptor
BAD	Bcl-2 associated death promoter
Bcl-2	B-cell lymphoma 2
bFGF	basic fibroblast growth factor
BNP	brain natriuretic peptide
Ca^{2+}	calcium
cGMP	cyclic guanosine monophosphate
CHF	chronic heart failure
COX-2	cyclooxygenase-2
CSF	colony-stimulating factors
CVD	cardiovascular disease
DAG	diacyglycerol
DOCA	deoxycorticosterone acetate
ECE	endothelin converting enzyme
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
Egr-1	early growth response-1
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
ET	endothelin
ET_A	endothelin receptor type A
ET_B	endothelin receptor type B
FGF	fibroblast growth factor

G protein	guanosine nucleotide binding protein
GDP	guanosine diphosphate
GPCR	guanosine nucleotide binding protein-coupled receptor
GPx	glutathione peroxidase
Grb2	growth factor receptor-bound protein 2
GSK-3	glycogen synthase kinase 3
GSH	glutathione
GST	glutathione-s-transferase
GTP	guanosine triphosphate
H_2O_2	hydrogen peroxide
HAT	histone acetyltransferase
HDL	high density lipoprotein-cholesterol
HIF	hypoxia inducible factor
HO-1	heme oxygenase-1
IGF-1	insulin-like growth factor type 1
IGF-1R	insulin-like growth factor type 1 receptor
IL	interleukin
iNOS	inducible nitric oxide synthase
ISO	$_L$-isoproterenol
IR	insulin receptor
IRS	insulin receptor substrate
IP_3	inositol triphosphate
JNK	c-Jun N-terminal kinase
kDa	kiloDalton
LDL	low-density lipoprotein-cholesterol
$_L$-NAME	N^{ω}-nitro-L-arginine methyl ester
LOX	lipoxygenase
LOX-1	oxidized low density lipoprotein receptor 1
MAPK	mitogen activated protein kinase
MEF2	myocyte enhancer factor 2

MEK	mitogen extracellular regulated kinase
MI	myocardial ischemia
MMP	matrix metalloproteinase
mTOR	mammalian target of rapamycin
NADPH oxidase	nicotamide adenine dinucleotide phosphate oxidase
NF-1	nuclear factor 1
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
($O_2^{\cdot -}$)	superoxide anion
($OH^{\cdot}$)	hydrogen radicals
($ONOO^-$)	peroxynitrite
p53	tumor protein 53
$p70^{s6k}$	p70 ribosomal S6 kinase
$p90^{rsk}$	p90 ribosomal kinase
PDGF	platelet derived growth factor
PDGF-R	platelet derived growth factor receptor
PDK	phosphoinositide-dependent kinase
PH	pleckstrin homology
PI3-K	phosphatidylinositol 3-kinase
PI	phosphatidylinositol
PI (4,5)P_2	phosphatidylinositol 4, 5 triphosphate
PI (3, 4,5)P_3	phosphatidylinositol 3, 4, 5 triphosphate
PLC β	phosphoinositide-specific phospholipase C β
PKB	protein kinase B
PKC	protein kinase C
PTK	protein tyrosine kinase
PTPase	phosphatase
PYK-2	proline-rich tyrosine kinase
R-PTK	receptor protein tyrosine kinase
ROS	reactive oxygen species

SH2	src homology 2
Shc	src homology collagen
SHR	spontaneously hypertensive rat
SHP-2	SH2 domain-containing tyrosine phosphatase-2
SOD	superoxide dismutase
SOS	son of seven less
STAT	signal transducer and activator of transcription
TNF-α	tumour necrosis factor-α
TGF-β	transforming growth factor-β
VSMC	vascular smooth muscle cell

Remerciements

First and foremost, I owe my deepest gratitude to my supervisor Dr Ashok K. Srivastava whose endless encouragement, guidance, support and sincerity has been my inspiration as I hurdle all the obstacles in the completion of this research work. One could not wish for a better or friendlier supervisor. His timely advice, his kind and patient instructions, and his commitment towards his students are exemplary of true mentorship.

I thank my fellow labmates George Vardatsikos, Ali Bouallegue, Estelle Rolande Simo, Viktoria Youreva and Ahmed Mir, to whom I am very grateful for their unconditional support and help. Their friendship and support has truly made a difference in my life. I would also like to thank all other labmates at the MDRC for making this experience enriched through their suggestions and assistance.

Lastly I would like to thank my family for all their love, encouragement and support in all my pursuits.

CHAPITRE 1

INTRODUCTION

Cardiovascular diseases (CVDs), including hypertension, atherosclerosis and heart failure, are among the most encountered pathologies that affect developed countries. Even though significant advances in diagnosis and treatment have been introduced, CVDs remain one of the leading causes of mortality and morbidity. Many pathways are implicated in the regulation of cardiovascular homeostasis and a variety of factors can contribute to the onset of CVDs. Among those factors are two important vasoactive peptides, endothelin-1 (ET-1) and angiotensin II (Ang II), that play an important physiological role in regulating vascular tone and blood pressure. Alterations in the regulation of these vasoactive peptides can contribute to the pathogenesis and progression of numerous CVDs. High levels of ET-1, associated with increased activation of growth promoting and proliferative signaling, are present and implicated in several CVDs, including hypertension, atherosclerosis and heart failure. Therefore, targeting the ET-1 system may be helpful for the management of these conditions and their associated complications. In this regard, the last decade has witnessed a surge of interest in investigating the potential role of various plant derived substances, such as curcumin that exhibit cardiovascular protective effects. Therefore, the objective of this section is to provide a brief overview on the aspects of the ET-1 system and its contribution to the pathophysiology of CVDs, as well as a brief overview on curcumin and its mechanism of action at the molecular level, with particular interest to its role in the cardiovascular system.

1.1. Endothelin

Endothelin (ET), discovered by Dr. Yanagisawa's group in 1988 (1), is one of the most prominent known vasoconstrictors that also exhibits inotropic, chemotactic and mitogenic properties, influences salt and water homeostasis, and stimulates the renin-angiotensin-

aldosterone as well as the sympathetic nervous systems (1, 2). ET plays a crucial role in cardiovascular physiology. The overall physiologic action of ET is to increase vascular tone and blood pressure (2). Alteration of this system has been associated with several CVDs (3). These events establish ET as a potentially important mediator in the pathogenesis of cardiac, vascular and renal abnormalities such as hypertension, atherosclerosis, hypertrophy, and restenosis (4). ET exerts its physiological action through the activation of several signal transduction pathways linked to cellular hypertrophy, migration and proliferation in several cell types, including vascular smooth muscle cells (VSMC) (5).

1.2 Structure of endothelins

ET exists as three 21 amino acid isopeptides named ET-1, ET-2 and ET-3. Three independent genes, located on chromosome 6, 1, and 20, respectively, encode the three structurally and pharmacologically distinct isopeptides (6). A proposed fourth member was later shown to be rodent homologue to human ET-2, rather than a truly novel isoform (7). ETs possess a great sequence homology with four further peptides, known as sarafotoxins, extracted form the venom of a snake, Atractaspis engaddensis (8). All ET isopeptides share a common cyclic structure that consists of two disulfide bridges joining the cysteine amino acids (positions 1-15 and positions 3-11) at the N-terminal end, a cluster of three polar charged side chains on amino acid residues 8-10 and a hydrophobic C-terminus containing an aromatic indole side chain at Trp^{21} (Figure 1.1). The C-terminal end contains amino acids that bind to the ET receptor while the N-terminal determines peptide affinity to the receptor (9). ET-2 contains two amino acid substitutions and shares 90% sequence homology with ET-1. ET-3 contains six amino acid substitutions and shares 71% sequence

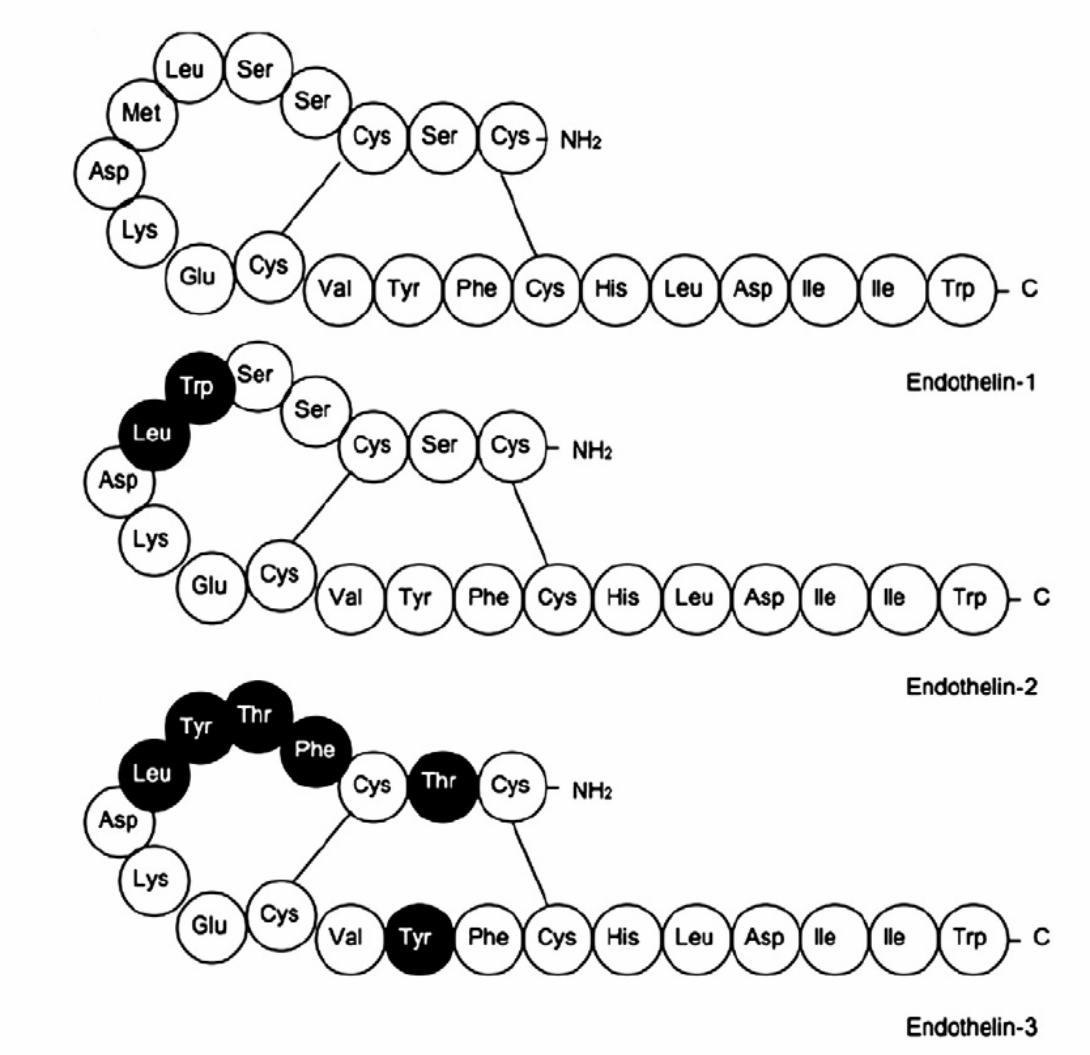

Figure 1.1 Structure of the isoforms of endothelin (ET).

Endothelin (ET) is a 21 amino acid cyclic peptide with two disulphide bridges joining the cysteine residues at positions 1-15 and 3-11. The amino acids highlighted in black represent the differences in between ET-2 and ET-3 compared to ET-1. (Khimji AK : *Cellular Signalling* 22: 1615-1625, 2010).

homology with ET-1 and ET-2. Amongst the three ETs, ET-1 is regarded as the most prominent isoform synthesized by the vasculature (1) and accounts for the majority of the pathobiological effects exerted by the ETs (10).

1.3 ET-1 regulation of generation and sites of generation

The regulation of ET-1 production is achieved at the transcriptional level of the preproET-1 gene (11). ET-1 expression is regulated at the promoter region (5') of the preproET-1 gene where there are binding sites for activator protein 1 (AP-1) and nuclear factor 1 (NF-1) transcription factors, which mediate the induction of mRNA for ET-1, through regulation by Ang II and transforming growth factor-β (TGF-β), respectively (12-14). Further post-transcriptional modulation occurs through selective destabilization of the preproET-1 mRNA via adenine-uracil-rich sequences, present in the non-translated 3' region, accounting for its selectively short biological half life of 15-20 minutes (15). ET-1 mRNA is upregulated by various stimuli, including vasoactive hormones, growth factors, hypoxia, low vascular shear stress, lipoproteins, free radicals, endotoxin and cyclosporin (16). However, ET-1 production is downregulated by high shear stress and by compounds that increase intracellular levels of cyclic guanosine monophosphate (cGMP), such as endothelium-derived nitric oxide (NO), nitrovasodilators, natriuretic peptides, heparin and prostaglandins (Figure 1.2) (16).

Vascular endothelial cells are the main site of ET-1 synthesis (6), which also correlates with the high expression levels of mRNA for ET-1 precursor molecule preproET-1 and its converting enzyme. VSMC also express ET-1 mRNA, but its production is 100 fold less than that of endothelial cells (15). Even in very low concentrations, ET-1 is also

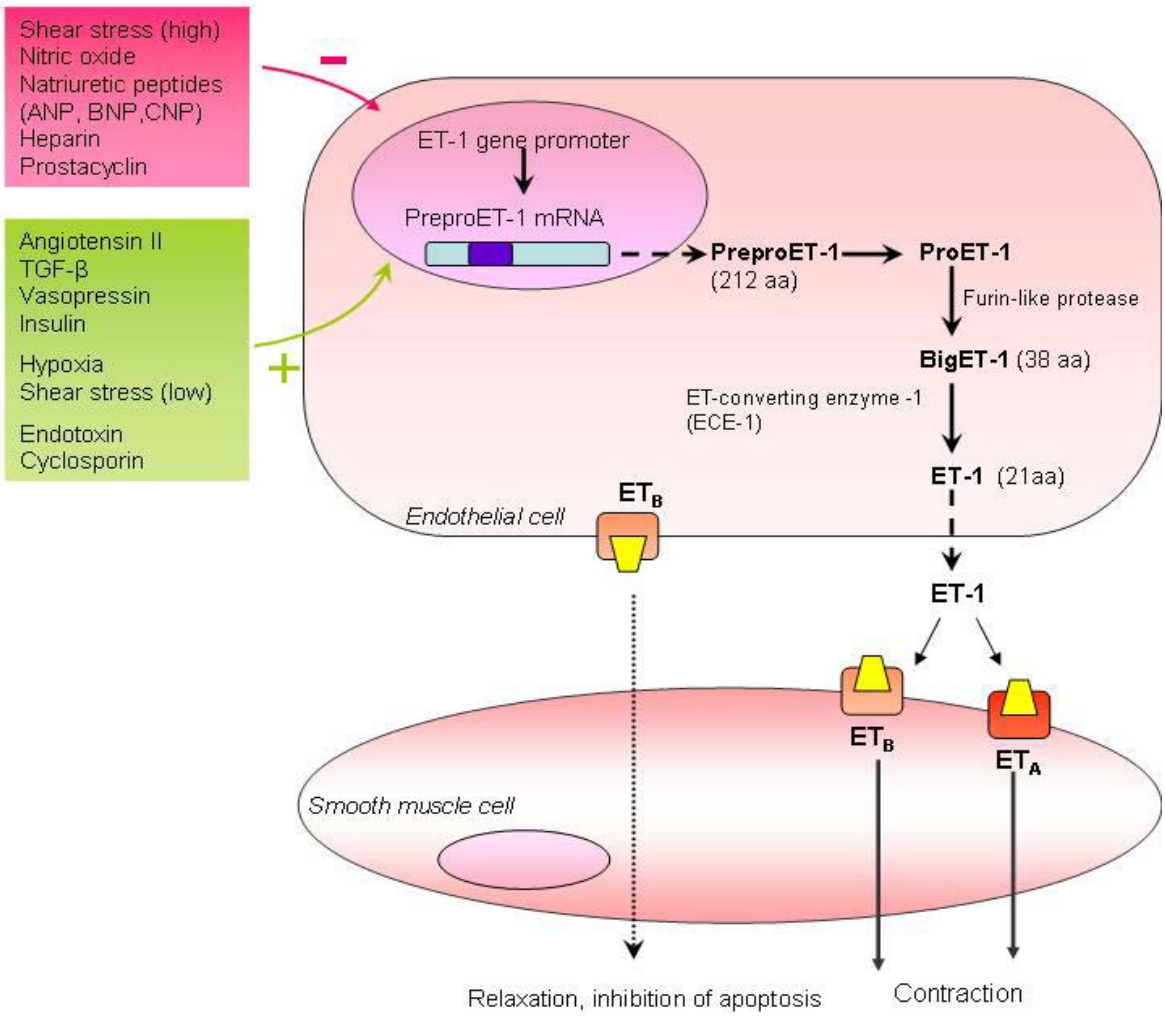

Figure 1.2 Factors regulating ET-1 synthesis, pathway of ET-1 generation and ET receptor-mediated actions on smooth muscle cells.

ET-1 synthesis is regulated by multiple factors; stimulators are highlighted in green and inhibitors in red. The synthesis of ET-1 begins with the transcription product preproET-1 (212 aa), which is cleaved to form proET-1. Pro-ET-1 undergoes cleavage by a furin-like protease to form bigET-1 (38aa) that is converted to mature ET-1 (21 aa) by endothelin converting enzyme-1 (ECE-1). ET type-A (ET_A) receptors are found in vascular smooth muscle cells and mediate vasoconstriction and cell proliferation, while ET_B receptors reside on endothelial and smooth muscle cells and mediate vasodilatation. ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; TGF-β, transforming growth factor β. (Remuzzi et al: *Nature Reviews Drug Discovery* 1: 986-1001, 2002).

produced by the heart, kidney, lung, posterior pituitary and central nervous system (10, 16, 17). In the cardiovascular system, ET-2 and ET-3 are expressed at very low levels.

1.4 Biosynthesis of ET-1

Several processing steps are required before the mature ET-1 peptide is formed (Figure 1.2). ET-1 synthesis begins with the transcription of the preproET-1 gene. The translation of the preproET-1 mRNA yields a large 212 amino acid precursor molecule, preproET-1. The latter is converted to proET-1 through the removal of its signal sequence and secreted into the cytoplasm (2). ProET-1 then undergoes a proteolytic cleavage at dibasic sites by a furin-like protease to form a biologically inactive 38 amino acid peptide, bigET-1 (18). BigET-1 is cleaved between Trp^{21} and Val^{22}, in the rate limiting step in ET-1 synthesis, by the ET-converting enzyme-1 (ECE-1) metalloproteinase to yield the mature 21 amino acid bioactive ET-1 peptide (9). ET-1 has been known to be synthesized in a constitutive *de novo* manner and mature ET-1 is directly released into the bloodstream (11). Evidence suggests that in certain cells it is also secreted via pathways involving secretory granules (19). In fact, secretory vesicles containing both mature ET-1 and ECE have been identified in endothelial cells (20). ET-1 secretion by endothelial cells occurs in a polar manner, where most of ET-1 is secreted on the basal side, resulting in 100 fold higher concentrations within the vascular wall compared to plasma levels (21). Thus, under normal physiological conditions, ET-1 is not a circulating hormone but rather a locally-acting autocrine/paracrine factor (10).

1.5 Bioavailability and clearance of ET-1

In healthy subjects, the circulating concentrations of ET-1 in venous plasma are in the range of 1-10pmol/L (15). Circulating concentrations of ET-1 are lower than those which cause

vascular constriction *in vitro* and *in vivo,* although concentrations at the interface between an endothelial cell and VSMC are likely to be higher (15). Indeed, endothelial cells secrete substantially more ET-1 towards VSMC than they do luminally (21). Thus, the circulating level of ET-1 might not directly reflect the full physiological impact of ET-1. Venous plasma ET-1 concentrations of bigET-1 and C-terminal fragments, when they are cleaved by ECE-1, appear to reflect generation of ET-1 more accurately (22). The low plasma levels of ET may be related to rapid elimination from the bloodstream.

ET levels are controlled by continuous metabolism/clearance. Clearance of ET-1 from circulation is very rapid, and its biological half life is about 1 minute, owing to its efficient extraction in the pulmonary and renal vascular beds (23). This extraction involves binding to the cell surface ET_B receptors, followed by internalization and degradation, probably within lysosomes (24). ETs are also degraded by neutral endopeptidases found in kidney proximal tubules (24). The liver is also a site of ET-1 clearance (25). On the contrary to its rapid elimination, its biological effects last considerably longer, possibly due to the almost irreversible binding of ET-1 to its receptor (26). For instance, its pressor effects are sustained for up to an hour (27) and it has been shown that ET-1 remains associated with ET receptor up to two hours after endocytosis (28).

1.6 ET-1 receptors

ET-1 exerts its biological effects in a paracrine/autocrine fashion through the activation of its receptors. Two main ET receptor subtypes, ET_A and ET_B, have been cloned in humans (29, 30). These receptors belong to the rhodopsin class A of seven transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs), which stimulate cellular events by

activation of heterotrimeric guanine nucleotide binding proteins (G proteins). They contain seven transmembrane domains of 22-27 hydrophobic amino acids in their estimated 400-amino acid sequences with an N-terminal extracellular region and C-terminal intracellular region (29, 30). Each receptor is capable of coupling to different G protein families. ET_A and ET_B receptors share 59% polypeptide sequence identity and are encoded by distinct genes located on chromosomes 4 and 13, respectively (31, 32). ET-1 receptors have different molecular and pharmacological characteristics and exhibit somewhat different physiological roles based on their location. ET_A receptors contain 427 amino acids (31) and are predominantly expressed in VSMC but are also found in cardiomyocytes, fibroblasts, hepatocytes, adipocytes, osteoblasts and brain neurons (29, 33), and exhibit higher affinity for ET-1 and ET-2 than for ET-3 (29). ET_B receptors contain 442 amino acids (31) and are predominantly expressed in endothelial cells, however, a relatively low level of expression in other cells including VSMC, cardiomyocytes, hepatocytes, fibroblasts, osteoblasts, different types of epithelial cells and neurons has been reported (30). ET_B receptors have equal subnanomolar affinities for all ET peptides (30).

Stimulation of ET_A and ET_B receptors elicits diverse physiological responses. ET-1 binding to ET_A and ET_B receptors on VSMC produces vasoconstriction, cell growth and cell adhesion (18). Conversely, ET-1 binding to endothelial ET_B receptors causes vasorelaxation through the release of vasodilators acting on VSMC, prevents endothelial cell apoptosis, inhibits ECE-1 expression within endothelial cells and is involved in ET-1 clearance (18). Thus, the net effect produced by ET-1 on the vasculature is determined on the receptor localization and the balance between ET_A and ET_B receptors.

1.7 Biological actions of ET-1 in the cardiovascular system

In the vasculature, ET-1 is involved in the basal vascular tone regulation. It induces a biphasic response represented by a transient vasodilatation followed by a sustained vasoconstriction. Endothelial cell ET_B receptors mediate vasodilatation through the production of endothelium-derived vasodilators, such as NO and prostacyclin, before the development of sustained vasoconstriction. Vasoconstriction is mediated by ET-1 activation of ET_A and ET_B receptors located on VSMC. The prolonged vasoconstriction is not dependent on ET-1 plasma levels but rather on the slow dissociation from its receptors (34). The vasoconstrictor effect is selective for some vascular beds. In animals, the coronary and renal vascular beds exhibit higher vasoconstriction to systemic administration of ET-1 (35). Systemic administration of ET-1 in humans induces sustained vasoconstriction in coronary vessels (36), but also causes renal and splanchnic vasoconstriction (37, 38). The differences among beds may be related to the balance between ET_A and ET_B-mediated effects.

ET-1 has been also shown to increase blood pressure *in vivo* in both animals and humans. In animals, bolus administration and intravenous infusion of ET-1 increased arterial pressure and this effect lasted for at least one hour before arterial pressure returned to base-line levels (39). In humans, intravenous infusion of ET-1 increased blood pressure in healthy volunteers (36, 40). Bolus administration of ET usually causes a preceding transient hypotension, which is mostly marked for ET-3 (6) and is mediated by endothelin ET_B receptors. This does not occur when ET concentrations rise more slowly, which is more likely to represent physiological conditions (39).

ET-1 is also synthesized in the heart by cardiomyocytes, cardiac fibroblasts and cardiac endothelial cells (41). The reported biological effects of the ET system on the healthy heart are

mainly positively inotropic in association with a negative lusitropic effect (33), positively chronotropic and hypertrophic (42). However, the ET system is upregulated in chronic heart failure, and ET-1-induced sustained coronary vasoconstriction may result in the deterioration of cardiac function causing myocardial ischaemia leading to fatal ventricular arrhythmias and cardiac hypertrophy (43, 44). Under such conditions, ET-1 exerts systemic and coronary vasoconstriction leading to increased afterload and reduced cardiac output (45). ET-1 induces cardiac hypertrophy, through its mitogenic properties, as an adaptational mechanism to stresses such as pressure overload (46-48).

ET-1 also exerts long-term effects in the vasculature, such as vascular remodeling, by promoting smooth muscle proliferation (49), protein synthesis (50), and production of a variety of cytokines (51) and growth factors (52). ET-1 also potentiates the mitogenic effects of growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) and is considered as a co-mitogen with these growth factors (53).

1.8 Role of ET-1 in cardiovascular diseases

Dysregulation of the ET system is significantly involved in the development of cardiovascular pathologies, such as hypertension, atherosclerosis, cardiac hypertrophy, congestive heart failure and coronary artery disease, as well as CVDs linked to diabetes, pulmonary hypertension, pulmonary fibrosis, kidney failure and other important processes. ET-1 is believed to play a role in vascular remodeling associated with experimental and human hypertension (54). ET receptor antagonists that have been developed help to investigate the role of ET-1 in CVDs (48).

1.8.1 ET-1 in hypertension

1.8.1.1 ET-1 in experimental hypertension

Evidence of pressor action of ET-1 has led to the speculation that ET-1 is involved in the pathogenesis of hypertension (1). In fact, ET-1 plasma levels are found to be significantly increased in certain animal models of hypertension (55). Deoxycorticosterone acetate (DOCA) salt-hypertensive rats, DOCA salt-treated spontaneously hypertensive rats (SHR), Dahl salt-sensitive rats, Ang II-induced hypertension, 1-kidney 1-clip Goldblatt hypertensive rats, and stroke-prone SHR are all experimental models of hypertension that exhibit an increase in systemic levels of ET-1. The increased production of vascular ET-1 in these hypertensive models is associated with hypertrophic remodeling of resistance arteries, and a response to both selective and mixed ET receptor antagonism resulted in a reduction in blood pressure as well as a regression of vascular growth (55). ET-1 has been shown to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and increase reactive oxygen species (ROS) production in VSMC, in blood vessels and in hypertensive rats (56, 57). The growth-promoting remodeling action of ET-1 may be partly mediated by this increase in ROS production (58). In aldosterone-induced hypertension, systolic blood pressure, ET-1 plasma levels, systemic oxidative stress, and vascular NADPH activity in association with small artery hypertrophic remodeling was increased (59). However, all these aberrant parameters were normalized in this model by treatment with ET_A receptor antagonist (59).

ET-1 is involved in renal and cardiac target organ damage in hypertension. An increase in renal ET-1 in hypertensive rats is involved in renal fibrosis through the activation of growth factors and by inducing inflammation (60). These events were normalized when treated with a selective ET_A receptor antagonist, and kidney function was restored (60). Rats made hypertensive

through exogenous ET-1 infusion showed changes in renal function, and signs of oxidative stress that were reduced by a free radical scavenger further imply a role of ROS in this process (61). In the heart, ET_A receptor (62), ET_A/ET_B receptor (63) and ECE antagonists (64) prevented target organ damage in hypertensive animal models. Blockade of the ET system in transgenic mice with inducible malignant hypertension prevented an increase in cardiac ET-1 concentrations, suggesting that ET receptor blockade may provide as a protecting tool against hypertensive cardiac damage (65). It should, however, be noted that all animal hypertensive models are not the same, and that different hypertensive diseases have different etiologies in which ET-1 plays a different role within each form of hypertension.

1.8.1.2 ET-1 in essential hypertension

The hallmark of hypertension is an increase in peripheral vascular resistance displayed by increased vascular tone of small arteries and vascular remodeling (66, 67). In comparison to normotensive patients, there is no significant change in plasma concentrations of ET-1 in hypertensive patients from studies that have investigated the role of ET-1 in essential hypertension (54). As such, plasma concentrations of ET-1 do not reflect the presence of essential hypertension, presumably due to the fact that ET-1 is generated and is biologically active in a paracrine and/or autocrine manner. Increases in ET-1 levels seem to be related to age, smoking and renal dysfunction rather than to hypertension (68). However, in certain ethnic groups, such as African American hypertensive subjects, increased plasma ET-1 levels have been observed (69). Interestingly, increased preproET-1 is found in the endothelium of small arteries of patients with moderate-to-severe hypertension (70) and enhancement of ET-1 generation plays a role in hypertrophic signaling in these patients (70). Increased ET-1 messenger and protein levels in VSMC cause the formation of larger elastic and muscular arteries of hypertensive

patients (71). Increased ET system activity has been suggested in patients with hypertension in comparison to normotensive patients. This is based from studies where ET_A receptor antagonism causes vasodilation in forearm vessels of essential hypertensive patients compared with normotensive subjects (72). Conversely, ET_B receptor antagonism induced vasoconstriction on forearm resistance arteries in normotensive patients (73) and is shown to have a vasodilator action on hypertensive subjects (73), indicating a vasoconstricting effect of ET_B receptors in hypertensive but not in normotensive states. African Americans appear to have an increased expression of VSMC ET_B receptors that might mediate the ET-1 response (69). Other studies have investigated a long-term antihypertensive effect of ET receptor antagonism. A four-week treatment with ET_A/ET_B receptor antagonist, bosentan, significantly lowered diastolic pressure in a study with 293 patients with mild-to-moderate essential hypertension (74). Darusentan, a selective ET_A receptor antagonist, also significantly reduced systolic and diastolic blood pressure in human hypertension (75).

1.8.2 ET-1 in atherosclerosis

In addition to its effects on blood pressure, ET-1 is pro-inflammatory and is implicated in the development of atherosclerosis (76). Evidence supporting a role for the effect of ET-1 in the pathogenesis of atherosclerosis comes from data supporting that ET-1 and its receptor levels are elevated in experimental models of atherosclerosis (77, 78), and in human coronary artery atherosclerotic plaques (79). Additionally, ECE-1 has been shown to be upregulated in VSMC and in macrophages from atherosclerotic plaques (80). Further evidence is demonstrated in experiments showing that ET_A receptor antagonism inhibits the formation of early atherosclerotic lesions in hyperlipidaemic hamsters, by decreasing the number and size of macrophage-foam cells (81), suggesting a pro-inflammatory role of ET_A activation. ET_A receptor antagonism also

reduced the development of atherosclerosis in apolipoprotein E (ApoE) deficient mice, an animal model for atherosclerosis (82). Bosentan inhibited neointimal development in collared carotid arteries of rabbits, another known model of atherosclerosis (83). Bosentan also significantly attenuated the development of graft atherosclerosis in rat cardiac allografts (84). In humans, infusion of selective ET_A receptor antagonist BQ123, improves coronary vascular function in patients with atherosclerosis (85).

ET-1 has been associated with the development of atherosclerosis mainly through its actions on VSMC. ET-1 stimulates VSMC proliferation (86), migration (87), contraction (88), matrix remodeling (89), synthesis of extracellular matrix (90) and the expression of other pro-atherogenic growth factors such as PDGF and TGF-β (91).

1.8.3 ET-1 in heart failure

Circulating ET levels have been shown to be increased in both experimental and human heart failure (92, 93). ET-1 is proposed to contribute to acute and chronic increases in vascular resistance, ventricular and vascular remodeling, inflammation, and arrhythmogenesis in models of heart failure (94). ET-1 stimulates the secretion of other neurohormones and potentiates their effects, resulting in long-term effects on the heart and contribution to the progression of chronic heart failure (CHF) (47). The gene expression of ET-1 precursor and ECE-1 is up-regulated four and three-fold respectively, in the failing human heart (95). This increase is attributed to the production from cardiac as well as extra-cardiac tissues such as the lungs (93). The increased levels of ET-1 correlate with haemodynamic severity and symptoms (96, 97). Evidence is accumulating mostly from animal studies, that ET receptor antagonism can ameliorate the deleterious haemodynamics and structural changes associated with heart failure.

ET_A receptor antagonism in hamsters with CHF and in rat coronary ligation models improves their survival (98, 99). In heart failure in dogs, bosentan decreased aortic pressure and increased stroke volume (100). Dual ET receptor antagonist TAK-044 and ET_A receptor antagonist FR 139317 also decreased cardiac pressures, and increased cardiac output (101). In a pig model of CHF, combined Ang II receptor blockade with bosentan resulted in greater improvement of ventricular function (102). Recently, it has been shown in an acute model of rat heart failure that myocardial contractility was restored and cardiac relaxation significantly improved after application of PP36, an inhibitor of ECE, suggesting a crucial role of ET production in this pathophysiological state (103).

In clinical trials, short term administration of bosentan or ET_A receptor antagonist LU135252 exhibited beneficial haemodynamic effects of patients suffering with CHF (104-106). In another clinical trial, intravenous infusion of the dual ET receptor antagonist tezosentan rapidly and effectively improved haemodynamics in patients suffering from acute decompensated heart failure (107). Despite these results and the clear rationale for the use of ET antagonists in heart failure, human clinical trials have generally not been proven to be very effective. The beneficial haemodynamic effects have been found to be short lived and treatment with ET antagonists has been associated with adverse side effects, including hypotension and abnormal liver function (108, 109).

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

ET-1 exerts its physiological actions through the activation of multiple signaling pathways which include the PLC/DAG/IP3, MAPKs and PI3-K/PKB pathways. Receptor and non-receptor tyrosine kinases also play a role in mediating ET-1-induced signaling events. The

cellular events triggered by the activation of these pathways are involved in regulating cellular growth, proliferation, contraction and survival of VSMC (110).

1.9.1 Activation of the phosphoinositide cascade by ET-1 in VSMC

The binding of ET-1 to its receptor induces a conformational change in the heterotrimeric G protein, that consists of an α, β and γ-subunit (111). In its inactive form, the α-subunit is ligated to guanosine diphosphate (GDP) but when activated, GDP is exchanged for guanosine triphosphate (GTP), causing the α-subunit to dissociate from the βγ dimmer complex, while they all remain associated to the membrane (111). Activation of the Gα subunit following ET-1 binding to its receptor initiates downstream G-protein signaling. This leads to the activation of phosphoinositide-specific phospholipase C β (PLC β), which then hydrolyzes the membrane phospholipid phosphatidylinositol-4'-5'-biphosphate (PIP_2) to generate two second messengers: hydrophobic diacylglycerol (DAG), and soluble inositol-1',4',5'-triphophate (IP_3) (112). IP_3 stimulates the release of calcium (Ca^{2+}) from intracellular stores, which plays an important role in regulating the contractile response of the cell (113). DAG, together with Ca^{2+}, activate the phosphatidylserine-dependent protein kinase, protein kinase C (PKC) (114) (Figure 1.3).

PKC is a family of serine/threonine kinases, comprised of 12 currently identified isoforms (114) that translocate from the cytosol to the cell membrane, where they become activated and phophorylate several cytosolic proteins (115). Many studies have implicated PKC in deleterious vascular effects of several pathologies including diabetes and hypertension (116). Growing evidence suggest that ET-1-induced activation of PKC in VSMC leads to protein synthesis (117), cellular proliferation (115, 118) and contraction (119). Thus, ET-1-induced activation of PKC and its downstream effects appears to be important in regulating vascular function.

1.9.2 ET-1-induced activation of MAPK cascade in VSMC

Downstream of PKC, ET-1 receptor activation also results in stimulation of mitogen activated protein kinase (MAPK) cascades. MAPK are a family of serine/threonine protein kinases which are classically associated with cell growth, proliferation, differentiation, death, and contraction (120, 121). Extracellular signal-regulated kinase 1 and 2 (ERK1/2), p38 MAPK and c-Jun N-terminal kinases (JNK) are the main groups of MAPKs. These MAPK all follow a similar system of sequential activation by several upstream signaling components, in which a stimulus activates a MAPKKK, which will then phosphorylate and activate a MAPKK, which will at last phosphorylate Thr and Tyr residues in the activation loop of the final effector, MAPK, leading to its activation and hence cellular response. ET-1 activates JNK and p38 cascades but to a lesser degree than ERKs in VSMC (122). Signals from GPCR to ERK1/2 are transmitted via Ras, a small membrane-bound GTP-binding protein. Ras cycles between an active GTP-bound conformation and inactive-GDP-bound

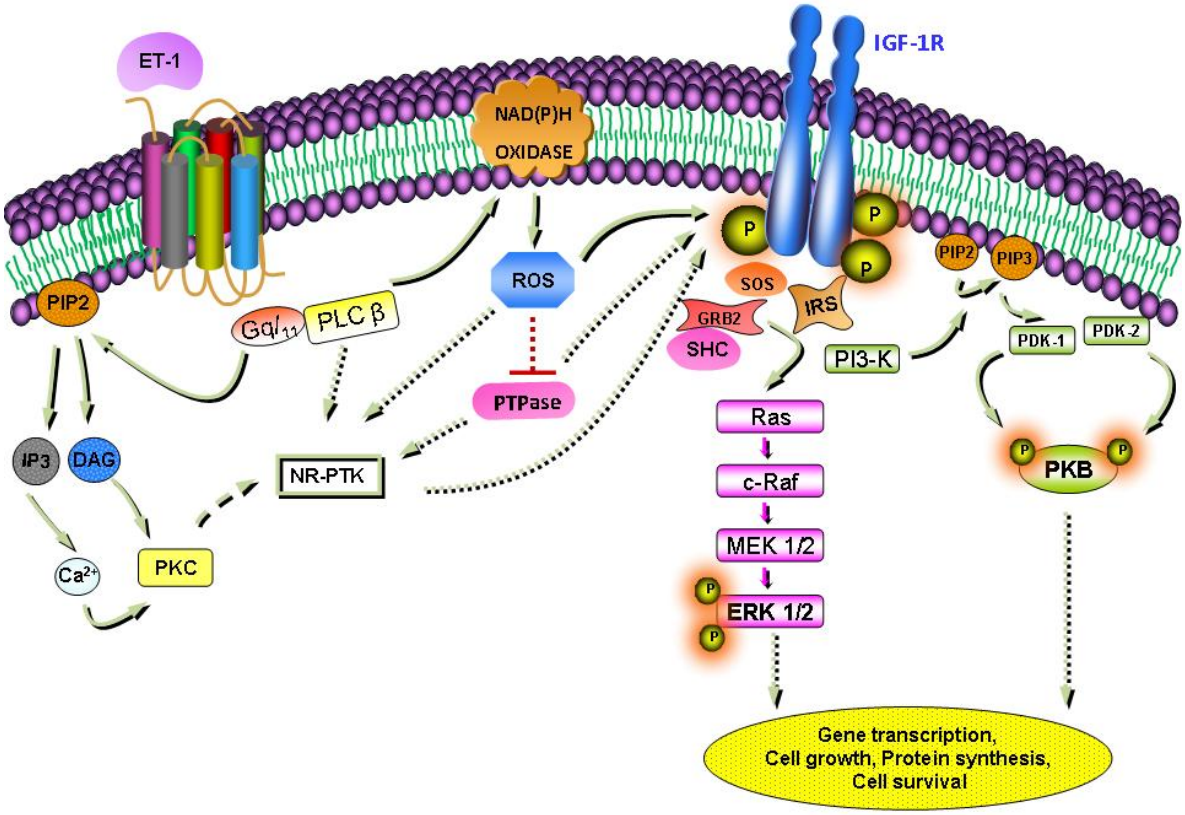

Figure 1.3 Schematic model showing the activation of ERK1/2 and PI3-K/PKB signaling pathways through IGF-1R phosphorylation by ET-1 in VSMC.

ET-1 receptor stimulation leads to both Gqα, as well as βγ activation, which in turn activate PLC β. PLC β converts PIP2 to IP3 and DAG. IP3 is responsible for elevating intracellular Ca^{2+} concentrations. DAG activates PKC. Through the activation of several downstream intermediates, Ca^{2+} alone or in partnership with PKC or other intermediates triggers the activation of NR-PTKs, by inducing their phosphorylation on target tyrosine residues. Activation of these PTKs leads to the phosphorylation of docking proteins, such as IRS-1, which serve as docking sites for Grb-2/SOS. The latter can lead to activation of Ras/Raf/MEK/ERK1/2 pathway. Phosphorylated IRS-1 activates PI3-K which goes on to catalyze the phosphorylation of PIP2 to PIP3, allowing PKD-1/2 recruitment to the plasma membrane, where PDK-1/2 will phosphorylate PKB on threonine and serine residues. ET-1 also increases ROS generation by activating NADPH oxidase, through an unknown mechanism, in VSMC. Endogenously produced ROS are able to inhibit PTPases through the oxidation of cysteine residues in its catalytic domain. Inhibition of these PTPases favours tyrosine phosphorylation of NR-PTKs and R-PTKs, resulting in the ligand-independent activation of R-PTK, such as IGF-1R, that act upstream of Ras/Raf/MEK/ERK1/2 and PI3-K/PKB pathways. Activation of ERK1/2 and PKB signaling cascades plays a role in mediating various cellular responses such as gene transcription, protein synthesis, cell growth and cell survival.

form (120). Activated Ras recruits and activates Raf, a MAPKKK, that phosphorylates MEK, a MAPKK, at specific serine/threonine residues, which in turn phosphorylates ERK1/2 in threonine and tyrosine residues (120). Chen et al. have reported an implication of PKC in ET-1-induced ERK activation through ET_A receptors in human VSMC (123). Activation of ERK1/2 leads to the phosphorylation of downstream cytosolic target substances on serine and threonine residues. ERK1/2, along with other MAPK family members, can also be translocated from the cytosol to the nucleus where they can phosphorylate and activate several transcription factors which lead to the activation of genes involved in cell growth and differentiation (120) (Figure 1.3).

Several reports have demonstrated that ET-1 activates ERK1/2 signaling pathways in cardiomyocytes (124), fibroblasts (125), glomerular mesangial cells (126) and VSMCs (5). Activation of ERK1/2 can potentially result to an increased proliferation and hypertrophy of VSMC in response to ET-1 given that inhibition of its upstream mediator, MEK1/2, resulted in inhibition of ET-1-induced DNA and protein synthesis (127, 128).

1.9.3 ET-1-induced phosphatidylinositol-3-kinase cascade activation in VSMC

Phosphatidylinositol-3-kinases (PI3-K) are a family of lipid kinases that have emerged as important effectors of ET-1 action (110). PI3-K phosphorylates the 3' position of the inositol ring of the membrane-bound phosphoinositides, phosphatidylinositol (PI), PI 4-phosphate, and PI 4,5-phosphate. This reaction generates biologically active lipids PI 3-P, PI 3,4-biphosphate (PI(3,4(P_2) and PI(3,4,5)triphosphate (PI(3,4,5)P_3), respectively (129). These phospholipids act as second messengers to activate several protein kinases such as PI(3,4,5)P_3 dependent protein

kinase (PDK), protein kinase B (PKB) and 70kDa ribosomal protein S6 kinase ($p70^{S6K}$) (129) (Figure 1.3).

PI3-Ks are divided into three classes based on their substrate specificity, molecular structure and mode of regulation (129). Class I PI3-Ks generates PI(3)P, PI(3,4)P_2, PI(3,4,5)P_3 and are activated by receptor protein tyrosine kinases (R-PTK) and GPCRs (129). Class II PI3-Ks generate PI(3)P, PI(3,4)P_2, and possess a lipid binding domain, whereas, class III PI3-Ks only generate PI(3,4,5)P_3 (129). Class I PI3-Ks are further subdivided into class IA and IB PI3-Ks and are heterodimeric proteins composed of a catalytic and regulatory subunit. Class IA proteins consist of a 110 kDa (p110) catalytic subunit and an associated 85 kDa (p85) regulatory subunit. Class IA has three isoforms of the catalytic subunit, p110α, p110β and p110δ, and several isoforms of the regulatory subunit, p85α, p55α, p50α, p85β and p55γ. In contrast to class IA, class IB has only one catalytic member, p110γ, and one form of the regulatory subunit, p101. The class IA PI3-Ks are activated by R-PTK, while class IB is activated by GPCR (130).

The PI3-K pathway plays a pivotal role in cell migration, differentiation, proliferation and survival (130). Several studies have reported an involvement of PI3-K as an upstream mediator of several ET-1 induced responses. However, except for rat glomerular mesangial cells, where ET-1-induced PI3-K activation is necessary for hypertrophy (131), no direct activation of PI3-K by ET-1 in VSMC has been documented. However, the PI3-K inhibitor wortmannin has been shown to have an inhibitory effect on ET-1-stimulated cell proliferation and protein synthesis in A-10 VSMC (127, 128).

1.9.4 ET-1-induced PKB activation in VSMC

Several downstream targets of PI3-K have been documented, the most studied being PKB, also known as Akt (a product of akt proto-oncogene). PKB is a 57 kDa serine/threonine kinase with three identified isoforms in the mammalian system, PKBα/Akt1, PKBβ/Akt2 and PKBγ/Akt3 (132). All isoforms possess an N-terminal pleckstrin homology (PH) domain that binds to phospholipids generated by PI3-K, a central catalytic kinase domain with specificity for serine or threonine residues on substrate proteins, and a C-terminal regulatory domain containing a hydrophobic motif (132). Full activation of PKB is a sequential two step process where the lipid products generated from PI3-K are recognized by the PH domain of PKB, translocating it to the plasma membrane, where it then gets phosphorylated on Thr308 by PDK-1 and on Ser473 by a putative kinase termed PDK-2 for its complete activation (133) (Figure 1.3). Phosphorylation of both sites is mitogen-and PI3-K-dependent (133).

PKB has been shown to promote a variety of cellular responses including inhibition of apoptosis and promotion of cell survival, regulation of cellular proliferation, metabolism and hypertrophy (132). PKB substrates include members of cell survival and apoptosis cascade such as Bcl-2, BAD (134, 135), caspase-9 (136) and glycogen synthase kinase-3 (GSK-3) (137), as well as regulators of protein synthesis and cell growth such as mammalian target of rapamycin (mTOR) (138). ET-1 has been shown to activate PKB in cardiomyocytes (139), myofibroblasts (140), human umbilical vein endothelial cells (141) and A-10 VSMC (5, 127, 128).

1.10 ET-1-induced growth factor transactivation

Recent findings suggest that ET-1 and other vasoactive peptides, such as Ang II, whose receptors belong to GPCR family, stimulate intracellular signaling pathways through

transactivation of R-PTK, and thereby mediate the ERK1/2 and PKB signaling events. Among the main growth factor receptors implicated in this process, epidermal growth factor receptor (EGF-R), insulin–like growth factor type 1 receptor (IGF-1R) and platelet derived growth factor receptor (PDGF-R) have been investigated in some detail. EGF-R has been studied in some detail in response to Ang II and ET-1 (142, 143), as has PDGF-R in response to Ang II (144). However, only a few recent studies have investigated the involvement of IGF-1R.

1.10.1 Role of IGF-1R

The IGF-1R is a member of the tyrosine kinase class of growth factor receptors that shares structural and functional homology with the insulin receptor (IR). Human IGF-1R is a ubiquitously expressed product of a single-copy gene located on chromosome 15 (145). The IGF-1R is a transmembrane heterotetrameric structure that is comprised of two extracellular ligand-binding α-subunits that are linked by disulfide bonds to each other and to the two transmembrane β-subunits that contain intrinsic tyrosine kinase activity, which is believed to be essential for most of the receptors biological effects (145). Binding of the ligand to the α-subunit induces a conformational change, leading to the activation of the protein tyrosine kinase (PTK) domain of the IGF-1Rβ subunit that responds by *trans*-autophosphorylation of a cluster of tyrosine residues within the IGF-1Rβ to provide docking sites for effector proteins (145). Once these residues become phosphorylated, the intrinsic PTK activity of IGF-1R is enhanced, thus leading to the phoshorylation of several adaptor/scaffolding proteins, including insulin receptor substrate (IRS-1 or IRS-2) and Src homology collagen (Shc) (146). IRS-1 contains multiple tyrosine phosphorylation sites that recognize and bind to SH2 domain-containing signaling molecules, such as Grb2, Nck, the p85 subunit of PI3-K, and the SH2 domain-containing tyrosine phosphatase-2 (SHP-2) (147). Of these, the binding of Grb2 associated with SOS, a guanine

nucleotide exchange factor, to the tyrosine-phosphorylated IRS-1 activates Ras and initiates sequential phosphorylation that leads to the activation of ERK1/2 (148). Shc can also interact with IGF-1R, in an IRS-1-independent manner (149), to recruit the Grb2/Sos complex and activate Ras/Raf/MEK/ERK pathway (148). IGF-1R activation also leads to the activation of PI3-K/PKB pathway. Tyrosine-phosphorylated IRS-1 interacts with the p85 subunit of PI3-K, leading to the activation of the catalytic p110 subunit and to the subsequent activation of the downstream substrate PKB (reviewed in (150)).

In VSMC, a requirement of IGF-1R activity has been recently shown in ET-1-induced PKB phosphorylation. These studies demonstrate that ET-1 was able to phosphorylate tyrosine residues on IGF-1R β subunit and that use of AG1024, a selective pharmacological inhibitor of IGF-1R-PTK activity, attenuated IGF-1R and PKB phosphorylation, as well as protein and DNA synthesis induced by ET-1 (151). These results suggest an important role of IGF-1R in mediating PKB phosphorylation, as well as hypertrophic and proliferative responses induced by ET-1 in VSMC. The mechanism by which ET-1 induces IGF-1R transactivation is not fully understood. However, a potential role of ET-1-induced ROS generation in this process has been suggested since H_2O_2 and subsequent inhibition of PTPase have shown to contribute to the activation of IGF-1R-PTK in VSMC further resulting in activation of PKB (152, 153) (Figure 1.3).

1.11 ROS and ET-1 signaling

ROS are very small, rapidly diffusible, highly reactive molecules that take part in physiological reactions and signal transduction but an excessive synthesis can overcome antioxidant mechanisms and generate deleterious effects, often resulting in the development of CVDs (11). This is in part due to their physicochemical properties which allow them to disrupt

biological macromolecules such as lipids, DNA, carbohydrates and proteins. Hydrogen radicals ($OH^{\cdot}$), superoxide anions ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), reactive nitrogen radicals and its derivative peroxynitrite ($ONOO^-$) are amongst the most important ROS. They are generated both enzymatically and non-enzymatically by nearly every cell type including VSMC and endothelial cells (11).

ROS are potent stimulators of ET-1 synthesis in endothelial cells (154) and in human VMSC (155). Alternatively, studies demonstrate that ROS can be generated in response to ET-1. In fact, ET-1 has been shown to activate NADPH oxidase within several cell types, including VMSC (5, 156) and that *in vivo*, free radicals generated by ET-1 play important roles in mineralocorticoid-induced hypertension (57, 157). Both the pressure and vasoconstrictive effect of ET-1 were diminished in parallel with the normalization of $O_2^{\cdot -}$ levels after treatment of DOCA-salt rats with ET_A receptor antagonist (57).

Increased ROS generation promotes cell growth and proliferation, and is therefore associated with a variety of cardiovascular pathologies, including hypertension and atherosclerosis (158). The elevated levels of ET-1 during hypertension and atherosclerosis lead to excessive production of oxidative stress and a decrease in antioxidant status (159). The involvement of ROS in ET-1-induced activation of MAPKs including JNK, p38mapk and ERK1/2 has been demonstrated in cardiac fibroblasts (125). Moreover, a role of ROS in ET-1-induced activation of ERK1/2 and PKB signaling has been demonstrated in VSMC (5) (Figure 1.3).

1.12 Plant-derived cardiovascular protection

ET-1 is a key player in cardiovascular homeostasis through the activation of the previously mentioned signaling pathways, and though this activation is a physiological cellular event, aberrant signaling has been implicated in the development and progression of pathophysiological events. For instance, upregulation of ET-1-induced signaling through MAPK and PI3-K/PKB pathways, particularly in VSMC, is linked to vascular changes observed during CVDs such as atherosclerosis and hypertension (160, 161). Many pharmacological interventions that have been introduced to target this system failed to show optimal efficacy in diseases other than pulmonary hypertension, therefore, interventions are needed to directly target the cellular mechanism in order to prevent the effects of ET-1 on VSMC in the development of CVDs. In this regard, natural derived products have been extensively researched during the last few decades, due to their reported beneficial diverse biological and pharmacological activities on the cardiovascular system. Knowledge of traditional medicine has allowed us to identify plants and plant-derived substances believed to exhibit such health promoting effects. Plants produce a large and diverse array of bioactive substances, known as phytochemicals. These are non-nutritive substances that possess health-protective benefits (162). Some of these compounds have been shown to exhibit cardiovascular protective effects. Among them, the main constituent of the spice turmeric, curcumin, has attracted the attention of scientists and been the subject of their investigations. The putative therapeutic properties of curcumin are mainly attributed to its antioxidant, anti-inflammatory and anti-carcinogenic properties (163-165).

1.13 Historical aspects of curcumin

Curcumin is the main ingredient of the spice turmeric which is an ancient gold-coloured spice commonly used in Asiatic countries, mainly India and China, as a culinary ingredient for

food flavouring and preservation, as a yellow textile colouring agent, and as a natural remedy for medicinal purposes. Turmeric is derived from the rhizomes of the perennial plant *Curcuma longa* Linn, belonging to the Zingiberaceae family, which is distributed in tropical and subtropical regions of the world (Figure 1.4). Turmeric contains a wide variety of phytochemicals including curcuminoids, a group of polyphenolic compounds that have been shown to deliver beneficial effects on health and on events that help in preventing certain diseases. Curcuminoids are mainly comprised of three bioactive analogs, curcumin (curcumin I), demethoxycurcumin (curcumin II), and bisdemethoxycurcumin (curcumin III) (166) (Figure 1.4). They have all been isolated and differ in their methoxy substitution on the aromatic ring. Curcumin is the most active and abundant constituent of turmeric. It is estimated that approximately 2 to 5% of turmeric is composed of curcumin (167), while the remainder is made up of mineral matter, carbohydrates, proteins, fat and moisture (168). The isolation of pure curcumin is very tedious; consequently, the commercially available extracts consist of a combination of the three curcuminoids with curcumin as the main constituent (75-81%). Curcumin is responsible for the characteristic yellow pigmentation of turmeric and is now acknowledged as being responsible for most of its therapeutic effects (167).

In folk medicine, turmeric and natural curcuminoids have been applied for centuries in therapeutic household remedies throughout many parts of the world, particularly in the Orient, where it is widely consumed. Its therapeutic use can be traced back to ancient

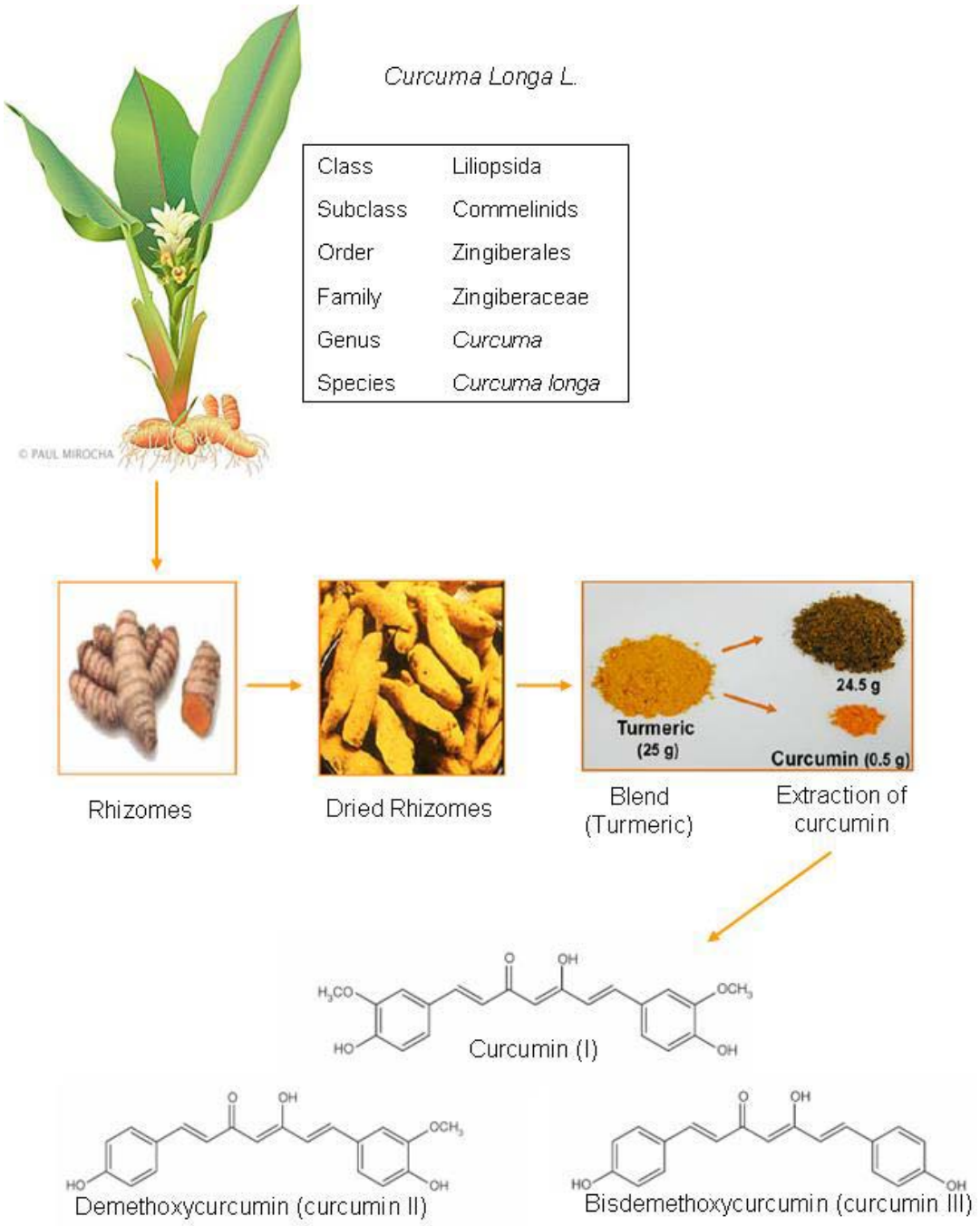

Figure 1.4 Taxonomic position of *Curcuma Longa Linn* and isolation, extraction and structure of parent curcuminoids. (Based on ref (169) and Chattopadhyay et al: *Current Science 1*: 44-53, 2004).

Indian and Chinese medicines, where it was used as an anti-inflammatory agent to treat ailments associated with abdominal pain, inflammation and pain caused by injury (170). In Ayurvedic medicine, it was used against pulmonary disorders, liver disorders, anorexia, rheumatism, diabetic wounds, runny nose, cough and sinusitis (171).

1.14 Chemical properties of curcumin

Curcumin was first isolated from turmeric in 1815 by Vogel and Pelletier, obtained in crystalline structure and identified as 1,6-heptadiene-3,5-dione-1,7-bis(4-hydroxy-3-methoxyphenyl)-(1E,6E) or diferuloymethane in 1870 by Daube (169). The hydrophobic polyphenol feruloymethane skeleton was later elucidated and synthesized in 1910 by J. Milodedzka and V. Lampe (169). In chemical terms, it is bis-α, β-unsaturated β-diketone, a linear diarylheptanoid compound, where two oxy-substituted aryl moieties are linked together through a seven carbon chain (172) (Figure 1.4). The aryl rings may be substituted by hydroxy or methoxy groups to produce analogues of curcumin or curcuminoids. It exhibits keto-enol tautomerism and in solution it exists predominantly in the enolic form, which is the more stable form (173). In neutral and acidic aqueous solutions, the keto form dominates (174). Curcumin has a molecular weight of 368.37g/mol, a melting point of 183^{o}C, and its molecular formula is $C_{21}H_{20}O_6$. It is insoluble in water or aqueous solvents but soluble in organic solvents such as dimethyl sulfoxide, methanol, ethanol and acetone. Spectrophotometrically, curcumin has a maximum absorption in methanol at 430nm and it absorbs maximally at 415 to 420nm in acetone, and a 1% solution of curcumin has 1650 absorbance units (175). It has a bright yellow colour at pH 2.5 to 7 and takes a red hue at pH higher than 7 (175).

1.15 Curcumin bioavailability, metabolism, biotransformation and pharmacokinetics

The bioavailability of curcumin is limited by a number of factors, which may be an obstacle to its utility as a therapeutic agent. Once absorbed, polyphenolic compounds can directly undergo conjugation reactions, predominantly methylation, glucuronidation, and sulfation (176). Based on animal studies, poor gastrointestinal absorption, poor water solubility, molecular instability, and rapid and efficient metabolism, mainly by the liver and intestine into metabolites that are rapidly excreted, are a combination of reasons that contribute to the notion that curcumin exhibits poor systemic bioavailability (177, 178). In order to enhance its bioavailability, the co-administration of curcumin with piperine or its complexation with phospholipids, liposomes and micelles has been proposed. Adjuvant co-administration prevents its rapid metabolism by interfering with enzymes that catalyze the metabolism of curcumin, while liposomes, micelles and phospholipid complexes can reduce the hydrophobicity of curcumin and increase the permeability of membrane barriers (166). Other strategies include employing nanoparticle technology providing easier penetration through membrane barriers because of their small size (166).

Data on the pharmacokinetics, metabolites, and systemic bioavailability of orally administrated curcumin in humans, provided from trials mainly conducted on cancer patients, showed that after oral administration of curcumin, no curcumin excretion was detected and that serum concentration peaks observed at one to two hours were undetectable at twelve hours (179). However, most curcumin conjugates produced by *in vivo* human metabolism are detectable in plasma at greater concentrations than free curcumin with a peak after four hours of dosing (180). Although, it has not yet been established if these metabolites are as bioactive as their parent

compound, some curcumin metabolites, such as the reduction metabolite tetrahydrocurcumin, have been shown to be biologically active in some systems (163).

An important feature of curcumin is that despite being consumed daily for centuries, it has not been shown to cause any toxicity (172). Clinical trial results show that curcumin is well tolerated, even at high doses, where it appears non-toxic to animals or humans (179, 181). In human trials, only minor side effects of curcumin, namely diarrhoea, have been reported (182). These trials however have examined the short term outcome. There is some evidence that long-term high-dose curcumin administration in rodents can be tumourigenic (183). It has also been shown that curcumins predominant activity changes from antioxidant to pro-oxidant with increasing concentration (184).

1.16 Biological actions of curcumin

Curcumin has been demonstrated to have a wide range of beneficial effects through its most pronounced properties which encompass its anti-inflammatory, antioxidant and anti-carcinogenic activities (177). Modern science has revealed that curcumin is a highly pleiotropic molecule that mediates its effects by modulation of several important molecular targets, including transcription factors, enzymes, growth factors and their receptors, cell cycle proteins, cytokines, cell surface adhesion molecules, and genes regulating cell proliferation and apoptosis (185). However, the cellular and molecular mechanisms of the favourable effects of curcumin on human health are not fully understood.

1.16.1 Anti-inflammatory properties of curcumin

Most chronic illnesses are believed to be caused by dysregulated inflammation (186). For instance, inflammation is involved in cancer, cardiovascular diseases, pulmonary diseases, metabolic diseases and neurological diseases (187-191). Although, the mechanism of its anti-inflammatory action remains unclear, curcumin has been shown to negatively modulate several pro-inflammatory responses. Several studies have demonstrated that curcumin was able to modulate the production of various inflammatory cytokines, thereby exhibiting potent anti-inflammatory activity. It has been shown to downregulate nuclear factor-κB (NF-κB), a transcription factor that plays a critical role in the induction of many pro-inflammatory mediators involved in chronic and acute inflammatory diseases and various cancers (186). Aggarwal and Singh were the first to demonstrate that curcumin suppressed NF-κB activation induced by different inflammatory stimuli (192), resulting in the suppression of NF-κB-dependent gene products that suppress apoptosis and mediate proliferation, invasion, and angiogenesis (186). The downregulation of NF-κB by curcumin results in a decrease in the expression of TNF-α, interleukin-1 (IL-1), interleukin-6 (IL-6) (169). Therefore, inhibition of pro-inflammatory cytokine production by regulation of transcription factors, such as NF-κB, is a potential strategy for controlling inflammatory responses. In addition, curcumin has been reported to inhibit the activities of cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) enzymes that are involved in generating lipid mediators intimately implicated in inflammation, thereby indicating that the anti-inflammatory actions of curcumin can also modulate arachidonic acid metabolism (193).

The nuclear transcription factor early growth response-1 (Egr-1) is implicated in the regulation of a number of genes involved in inflammation as well as growth and development, and is also a target of curcumin. Egr-1 regulates several pathophysiologically relevant genes in

the vasculature that are involved in differentiation, wound healing and blood clotting (194). It has been shown that curcumin suppressed the induction of Egr-1 in endothelial cells and fibroblasts, thereby modulating the expression of Egr-1-regulated genes in both these cell types (195). A recent study also showed that curcumin inhibits the inflammatory response and chemotaxis of monocytes by inhibiting Egr-1 (196).

Curcumin also downregulates MAPK pathways, which are activated by many inflammatory stimuli (194). Curcumin can contribute to the protection against the adverse vascular effect of the pro-inflammatory response through the suppression of TNF-α-stimulated ROS generation, monocyte adhesion, phosphorylation of JNK and p38 MAPK, and signal transducer and activator of transcription (STAT)-3 in endothelial cells (197). *In vitro* studies are somewhat contradictory since other investigators paradoxically show an activation of MAPK by curcumin (198, 199). The mechanism is unexplained, nevertheless; in both cases its final effects appear to be anti-inflammatory (172).

Curcumin thus exerts a protective role against inflammatory responses, through modulation of inflammatory mediators, and may therefore represent a therapeutic agent targeting the cardiovascular system since the inflammatory process plays a crucial role in the pathogenesis of some cardiovascular diseases, mainly atherosclerosis and acute coronary syndrome (200).

1.16.2 Antioxidant activity of curcumin

The antioxidant activity of curcumin was reported as early as 1975 (201). Oxidative stress associated with overproduction of ROS plays a major role in the pathogenesis of various diseases, including CVDs (158). Even though the antioxidant properties of curcumin have been shown to have several therapeutic advantages, its antioxidant mechanism remains poorly

understood. It has been shown to be a potent scavenger of a variety of ROS, including $O_2^{\cdot -}$ (174), OH^- (202), nitrogen dioxide radicals (203) and non-free radical species such as H_2O_2 (174). Curcumin has also been shown to enhance the activity of antioxidant enzymes and to counteract the activity of ROS generating enzymes (170, 204). Sreejayan and Rao first claimed that the presence of phenolic groups in the structure of curcumin was fundamental in its ability to eliminate ROS (205). Recent literature proposes that the phenolic and methoxy groups on the phenyl ring and the 1,3-diketone system are important structural features that contribute to the antioxidant effects. Evermore so, the antioxidant activity increases when the phenolic group with a methoxy group is at the ortho position (206). A more recent study concluded that the H-atom donation from the phenolic group is responsible for the strong antioxidant properties of curcumin (174). Curcumin can also indirectly increase the endogenous cellular antioxidant defenses through alternative antioxidant mechanisms. Heme-oxygenase-1 (HO-1) is a widely distributed mammalian enzyme that is one of the most prominent protective genes proven to be effective in ameliorating cardiovascular problems associated with increased oxidative stress (158), and is yet another target of curcumin (207). Curcumin has been shown to protect against oxidative stress through an increase in HO-1 production in endothelial cells (206).

Curcumin has the ability to protect lipids and DNA against oxidative degradation by scavenging free radicals and inhibiting free radical generation (203, 208, 209). Lipids are the most susceptible macromolecules to oxidative stress (203) and lipid peroxidation consists of a series of free radical-mediated chain reaction processes that leads to the damage of cell membranes. Many studies demonstrate the potential of curcumin to reduce lipid peroxidation, a key process in the onset and progression of many diseases, including atherosclerosis, heart diseases and cancer (210-213). Curcumin has been shown to reduce lipid peroxidation by

augmenting the activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase, glutathione (GSH), glutathione-s-transferase (GST) and glutathione peroxidase (GPx) (170).

Curcumin also exhibits a protective role against oxidative ischemic injury through inhibition of free radical propagation with subsequent inhibition of ROS generating enzyme activity, resulting in decreased ROS production (214).

Although the exact mechanism by which curcumin promotes these effects remains to be elucidated, *in vivo* and *in vitro* studies suggest that its antioxidant properties appear to underlie its pleiotropic biological activities.

1.16.3 Anticarcinogenic effects of curcumin

In vivo and *in vitro* studies have demonstrated the ability of curcumin to inhibit carcinogenesis at three stages: tumour initiation, promotion and metastasis (215). Its anti-carcinogenic effect has been studied in gastrointestinal, liver, lung, blood, breast, oral, prostate and skin cancers. The molecular basis of the anti-carcinogenic and chemopreventive effects of curcumin is attributed to its effect on several targets including transcription factors, growth regulators, apoptotic genes, angiogenesis regulators and cellular signaling molecules (215). Cancer cells are able to evade apoptosis and grow in a rapid and uncontrolled manner. Curcumin has been shown to suppress the proliferation of tumor cells through the activation of caspases, the induction of tumor suppressor genes, such as *p53*, the upregulation of proapoptotic proteins and the downregulation of anti-apoptosis proteins (216, 217). Curcumin has been shown to induce *in vitro* and *in vivo* apoptosis of various tumour cell lines such as breast cancer cells, lung cancer cells, human melanoma cells, human myeloma cells, human leukemia cells, human neuroblastoma cells, oral cancer cells and prostate cancer cells (reviewed in (215)). Besides

inducing apoptosis, curcumin also inhibits the expression of PI3-K and the phosphorylation of the pro-survival kinase Akt/PKB (217).

Curcumin has also been shown to suppress growth factor-induced actions on human cancer cell growth. Recent *in vitro* studies have shown that treatment of cancer cells with curcumin can inhibit the intrinsic EGF-R tyrosine kinase activity, EGF-R ligand-induced activation, as well as the expression of EGF-R (218). Curcumin has been shown to inhibit human colon cancer cell growth by suppressing gene expression of EGF-R through reducing the activity of transcription factor Egr-1 (219). Recent data further demonstrate that curcumin also inhibits the expression and activation of the IGF-1R tyrosine kinase, thereby reversing the IGF-1-induced cell growth and apoptosis resistance (220). Moreover, curcumin inhibits PDGF-R-induced proliferation and PDGF-induced ERK1/2 phosphorylation of pancreatic stellate cells, through the induction of HO-1 gene expression (221). Additional growth factor pathways modulated by curcumin include TGF-β, fibroblast growth factor (FGF), hypoxia-inducible factor (HIF)-1α, and colony-stimulating factors (CSFs) (reviewed in (175)).

MAPK signaling is a major pathway used by growth factors to trigger cell proliferation and differentiation. In tumor cells, curcumin, through inhibition of JNK phosphorylation, was shown to effectively block AP-1 and NF-κB signaling pathways (222). Furthermore, curcumin strongly repressed tumor promoter-induced phosphorylation of ERK, JNK and p38 MAPK in brain tumor cells (223). Curcumin inhibition of gene expression of Egr-1 also requires interruption of ERK MAPK signal pathway (219). Several ongoing clinical trials still remain inconclusive, yet curcumin appears to have therapeutic potential for both prevention and treatment of cancer. Pharmacologically, it is safe in humans but its limited bioavailability interferes with its therapeutic potential.

1.17 Curcumin and cardiovascular diseases

Most of the earlier studies on curcumin focussed on its beneficial effects in various models of cancer, however, its anti-inflammatory and antioxidant properties have generated interest to investigate if this compound can also exert cardiovascular protective effects. Even though the underlying mechanism of action is not fully understood, evidence that curcumin exerts protective effects on the cardiovascular system has been previously described and its use as a therapeutic agent to mitigate CVDs is currently being investigated. Evidence indicates that curcumin exhibits protective effects against cardiovascular pathologies such as atherosclerosis, cardiac hypertrophy and heart failure.

1.17.1 Curcumin and atherosclerosis

Atherosclerosis is the most common form of heart disease. It is considered a chronic and progressive disease arising from the inflammatory processes and oxidative stress within the vessel wall (224). Curcumin has been shown to exhibit anti-atherosclerotic action through inhibition of platelet aggregation (225), protection against inflammation and oxidation, and modulation of cholesterol homeostasis. Several lines of evidence strongly suggest that curcumin may prevent atherosclerosis by regulating some elements in cholesterol homeostasis. Studies have reported that curcumin is beneficial in lowering low-density lipoprotein-cholesterol (LDL) and raising high-density lipoprotein-cholesterol (HDL) while reducing lipid peroxidation. Animal studies conducted in experimental atherosclerotic rabbits reported that curcumin effectively inhibited LDL oxidation, and decreased cholesterol and triglycerides levels (211). Supplementation with curcumin also demonstrated a significant prevention of early atherosclerotic lesions in thoracic and abdominal aorta, through reduction in oxidative stress and decreased lipid peroxidation, resulting in reduction of aortic fatty streak formation, in

experimental model of atherosclerosis where rabbits were fed a high cholesterol diet for 30 days (226). Orally administered curcumin also decreased the formation of atherosclerotic lesions by 20% in apoE and LDL receptor-double knockout mice model, an animal model developed for experimental atherosclerotic research (227). In a recent study, Liao et al. demonstrated that curcumin induced a 50% reduction in atherosclerotic lesion reduction in apoE knockout mice and inhibited oxidized-LDL-induced cholesterol accumulation in cultured VSMC (228). Curcumin has also proven to be an effective antioxidant through the prevention of oxidation and modification of LDL, and the subsequent restoration of prostacyclin release in human endothelial cells, thereby indicating a protective role in preventing pathological conditions related to oxidative stress and the development of atherosclerosis (229).

In humans, a study involving the administration of 500 mg of curcumin for 7 days to 10 healthy volunteers revealed a 29% increase in HDL cholesterol, a 12% decrease in total serum cholesterol and a 33% decrease in serum lipid peroxidases (230). The administration of curcumin also reduced total and LDL cholesterol levels in patients with acute coronary syndrome (231). Another study showed that 10 mg of curcumin given twice daily during 30 days significantly lowered the serum LDL levels and increased the serum HDL levels in healthy patients (232). The same group reported that 10 mg of curcumin administered twice daily for 15 days significantly lowered plasma fibrinogen levels in humans with atherosclerosis (233).

Abnormal proliferation of VSMC and mononuclear cells also contributes to the progression of cardiovascular diseases, including atherosclerosis. Curcumin suppressed mitogen-induced proliferation of human blood mononuclear cells, inhibited neutrophil activation and mixed neutrophil reaction, and also inhibited serum-induced as well as PDGF-dependent proliferation of rabbit VSMC (234). VSMC migration and collagen synthesis are also key events

involved in the pathological changes occurring with atherosclerosis. Curcumin has potent inhibitory effects on PDGF-induced VSMC proliferation, migration and collagen synthesis (235). This inhibitory effect on vascular remodeling is attributable to the attenuation by curcumin of PDGF-induced PDGF-R, ERK1/2 and Akt signaling in VSMC (235). Using an animal arterial balloon-injury model characterized by PDGF-R upregulation to substantiate the *in vitro* results, curcumin significantly inhibited neointima formation, collagen synthesis, cell proliferation and the overexpression of PDGF-R (235). Curcumin also decreased cholesterol-induced proliferation of aortic rat VSMC and suppressed the phosphorylation of ERK1/2 as well as its translocation to the nucleus (236). The vascular anti-proliferative effect of curcumin has also been demonstrated through the induction of HO-1 expression in rat and human VSMC (237). Curcumins potential role in the prevention of atherosclerosis is further supported through its inhibitory effect on VSMC migration (238). This inhibitory effect was observed on TNF-α-induced migration that was mediated by decreasing TNF-α-induced ROS production, leading to suppression of matrix metallopeptidase 9 (MMP-9) activation and protein expression through the downregulation of NF-κB (238). Synthetic curcuminoids have also been shown to exert anti-proliferative effects on cell growth. Dehydrozingerone, a biosynthetic structural analogue of curcumin, inhibited PDGF-stimulated VSMC migration, proliferation, collagen synthesis, and PDGF/H_2O_2-stimulated phosphorylation of PDGF-R and downstream Akt (239). HO-3867, another synthetic curcuminoid, significantly inhibited the proliferation of serum-stimulated VSMC (240). Hydrazinocurcumin, yet another synthetic curcumin derivative, potently inhibited the proliferation of bovine aortic endothelial cells (241). These studies support the notion that curcumin confers protection against pathological remodeling of blood vessels, a contributory mechanism of atherosclerosis.

Since atherosclerosis is a chronic inflammatory disease associated with increased oxidative stress in VSMC, it would be possible that the anti-atherogenic effects of curcumin are attributable to its antoxidant and anti-inflammatory properties (238). Curcumin modulates several targets that mediate an anti-atherosclerotic effect but the detailed anti-atherosclerotic mechanisms of curcumin still remain to be elucidated.

1.17.2 Curcumin effects on the heart

The effect of curcumin on cardiac hypertrophy and myocardial ischemia (MI) has been studied in both *in vivo* and *in vitr*o models. Cardiac hypertrophy is an adaptive enlargement of the myocardium in response to a variety of stresses, such as an increased workload or myocardial infarction, and is characterized by an increase in the size of the individual cardiomyocytes and the whole heart (242). Cardiac remodeling plays a critical role in the progression of pathologic cardiac hypertrophy to heart failure and death (242). Hypertrophic stimuli initiate several subcellular signaling pathways and these signals reach the nuclei of cardiomyocytes and activate a subset of hypertrophy-responsive transcription factors that change the pattern of the gene expression. Activation of these transcription factors is mediated, in part, through post-transcriptional modifications, such as acetylation by histone deacetylases and an intrinsic histone acetyltransferase (HAT), p300 (243). Nuclear acetylation by p300 is a critical event during cell hypertrophy. Activation of p300 is not only required for pathological myocyte growth but also for normal myocardial development and differentiation. p300 also induces the expression of genes encoding atrial natriuretic factor (ANF), ET-1, and β-myosin heavy chain (β-MHC), which are well established markers of myocardial cell hypertrophy (244-246). Curcumin was reported to be an inhibitor of p300-HAT and was found to repress the p300 induced hypertrophic responses in cultured neonatal cardiomyocytes, including the expression of both ANF and β-MHC genes

(247). It also inhibited the p300 HAT activity and thereby prevented the development of heart failure in two different heart failure models *in vivo*, a hypertensive heart disease in salt-sensitive Dahl rats and in a surgically-induced myocardial infarction in rats (247).

Heart failure is a process of systemic inflammation with overexpression of local inflammatory cytokines including TNF-α (248). Recent reports have shown that TNF-α contributes to the process of myocardial remodeling in evolving heart failure (249). Orally administered curcumin improved left ventricular function in rabbits exhibiting heart failure-induced by pressure overload that resulted from the inhibition of myocardial collagen remodeling associated with suppression of TNF-α expression (250).

As previously mentioned, curcumin also behaves as a free radical scavenger and antioxidant, inhibiting lipid peroxidation and oxidative DNA damage. Puvanakrishnan et al. reported that curcumin plays a protective role against isoproterenol-induced myocardial necrosis in rats and that this protective effect is attributed to its antioxidant properties (214). L-Isoproterenol (ISO) is a synthetic cathecholamine that causes myocardial damage when administered in large doses. Orally administered curcumin (200mg/kg) protected against ISO-induced oxidative myocardial injury in rats through enhancement of the antioxidant defense and thereby exhibited cardioprotective activity against ISO-induced cardiotoxicity (251). Furthermore, Venkatesan observed a protective effect of orally administered curcumin against cardiotoxicity produced by adriamycin in rats, showing a reduction in the parameters that indicate lipid peroxidation (252). Moreover, curcumin suppressed neutrophil infiltration into the human injured myocardium (164). Neutrophils are a major source of free radicals that characteristically invade the myocardial tissue during ischemia (164).

Ang II can play a critical role in the genesis of cardiac hypertrophy in hypertension and MI (253). Cardiac hypertrophy in response to Ang II is initiated though activation of its receptors, which results in marked oxidant stress via NADPH oxidase and NF-κB activation (254, 255). Downstream of NADPH oxidase lies the major redox-sensitive transcription factor NF-κB, which is perhaps the most critical transcription factor in mediating the transcription of a host of pro-inflammatory, pro-oxidant, and pro-growth genes, in response to Ang II (256). Freund et al. demonstrated the requirement of NF-κB in Ang II-mediated cardiac hypertrophy *in vivo* (257). Curcumin attenuates Ang II-mediated ROS generation, and the expression of NADPH oxidase and NF-κB in cardiomyocytes (253). Attenuation of the redox state by curcumin results in the abrogation of Ang II-mediated cardiomyocyte growth as well as the expression of the hypertrophic markers, ANP and BNP. Oxidized-LDL receptor-1 (LOX-1) upregulation has a central role in cardiomyocyte hypertrophy response to Ang II (253). Curcumin reduced the Ang II-mediated upregulation of Ang II type 1 receptor (AT1R) and LOX-1 expression and activation which translates into a strong inhibition of redox signaling resulting in a marked inhibition of cardiomyocyte growth (253).

1.17.3 Curcumin and hypertension

The effect of curcumin on hypertension has not been explored in detail, yet a recent study revealed for the first time, under *in vivo* conditions, that curcumin attenuated the development of hypertension in NO-deficient hypertensive rats (163). Curcumin suppressed blood pressure elevation, decreased vascular resistance and restored vascular responsiveness in rats with N^{ω}-nitro-L-arginine methyl ester ($_{L}$-NAME)-induced hypertension, through an antioxidant mechanism (163). Curcumin has also been shown to induce vasorelaxation on isolated porcine coronary arteries through its antioxidant capacity to promote NO release (258). Moreover,

curcumin supplementation showed significant attenuation of mean arterial blood pressure in streptozotocin-induced diabetic rats (259). In humans, oral turmeric supplementation decreased systolic blood pressure in patients with kidney disease, lupus nephritis (260). Curcumin supplementation may therefore be beneficial in improving vascular function and preventing cardiovascular complications.

1.17.4 Curcumin and diabetic cardiovascular complications

A pilot study done in 1972 reported that curcumin lowered blood sugar levels in human diabetic subjects (261). Since then, the modulation of curcumin has been extended to elucidate the molecular basis for obesity and obesity-related metabolic diseases, including type 2 diabetes and cardiovascular diseases. The anti-diabetic effects of curcumin are also linked with the inhibition of inflammatory and oxidative markers. Increased oxidative stress has been associated with the pathogenesis of chronic diabetic complications, including cardiomyopathy (262). It has been suggested that NO pathway is involved in augmenting oxidative stress (263). Many studies have shown the implication of eNOS and iNOS in the pathogenesis of cardiovascular complications in diabetes (264). In a study where myocardial tissue from diabetic rats exhibited increased levels of eNOS and iNOS mRNA, curcumin treatment prevented this NOS mRNA upregulation leading to a decrease in the oxidative DNA damage in association with a reduction in the expression of transcription factors NF-κB and AP-1 (265).

Diabetic cardiomyopathy eventually leads to heart failure (186). Transcriptional co-activator p300 and its interaction with myocyte enhancer factor 2 (MEF2) play a role in diabetes-induced cardiomyocyte hypertrophy. Curcumin treatment prevented diabetes-induced

upregulation of these transcripts, suggesting a protective mechanism in glucose-induced cardiomyocyte hypertrophy in diabetes (266).

NF-κB, TNF-α and Egr-1 have been closely linked with the induction of insulin resistance, a key feature in type II diabetes mellitus. Curcumin has been shown to downregulate these biomarkers (195, 267-270), suggesting a putative mechanism in overcoming insulin resistance as demonstrated by several animal studies (271-273).

Hyperglycemia leads to increased oxidative stress resulting in endothelial dysfunction. A randomized 8 week-study was performed on 72 patients with type II diabetes to evaluate the effects of curcumin on endothelial dysfunction in association with reductions in inflammatory cytokines and markers of oxidative stress. Patients receiving 150 mg of curcumin twice daily showed an improvement of endothelial function and significant reductions in the levels of malondialdehyde, ET-1, IL-6, and TNF-α (274). Curcumin supplementation also improved diabetes-induced endothelial dysfunction in streptozotocin-induced diabetic rats through decreased PKC expression and decreased ROS production (259).

The precise mechanism by which curcumin improves vascular homeostasis remains unclear. However, the ability of curcumin to ameliorate pathophysiological states, such as atherosclerosis, cardiac remodeling and myocardial ischemia, through antioxidant and anti-inflammatory properties, have been suggested to contribute to this response.

1.18 Goal of this study

The broad biological activity of curcumin, including antioxidant and anti-inflammatory effects, influences key cellular signal transduction pathways in multiple diseases. Curcumin has proven to be beneficial in studies within the cardiovascular system involving atherosclerosis,

ischemia-induced diseases, diabetic-induced cardiovascular complications, cardiac hypertrophy and myocardial infarction. However, very little is known regarding the molecular mechanisms involved in mediating these responses.

ET-1 is a very potent active molecule that plays an important role in health and disease through its ability to regulate various cardiovascular functions. ET-1 activates multiple signaling pathways including MAPK and PI3-K/PKB which mediate the hypertrophic and proliferative responses in VSMC. An important role of oxidative stress generation in mediating the effect of ET-1 has been demonstrated. Moreover, ROS are produced and act as second messengers as part of the signaling of receptor protein tyrosine kinases, which are activated after vascular injury. Curcumin has been shown to suppress ROS generation and to inhibit several of the mediators involved in the ET-1 signaling pathway in several cellular lines. Curcumin has also been shown to inhibit cell proliferation, arrest cell cycle progression and induced cell apoptosis in VSMC (275). However, the effect of curcumin in ET-1-induced signaling in VSMC has not been investigated. Therefore, the present study aims at examining the effect of curcumin on ET-1-induced phosphorylation of ERK1/2, c-Raf, PKB and IGF-1R. Since ET-1 action requires IGF-1R transactivation we have also investigated if curcumin modulates IGF-1-induced signaling in VSMC. In addition, we tested if the transcription factor Egr-1 is also a target of curcumin in response to ET-1 as well as IGF-1.

CHAPITRE 2

ARTICLE

Attenuation of Endothelin-1 and Insulin-like-Growth-Factor 1-induced Signaling by Curcumin in A-10 Vascular Smooth Muscle Cells

To be submitted to Journal of Canadian Physiology and Pharmacology

Attenuation of Endothelin-1 and Insulin-Growth-Factor Type 1-induced Signaling by Curcumin in A-10 Vascular Smooth Muscle Cells

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Abstract

Despite recent advances, mortality from cardiovascular disease remains high, highlighting the requirement for improved therapies. Several studies have demonstrated a correlation between cardiovascular disease and an increased activation of ET-1-induced signaling in vascular smooth muscle cell (VSMC), including protein kinase B (PKB) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathways, as well as an increase in ET-1-mediated VSMC hypertrophy and proliferation. Oxidative stress has been suggested to play an intermediary role in mediating ET-1-induced cardiovascular pathophysiological effects. Curcumin (diferuloylmethane), a major component of the spice turmeric, has been shown to exhibit cardiovascular protective effects mainly through its antioxidant and anti-inflammatory properties. The current study was undertaken to examine the effectiveness of curcumin to inhibit ET-1-stimulated signaling events in A-10 VMSC. ET-1 (100nM) produced a marked phosphorylation of PKB, ERK1/2, c-Raf and insulin-like growth factor type 1 receptor (IGF-1R), in VSMC, that was dose dependently inhibited by pretreatment with curcumin. Curcumin also attenuated IGF-1-induced phosphorylation of PKB, ERK1/2 and c-Raf as well as phosphorylation of IGF-1R. Furthermore, curcumin also inhibited ET-1 and IGF-1-induced expression of early growth response-1 (Egr-1), a transcription factor downstream of ERK1/2 that plays a regulatory role in several cardiovascular pathological processes. In conclusion, these data provide evidence that curcumin is a potent inhibitor of ET-1 and IGF-1-induced mitogenic and proliferative signaling responses in A-10 VSMC and suggest that the ability of curcumin to attenuate these events may contribute as a potential mechanism for its cardiovascular protective effects.

Key words: Curcumin, Early growth response-1, Endothelin-1, Extracellular signal-regulated kinase 1 and 2, Insulin-like growth factor type 1, Insulin-like growth factor type 1 receptor, Protein kinase B, Vascular smooth muscle cells

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide that has been shown to exhibit mitogenic and hypertrophic responses in vascular smooth muscle cells (VSMC) (1, 2). A potential role of ET-1 in promoting the pathophysiology of cardiovascular diseases such as coronary artery disease (3), hypertension (4), heart failure (4) and vascular remodeling (atherosclerosis and restenosis) (5) has been suggested. VSMC play a critical role in the structural and functional characteristics of the vessel wall, including growth, repair and remodeling (6). ET-1 mediates its effects on VSMC through interaction with its two subtypes of heterotrimeric G protein coupled receptors (GPCR), ET_A and ET_B (1, 2). This leads to the activation of multiple signaling pathways, including growth promoting mitogen-activated protein kinases (MAPK), of which extracellular signal-regulated kinase 1 and 2 (ERK1/2) is the most well characterized, and phosphatidylinositol 3-kinase (PI3-K)/protein kinase B (PKB) pathways (1, 2). An intermediary role of transactivation of growth factor receptor protein tyrosine kinases (R-PTK) in transducing ET-1-induced signaling events has been proposed (7). In this regard, we have recently shown that insulin-like growth factor type 1 receptor (IGF-1R) plays a critical role in mediating ET-1-induced PKB activation, as well as hypertrophic and proliferative responses induced by ET-1 in VSMC (7). Although the mechanism of action responsible for R-PTK transactivation is not yet fully understood, several factors are suggested to be implicated in this process. Amongst them, the generation of reactive oxygen species (ROS) which has been shown to play an important role in inducing the activation of ERK1/2 and PKB signaling in VSMC in response to ET-1 (8), has also been shown to contribute to the activation of IGF-1R-PTK in VSMC (9). IGF-1R is classically activated by its growth factor ligand, insulin-like growth factor type 1 (IGF-1). IGF-1 can be synthesized and secreted in cultured VSMC (10), and has been shown to act as a potent mitogen for VSMC *in vitro* (11, 12). ERK1/2 and PI3-K/PKB signaling promotes IGF-1-induced

mitogenic and proliferative responses respectively, in VSMC (13, 14). Accordingly, inhibition of ET-1 and IGF-1-stimulated VSMC signaling pathways may serve as a potential therapeutic intervention to attenuate cellular manifestations associated with the progression of many vascular diseases.

Curcumin, a natural polyphenolic compound found in the spice turmeric (*Curcuma Longa*), has recently attracted much attention as it has been shown to exhibit a vast array of biological activities, including antioxidant, anti-proliferative, anti-tumour and anti-inflammatory properties (15). Several studies have suggested that curcumin may play a role in the protection against certain cardiovascular diseases, including arterial disease (16), atherosclerosis (17), cardiac hypertrophy (18-20), heart failure (20), and damage caused by myocardial ischemia (21-23). The precise mechanisms responsible for the cardiovascular protective effect remains obscure however, curcumin has been shown to exert its effects on several signaling pathways linked to growth, proliferation and gene expression (24-29). It has been shown to inhibit serum-induced and platelet-derived growth factor (PDGF)-dependent mitogenesis in VSMC (26). In a recent study, curcumin attenuated PDGF-induced signaling in VSMC through inhibition of PDGF receptor, ERK1/2 and PKB phosphorylation (25). Since curcumin exhibits anti-proliferative effects and has potent antioxidant properties with its ability to scavenge ROS (30-32), and ET-1 exerts its effects through the generation of ROS with an intermediary role of IGF-1R transactivation (7, 8), the purpose of this study was to investigate the effect of curcumin on ET-1 and IGF-1-mediated proliferative and hypertrophic signaling responses in VSMC. Therefore, we examined the effect of curcumin on ET-1 and IGF-1-induced phosphorylation of IGF-1R, c-Raf, ERK1/2 and PKB, key mediators involved in growth-promoting, proliferative and hypertrophic responses. In addition, we have also investigated the effect of curcumin on early growth response

(Egr)-1, a transcription factor which is a downstream target of ERK1/2 and is implicated in multiple cardiovascular pathological processes (33).

Materials and Methods

***Antibodies and reagents**:*

Cell culture reagents were procured from Invitrogen Corp. (Grand Island, NY). ET-1 was purchased from American Peptide Inc. (USA). IGF-1 was purchased from Peprotech Inc. (USA). Monoclonal phospho-specific-Tyr^{204} ERK1/2, polyclonal ERK1/2, anti-IGF-1R, Egr-1 and GAPDH antibodies, as well as horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were from Santa Cruz Biotech (Santa Cruz, CA). The phospho-Ser^{473}-specific-PKB, anti-PKB, phospho-Ser^{338}-specific-c-Raf, anti-c-Raf and anti-rabbit antibodies were from Cell Signaling (Beverly, MA). Curcumin was procured from Calbiochem (San Diego, CA). Anti-phosphospecific IGF-1R (phospho-Tyr$^{1158/1162/1163}$) antibody was obtained from Biosource. Hoechst 33342 (10mg/ml) was obtained from Invitrogen. The enhanced chemiluminescence (ECL) detection kit was from Amersham Pharmacia Biotech (Baie d'Urfé, Qc, Canada).

***Cell culture*:**

VSMC are derived from embryonic rat thoracic aorta A-10 cells and were maintained in culture with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin + streptomycin, at 37^{o}C in a humidified atmosphere of 5% CO_2. Cells were grown to 80-90% confluence in 60mm plates and incubated in serum-free DMEM 20 hours prior to treatment. They were passaged twice a week by harvesting with Trypsin/EDTA.

Immunoblotting:

Cells incubated in the absence or presence of various agents were washed twice with ice-cold PBS and lysed in 200µl of buffer (25nM Tris-HCl, pH 7.5. 25mM NaCl, 1mM Na orthovanadate, 10mM Na fluoride, 10 mM Na pyrophosphate, 2 mM benzamidine, 2 mM ethylenebis (oxyethylenenitrilo)-tetraacetic acid (EGTA), 2mM ethylenediamine-tetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 0.5 µg/ml leuleptin) on ice. The cells were scraped, collected and clarified by centrifugation at 12,000g for 10 minutes at 4^{o}C to remove insoluble materials. Protein concentrations were measured using Bradford assay. Equal amounts of protein were subjected to 7.5% SDS-polyacrylamide gel (SDS-PAGE), transferred to polyvinylidine difluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked 1 hour with PBS-Tween 20 containing 5% non-fat dry milk at room temperature and then incubated overnight at 4^{o}C with respective primary antibodies (Monoclonal phospho-specific-Tyr^{204} ERK1/2 antibody (1:4000), phospho-Ser^{338}-specific-c-Raf (1:2000), phospho-Ser^{473}-specific-PKB antibody (1:2000), phosphor-Tyr$^{1131/1135/1136}$-specific-IGF-1R (1:000), Egr-1 (1:1000), anti-PKB (1:2000), polyclonal ERK1/2 (1:4000), anti-c-Raf (1:2000), anti-IGF-1R (1:1000) and GAPDH (1:2000)). The antigen-antibody complex was detected by horseradish peroxidase-conjugated second antibody (1:4000), and protein bands were visualized by ECL. The intensity of the bands was quantified by densitometric analysis using Quantity One imaging and Graphpad Prism 5 software programs.

Nuclear extraction protocol

Cells incubated in the absence or presence of various agents were washed twice with ice-cold PBS and collected in 500µl of buffer solution containing 10mM Hepes, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM protease cocktail inhibitor and 1mM Na orthovanadate. Lysates were incubated in ice for 15 minutes before the addition of NP40 10% detergent and then they were vortexed for 10 seconds at highest setting before being centrifuged at 13000RPM for 4 minutes at 4^{o}C. The supernatant (that corresponds to the cytoplasmic fraction) was saved and transferred in a clean tube. The pellet was resuspended in 60µl, by pipeting up and down several times, in buffer containing 10mM Hepes, 400mM NaCl, 0.1mM EDTA, 0.1mM EGTA, 1mM PMSF, 1mM protease cocktail inhibitor and 1mM Na orthovanadate. Lysates were sonicated by performing 6 cycles at 10 seconds per cycle with 30 second intervals and then centrifuged at 13000RPM for 5 minutes at 4^{o}C. Pellet was discarded and the supernatant, which corresponds to the nuclear fraction, was collected. Protein concentrations were measured using Bradford assay.

Apoptosis assay

Curcumin-induced apoptosis was monitored by the extent of nuclear fragmentation. Nuclear fragmentation was visualized by Hoechst 33342 staining of apoptotic nuclei. Hoeckst 33342 is a fluorescent bisbenzimide that stains DNA and penetrates membranes of dead cells. It is excited by ultraviolet light of 350nm and emits blue light of 461nm wavelength. After treatment with varying concentrations of curcumin for 30 minutes cells were treated with 2% paraformaldehyde (diluted in PBS) in 2000µL basal medium. The adhered cells were then stained with 2.5µL of Hoechst 33342 (10mg/ml Invitrogen) in 2500µL medium and incubated at room temperature in the dark for 15 minutes. The cells were imaged under an Olympus BH-2 fluorescence microscope

using 350nm stimulation and 460nm emission. Triplicates samples were used for each treatment. Under our assay the treatment of A-10 VSMC for 30 minutes at 50 μM did not cause any significant cell death (data not shown).

***Statistics*:**

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

Results

Curcumin inhibits ET-1- and IGF-1-induced phosphorylation of PKB in A-10 VSMC

PKB activation has been shown to contribute to hypertrophic responses in VSMC (reviewed in (1)). Therefore, we investigated the effect of curcumin on PKB phosphorylation induced by ET-1 and IGF-1. As shown in Fig. 1 both ET-1 and IGF-1 enhanced the phosphorylation of PKB 3 fold and 15 fold, respectively. However, pretreatment of A-10 VSMC with curcumin for 30 minutes dose-dependently attenuated both ET-1 and IGF-1-induced phosphorylation of PKB. The attenuating effect of curcumin could be detected at 5μM but was more prominent at higher concentrations and at 25μM almost complete inhibition of ET-1-induced PKB phosphorylation was observed (Fig. 1A). Similarly, curcumin also inhibited IGF-1-induced phosphorylation of PKB in these cells but appeared to be slightly less potent as compared to its effect on ET-1-induced response (Fig. 1B).

Curcumin inhibits ET-1- and IGF-1-induced phosphorylation of c-Raf and ERK1/2 in A-10 VSMC

Activation of ERK1/2 signaling has been implicated in mediating hypertrophic and proliferative effects of ET-1 (34, 35). Therefore, we next investigated the effect of curcumin on ERK1/2 signaling (Fig 2). Treatment with ET-1 and IGF-1 increased ERK1/2 phosphorylation in A-10 VSMC (Fig. 2). However, pre-treatment of the cells with curcumin prior to stimulation with either ET-1 or IGF-1 resulted in a significant inhibition in ERK1/2 phosphorylation induced by both ET-1 and IGF-1 (Fig. 2). Curcumin had no effect on the expression of ERK1/2 protein (Fig. 2).

ERK1/2 activation requires sequential activation of Ras, Raf and MEK upstream signaling components (1). Therefore, we examined the effect of curcumin on the activated c-Raf levels by measuring the phosphorylation level of the c-Raf protein at serine 338 in A-10 VSMC treated with ET-1 and IGF-1. As shown in Fig. 3, curcumin pretreatment markedly inhibited the phosphorylation of c-Raf stimulated by both agents in a dose-dependent manner without altering the levels of this protein. The inhibitory effect of curcumin on both ET-1 and IGF-1-induced phosphorylation of c-Raf was quite significant at 25μM and became even more prominent at 50 μM, where almost complete inhibition was observed (Fig. 3). These data indicate that curcumin inhibits the phosphorylation and hence the c-Raf kinase activity of this upstream component of ERK1/2 signaling pathway and thereby inhibits ET-1 and IGF-1-induced phosphorylation of ERK1/2 in A-10 VSMC.

Attenuation of ET-1- and IGF-1-induced tyrosine phosphorylation of IGF-1R β subunit by curcumin in A10-VSMC

Since, we have previously reported that ET-1 is capable of increasing tyrosine phosphorylation of IGF-1R β subunit, which is required for its activation, and that IGF-1R-PTK is involved in mediating ET-1-induced PKB activation in A-10 VSMC (7), we next tested the effect of curcumin on the ET-1-mediated phosphorylation of IGF-1R. As shown in Fig. 4A, when compared with ET-1 alone, curcumin inhibited ET-1-induced phosphorylation of IGF-1R. A pronounced attenuating effect was detected at 5μM whereas almost complete inhibition of IGF-1R phosphorylation was observed at 25μM (Fig. 4A). In order to distinguish this ET-1-induced transactivation of IGF-1R with the ligand-dependent activation of IGF-1R, we investigated the effect of curcumin on IGF-1-induced phosphorylation of IGF-1R. As expected, IGF-1 increased

IGF-1R phosphorylation and curcumin dose-dependently attenuated this effect with a marked attenuation observed at 25μM and an almost complete inhibition observed at 50μM (Fig. 4B).

Inhibition of ET-1- and IGF-1-induced upregulation of Egr-1 by curcumin in A-10 VSMC

It has been suggested that Egr-1 plays a regulatory role in multiple cardiovascular pathological processes (33). It has a low basal level expression in normal vessels but is rapidly and transiently expressed in VSMC and endothelial cells in response to injury (36). Egr-1 protein is biologically active and has exerts profound chemotactic and mitogenic effects in injured vascular cells, which may contribute to the structural remodeling that typically occurs in the pathogenesis of vascular diseases (36). Therefore, we wished to determine if ET-1 or IGF-1 will upregulate Egr-1 expression in A-10 VSMC. We observed that Egr-1 protein expression was upregulated within 30 minutes and peaked at 60 minutes of ET-1 and IGF-1 exposure of A-10 VSMC (Fig. 5). Since Egr-1 gene expression has been shown to be dependent on ERK1/2 signaling (33), and curcumin exerts an inhibitory effect on the ERK1/2 pathway, we were further interested in investigating the effect of curcumin on ET-1 and IGF-1-induced Egr-1 expression in A-10 VSMC. As shown in Fig. 6, pre-treatment of VSMC with curcumin prior to stimulation with either ET-1 or IGF-1 inhibited the expression of Egr-1 induced by these two vasoactive agents.

Discussion

Curcumin has been suggested to exert cardiovascular protective effects in a variety of experimental models. However, the precise molecular mechanism responsible for this response remains poorly understood. ET-1 and IGF-1 are important vasoactive peptides with a key role in the pathogenesis of vascular disease. The current study has assessed the effect of curcumin, the major active ingredient of turmeric (*Curcuma Longa*) spice, on ET-1 and IGF-1-induced signaling events. Here, we demonstrate that curcumin treatment attenuated ET-1 and IGF-1-induced phosphorylation of IGF-1R, ERK1/2, c-Raf and PKB in VSMC. We also show that curcumin treatment also inhibited the expression of zinc finger transcription factor Egr-1 by these vasoactive agents.

There is growing evidence that curcumin has a potential role in the protection against cardiovascular diseases. It was reported that curcumin improved the development of cardiac hypertrophy, through deactivation of hypertrophic signaling, and heart failure in animal models (18, 20). A recent study demonstrated that curcumin attenuated the development of hypertension, improved hemodynamic status and restored vascular function in nitric oxide-deficient hypertensive rats through alleviation of oxidative stress (37).

Although, curcumin has been shown to attenuate the response of several growth factors and their signaling pathways in cancerous cell lines (24), there are only limited studies on its effect in VSMC. Yang et al. have reported an inhibitory effect of curcumin on PDGF signaling and PDGF-stimulated VSMC proliferation, migration, and collagen synthesis (25). They demonstrated that curcumin inhibits PDGFR phosphorylation as well as the PDGF-induced phosphorylation of ERK1/2 and PKB (25). Dehydrozinzerone, a structurally half-analog and biosynthetic intermediate of curcumin, also elicited a similar inhibition of PDGF-stimulated

phosphorylation of PDGFR, PKB and ERK1/2 in VSMC, leading to the inhibition of VSMC migration, proliferation and collagen synthesis (38). However, our work represents the first study to demonstrate that curcumin antagonizes both ET-1 and IGF-1-induced IGF-1R phosphorylation, as well as their ability to stimulate the phosphorylation of c-Raf, ERK1/2 and PKB in VSMC. Given that an upregulated ET-1 system has been linked to hyperproliferation in VSMC from SHR (39), it may be suggested that curcumin-induced attenuation of ET-1 signaling might serve as one of the mechanisms by which curcumin exerts anti-hypertrophic effects. Since curcumin is an antioxidant and ET-1 as well as IGF-1-induced signaling requires ROS generation (8, 40), it may be possible that the ability of curcumin to reduces ROS formation may be one of the mechanisms by which curcumin exerts its attenuating effect on these signaling events.

The zinc finger transcription factor Egr-1 plays an important role in vascular biology. The Egr family includes Egr-1, Egr-2, Egr-3, and Egr-4. Among the family members, the best characterized is Egr-1. Following activation, Egr-1 is primarily expressed in the nucleus and is capable of regulating the transcription of several genes implicated in the development of vascular disease (41). Egr-1 is weakly, if at all, expressed in normal vessel wall but has been shown to be highly expressed in response to acute injury (36, 42) and vasoactive agents, such as angiotensin II (Ang II) (43). It is highly expressed in VSMC of atherosclerotic lesions (44) and plays critical roles in regulating VSMC growth and intimal thickening after vascular injury (45). ERK1/2 plays a prominent role in activating Egr-1 expression in endothelial cells (46) and VSMC (43, 47). Studies have shown that attenuation of Egr-1 gene expression inhibits VSMC migration and proliferation (48-51). Thus, it may be suggested that curcumin-induced attenuation of Egr-1 expression may attribute to its cardiovascular protective role. This notion is further supported by studies in which antisense nucleotides against Egr-1 were found to block ET-1-induced cardiac protein synthesis, a marker of cardiac hypertrophy (52).

The antioxidant activity of curcumin was reported as early as 1975 (53). Oxidative stress associated with the overproduction of ROS plays a major role in the pathogenesis of various cardiovascular diseases, including hypertension and atherosclerosis (54). The antioxidant activities of curcumin may be attributable to many factors, including the ability to scavenge ROS, the increase in NO bioavailability, and the enhancement of the antioxidant defense system. Curcumin has been proven to be a potent scavenger of a variety of ROS including superoxide anion radicals (O_2^-) (30), hydroxyl radicals (OH^-) (31), nitrogen dioxide radicals (32) and non-free radical species such as hydrogen peroxide (H_2O_2) (30). It has also been shown to enhance the activity of antioxidant enzymes (55) and to counteract the activity of ROS generating enzymes (56). In human endothelial cells, curcumin was shown to abolish ROS production and attenuate NADPH oxidase activity (57). In cardiomyocytes, attenuation of the redox state by curcumin resulted in the abrogation of Ang II-mediated cardiomyocyte growth (19). In synergy with its anti-inflammatory properties, curcumin prevented VSMC migration through its ability to suppress ROS generation (58). Moreover, curcumin has been shown to inhibit H_2O_2-stimulated phosphorylation of PDGF receptor in VSMC (38). As previously mentioned, ET-1 has been shown to increase the production of ROS in VSMC and this ROS generation has been shown to be a critical mediator in ET-1-induced signaling events linked to growth-promoting proliferative and hypertrophic pathways in VSMC (8). Our earlier studies have also shown that tyrosine phosphorylation of β-subunit IGF-1R is an important step in transducing the effect of H_2O_2 on the phosphorylation of ERK1/2 and PKB in VSMC (9, 59). Since both ERK1/2 and PKB signaling pathways play a critical role in mediating hypertrophic and cell survival responses (1), it is reasonable to suggest that the ability of curcumin to inhibit the IGF-1 and ET-1-induced activation of these pathways may be due to its antioxidative properties. In light of these findings,

it may be suggested that curcumin-induced inhibition of both PKB and ERK1/2 signaling, and Egr-1 expression, may at least, partially contribute to the vascular protective effect of curcumin.

In summary, this is the first report demonstrating that curcumin attenuates the signaling responses of ET-1 and IGF-1 in A-10 VSMC. We demonstrated that curcumin attenuated both IGF-1 and ET-1-stimulated increase of PKB, c-Raf, ERK1/2 and IGF-1R activation and Egr-1 expression in A-10 VSMC. Since ERK1/2, PKB and Egr-1 play a key role in mediating VSMC growth and hypertrophy, it may be suggested that the ability of curcumin to attenuate these pathways may serve as a potential mechanism by which it counteracts the biological response of IGF-1 and ET-1 and exerts a vascular protective effect.

Acknowledgments

This work was supported by funding form the Canadian Institutes of Health Research (CIHR) to A.K.S.

Figure Legends

Figure 1. Curcumin attenuates ET-1 and IGF-1-induced PKB phosphorylation in A-10 VSMC. A. Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 100nM of ET-1 for 5 minutes. **B.** Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 6.5nM of IGF-1 for 5 minutes. Cell lysates were immunoblotted by phospho-specific-Ser^{473}-PKB antibody (top panels of each section). Blots were also analyzed for total PKB (middle panels of each section). Bottom panels (bar diagrams) represent average data quantified by densitometric scanning of protein bands shown in the top panel. Values are the means ± SE of 6 distinct western blots from 3 independent experiments that were performed in duplicate, and are expressed as fold increase over basal phosphorylation, which is taken as 1. No results were excluded. $P<0.05$ considered as statistically significance versus ET-1 or IGF-1 alone.* indicates that $P<0.05$, ** indicated that $P<0.005$, and *** indicates that $P<0.0005$.

Figure 2. Curcumin attenuates ET-1 and IGF-1-induced ERK1/2 phosphorylation in A-10 VSMC. A. Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 100nM of ET-1 for 5 minutes. **B.** Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 6.5nM of IGF-1 for 5 minutes. Cell lysates were immunoblotted by phospho-specific –Tyr^{204}ERK1/2 antibody as shown in the top panels of each section. Blots were also analyzed for total ERK1/2 (middle panels of each section). Bottom

panels (bar diagrams) represent average data quantified by densitometric scanning of protein bands shown in the top panel. Values are the means ± SE of 6 distinct western blots from 3 independent experiments that were performed in duplicate, and are expressed as fold increase over basal phosphorylation, which is taken as 1. No results were excluded. $P<0.05$ considered as statistically significance versus ET-1 or IGF-1 alone.* indicates that $P<0.05$, ** indicated that $P<0.005$, and *** indicates that $P<0.0005$.

Figure 3. Curcumin attenuates ET-1 and IGF-1-induced c-Raf phosphorylation in A-10 VSMC. A. Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 100nM of ET-1 for 5 minutes. **B.** Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 6.5nM of IGF-1 for 5 minutes. Cell lysates were immunoblotted by phospho-Ser^{338}-specific-c-Raf antibody as shown in the top panels of each section. Blots were also analyzed for total c-Raf (middle panels of each section). Bottom panels (bar diagrams) represent average data quantified by densitometric scanning of protein bands shown in the top panel. Values are the means ± SE of 6 distinct western blots from 3 independent experiments that were performed in duplicate, and are expressed as fold increase over basal phosphorylation, which is taken as 1. No results were excluded. $P<0.05$ considered as statistically significance versus ET-1 or IGF-1 alone.* indicates that $P<0.05$, ** indicated that $P<0.005$, and *** indicates that $P<0.0005$.

Figure 4. Curcumin attenuates ET-1 and IGF-1-induced IGF-1R tyrosine phosphorylation in A-10 VSMC. A. Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 100nM of ET-1 for 5 minutes. **B.** Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 6.5nM of IGF-1 for 5 minutes. Cell lysates were immunoblotted by phospho-specific IGF-1R (phospho-$Tyr^{1158/1162/1163}$) antibody as shown in the top panels of each section. Blots were also analyzed for total IGF-1R (middle panels of each section). Bottom panels (bar diagrams) represent average data quantified by densitometric scanning of protein bands shown in the top panel. Values are the means ± SE of 6 distinct western blots from 3 independent experiments that were performed in duplicate, and are expressed as fold increase over basal phosphorylation, which is taken as 1. No results were excluded. $P<0.05$ considered as statistically significance versus ET-1 or IGF-1 alone.* indicates that $P<0.05$, ** indicated that $P<0.005$, and *** indicates that $P<0.0005$.

Figure 5. Egr-1 is upregulated by ET-1 and IGF-1 in A-10 VSMC. A. Serum-starved quiescent A-10 cells were treated without or with 100nM ET-1 for the identified time periods. B. Serum-starved quiescent A-10 cells were treated without or with 6.5nM of IGF-1 for the identified time periods. Nuclear fractions of the cell lysates were immunoblotted by Egr-1 antibody as shown in the top panels of each section. Blots were also analyzed for protein loading, using GAPDH (middle panels of each section). Bottom panels (bar diagrams) represent average data quantified by densitometric scanning of protein bands shown in the top panel. Values are the means ± SE of 6 distinct western blots from 3 independent experiments that were performed in duplicate, and are expressed as fold increase over basal phosphorylation, which is taken as 1. No

results were excluded. P<0.05 considered as statistically significance versus control.* indicates that P<0.05.

Figure 6. Curcumin downregulates Egr-1 in response to ET-1 and IGF-1 in A-10 VSMC. A. Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 100nM of ET-1 for 60 minutes. B. Serum-starved quiescent A-10 cells were pretreated without or with the indicated curcumin concentrations for 30 minutes, followed by 6.5nM of IGF-1 for 60 minutes. Nuclear fractions of the cell lysates were immunoblotted by Egr-1 antibody as shown in the top panels of each section. Blots were analyzed for total nuclear protein by GAPDH (middle panels of each section). Bottom panels (bar diagrams) represent average data quantified by densitometric scanning of protein bands shown in the top panel. Values are the means ± SE of 6 distinct western blots from 3 independent experiments that were performed in duplicate, and are expressed as fold increase over basal phosphorylation, which is taken as 1. No results were excluded. P<0.05 considered as statistically significance versus ET-1 or IGF-1 alone. *** indicates that P<0.0005.

A.

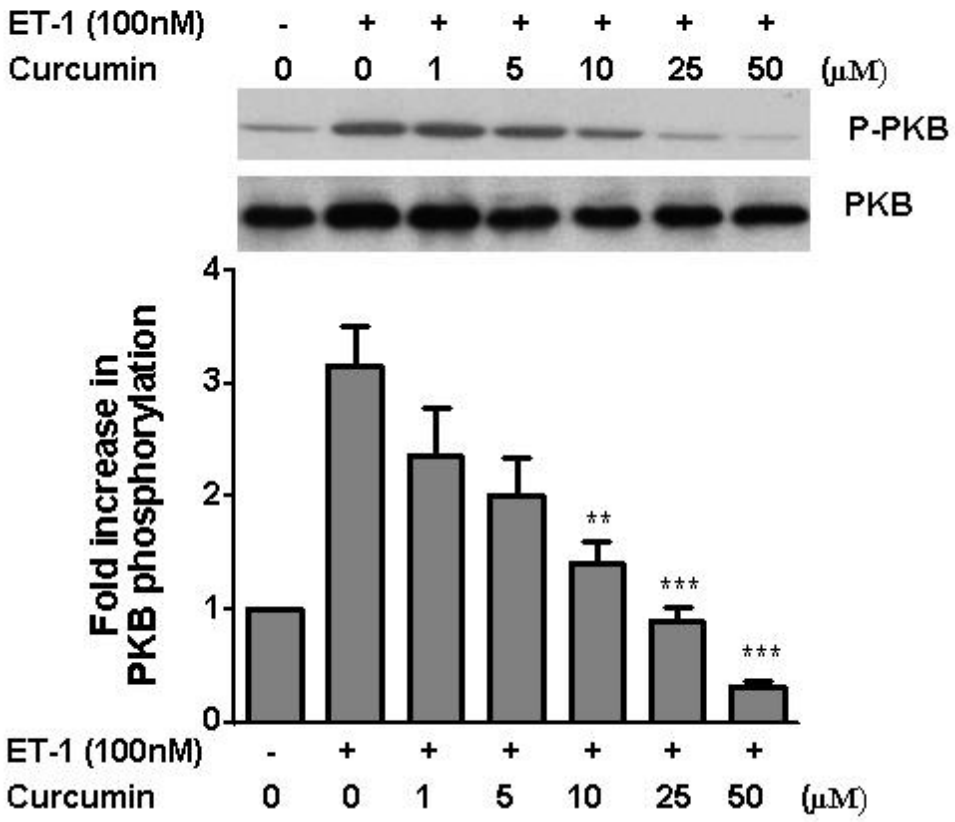

ET-1 (100nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)
P-PKB
PKB
Fold increase in PKB phosphorylation
0
1
2
3
4
**
***
***
ET-1 (100nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)

Figure 1

B.

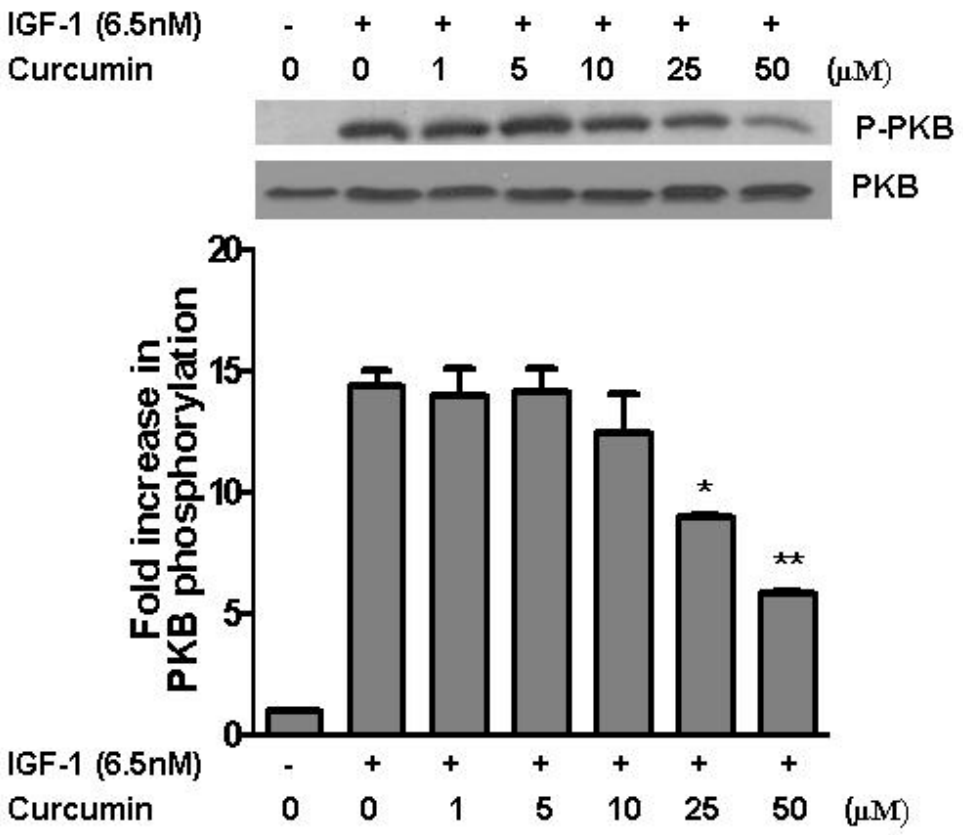

IGF-1 (6.5nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)
P-PKB
PKB
Fold increase in PKB phosphorylation
20
15
10
5
0
*
**
IGF-1 (6.5nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)

Figure 1

A.

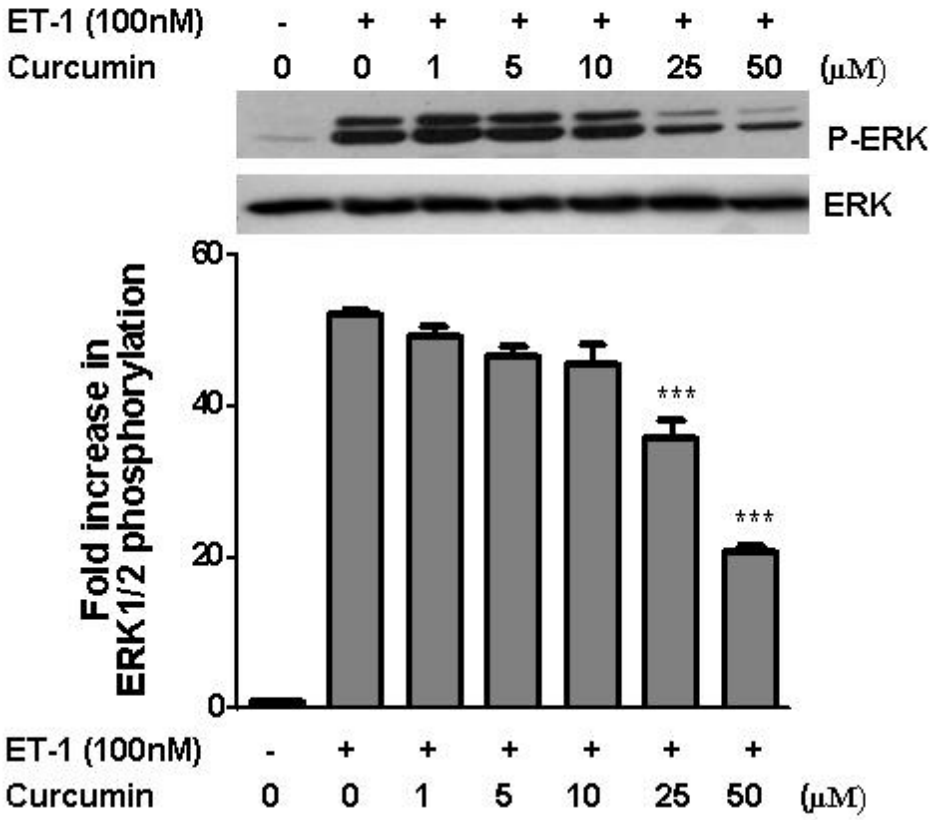
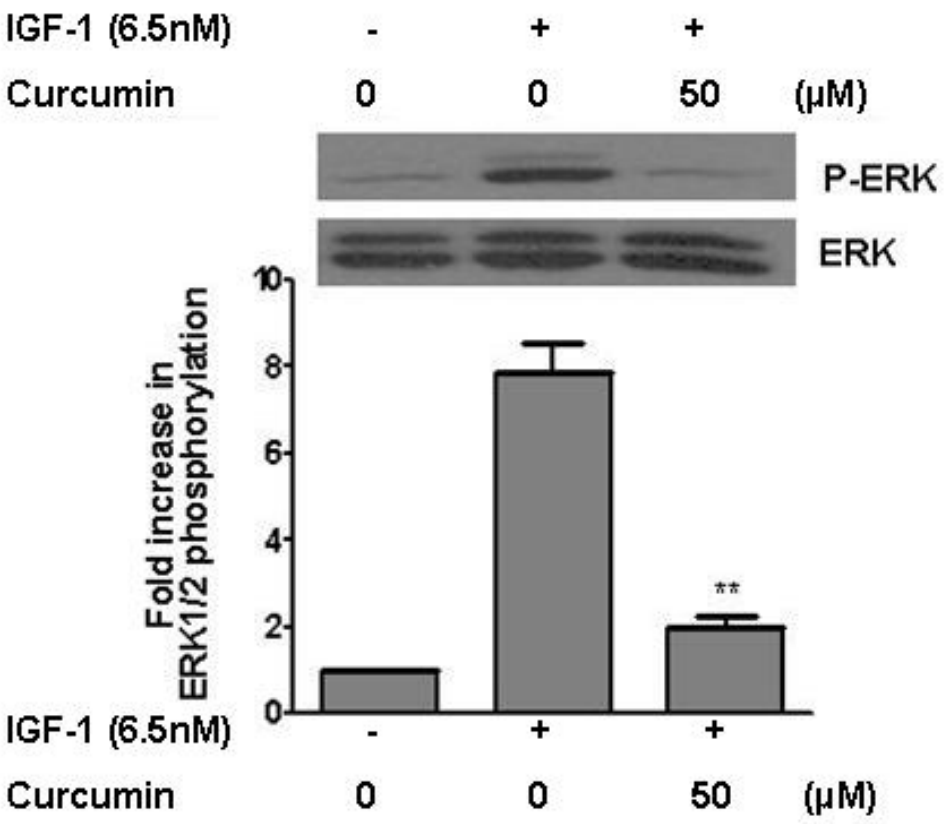

ET-1 (100nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)
P-ERK
ERK
Fold increase in ERK1/2 phosphorylation
60
40
20
0
***
***
ET-1 (100nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)

Figure 2

B.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Curcumin 0 0 50 (μM)
P-ERK
ERK
Fold increase in ERK1/2 phosphorylation
10
8
6
4
2
0
**
IGF-1 (6.5nM) - + +
Curcumin 0 0 50 (μM)

Figure 2

A.

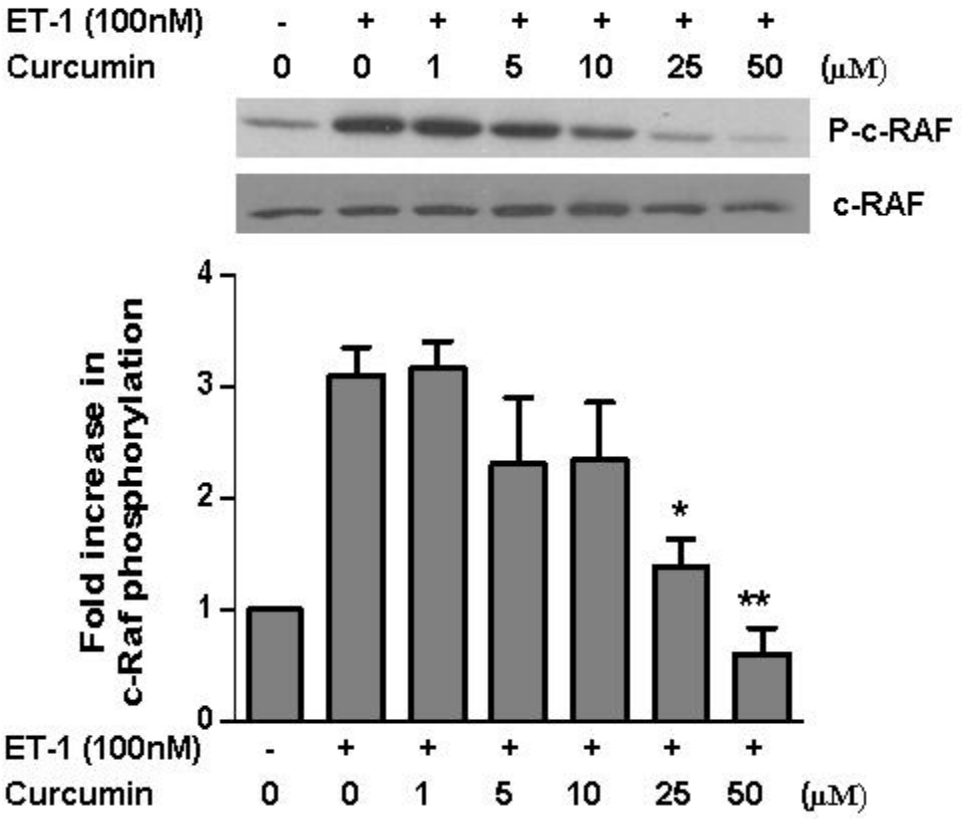

ET-1 (100nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)
P-c-RAF
c-RAF
Fold increase in c-Raf phosphorylation
0
1
2
3
4
*
**
ET-1 (100nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)

Figure 3

B.

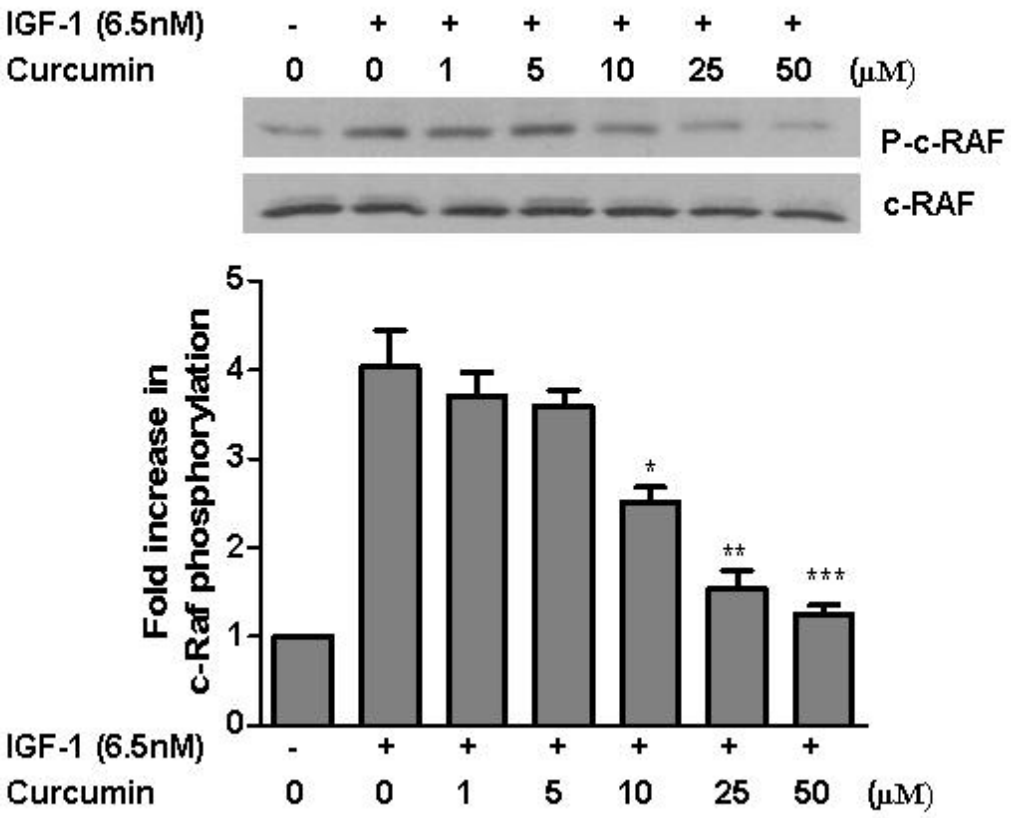
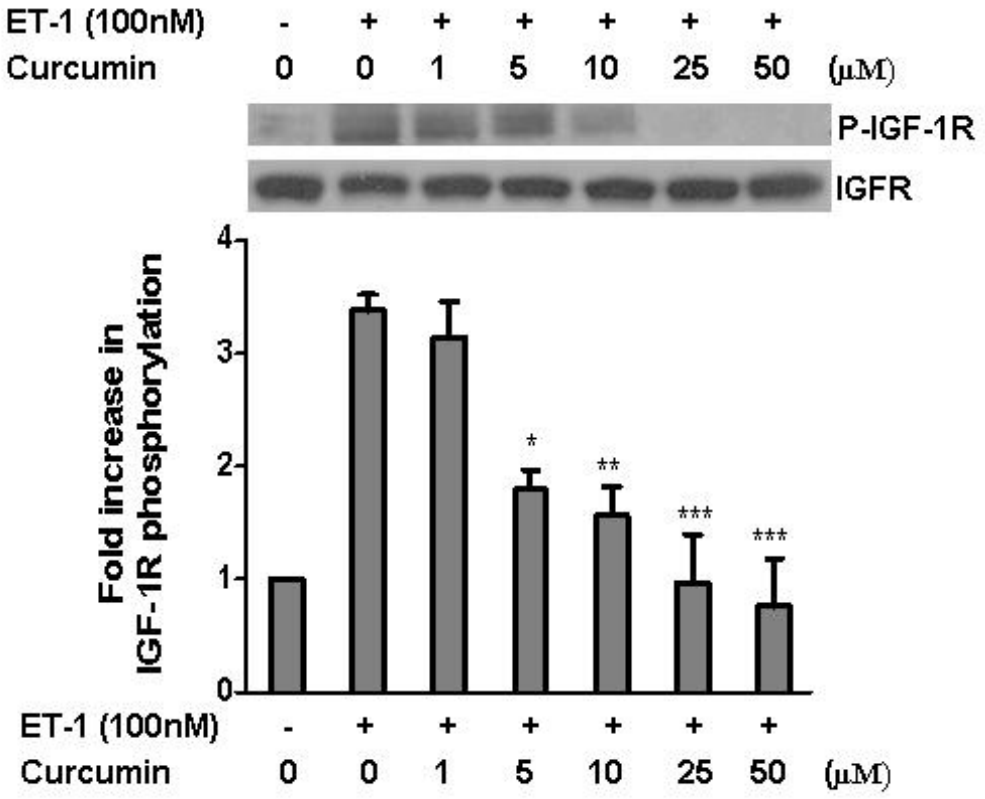

IGF-1 (6.5nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)
P-c-RAF
c-RAF
Fold increase in c-Raf phosphorylation
0
1
2
3
4
5
*
**
***
IGF-1 (6.5nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)

Figure 3

A.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Curcumin 0 0 1 5 10 25 50 (μM)
P-IGF-1R
IGFR
Fold increase in IGF-1R phosphorylation
0
1
2
3
4
*
**
***
***
ET-1 (100nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)

Figure 4

B.

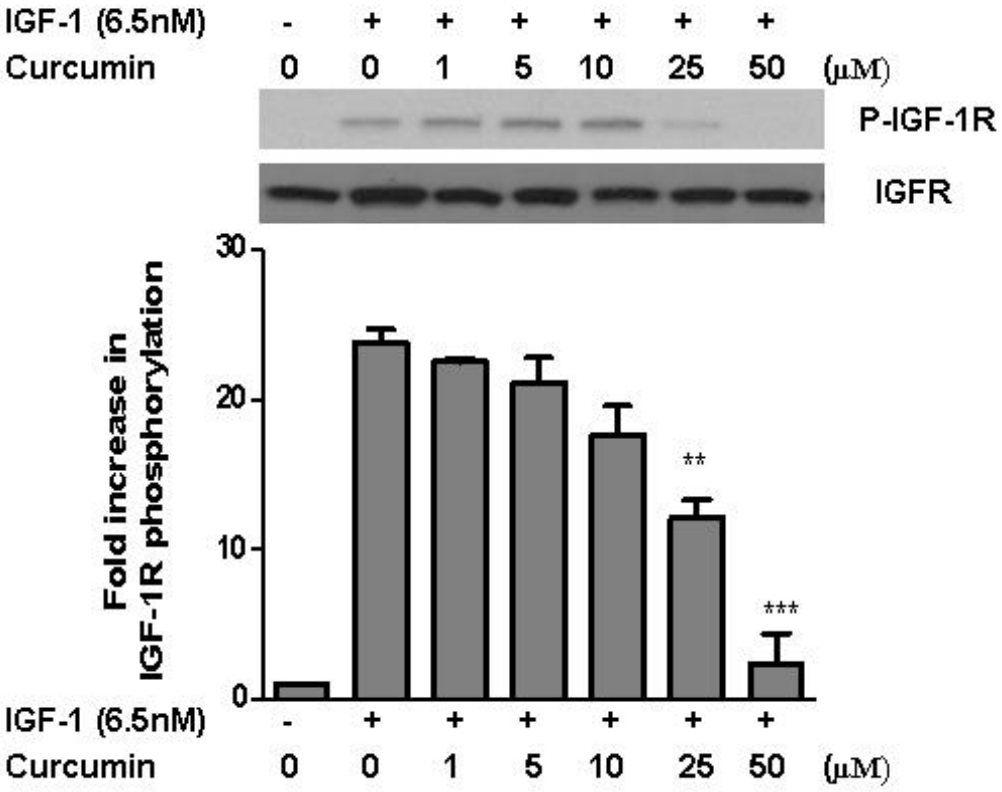
IGF-1 (6.5nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)
P-IGF-1R
IGFR
Fold increase in IGF-1R phosphorylation
30
20
10
0
**
***
IGF-1 (6.5nM) - + + + + + +
Curcumin 0 0 1 5 10 25 50 (μM)

Figure 4

A.

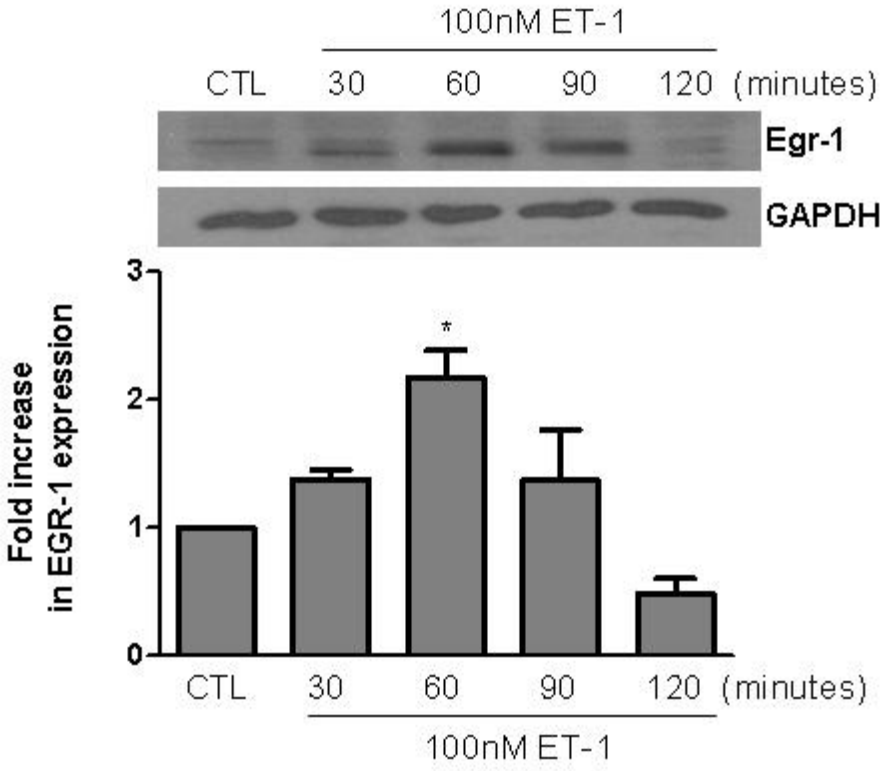
100nM ET-1
CTL
30
60
90
120
(minutes)
Egr-1
GAPDH
Fold increase in EGR-1 expression
3
2
1
0
*
CTL
30
60
90
120
(minutes)
100nM ET-1

Figure 5

B.

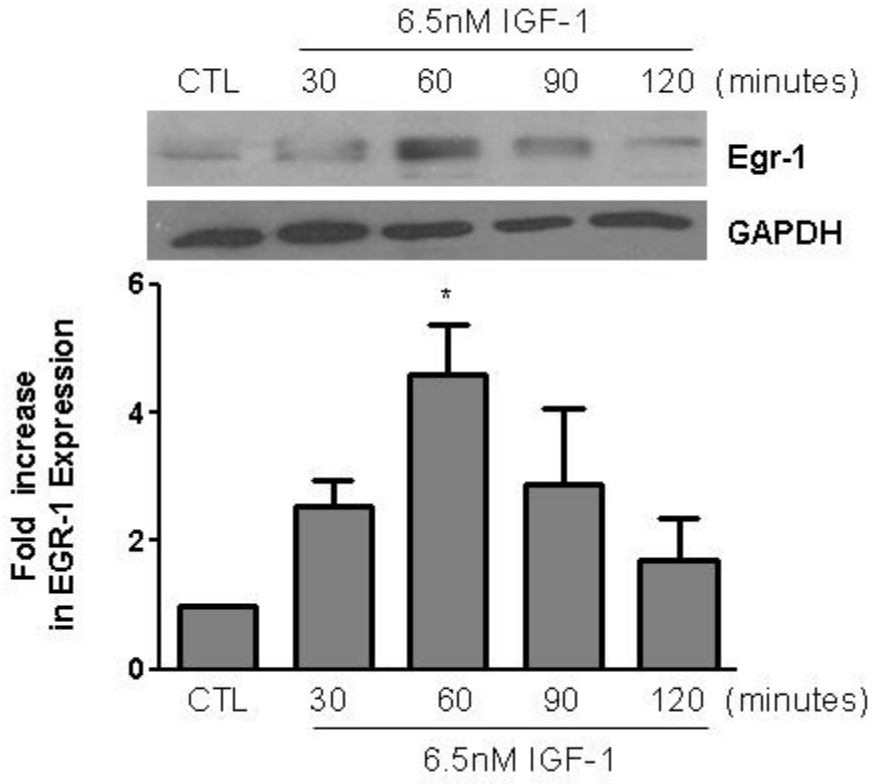
6.5nM IGF-1
CTL
30
60
90
120
(minutes)
Egr-1
GAPDH
Fold increase in EGR-1 Expression
6
4
2
0
*
CTL
30
60
90
120
(minutes)
6.5nM IGF-1

Figure 5

A.

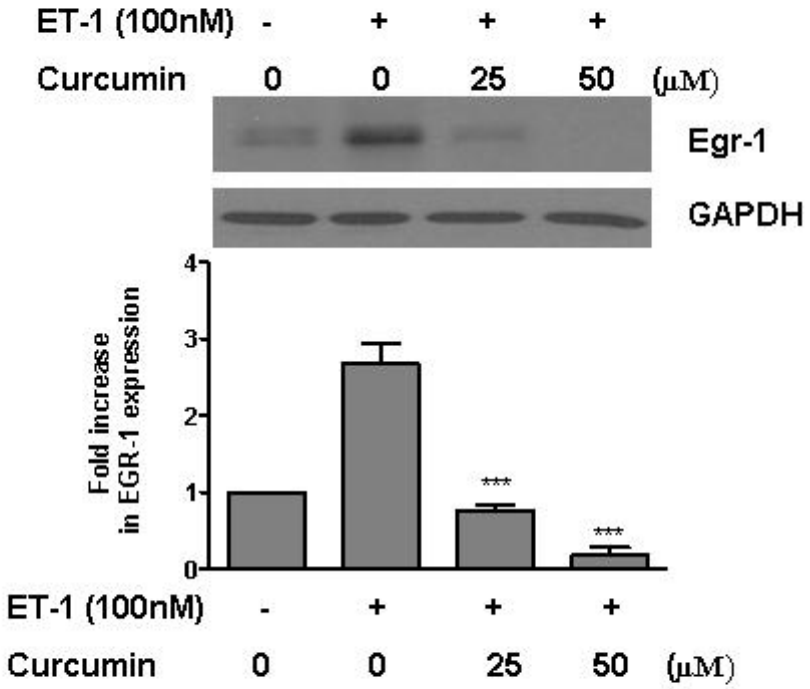
ET-1 (100nM) - + + +
Curcumin 0 0 25 50 (μM)
Egr-1
GAPDH
Fold increase in EGR-1 expression
0
1
2
3
4
***
***
ET-1 (100nM) - + + +
Curcumin 0 0 25 50 (μM)

Figure 6

B.

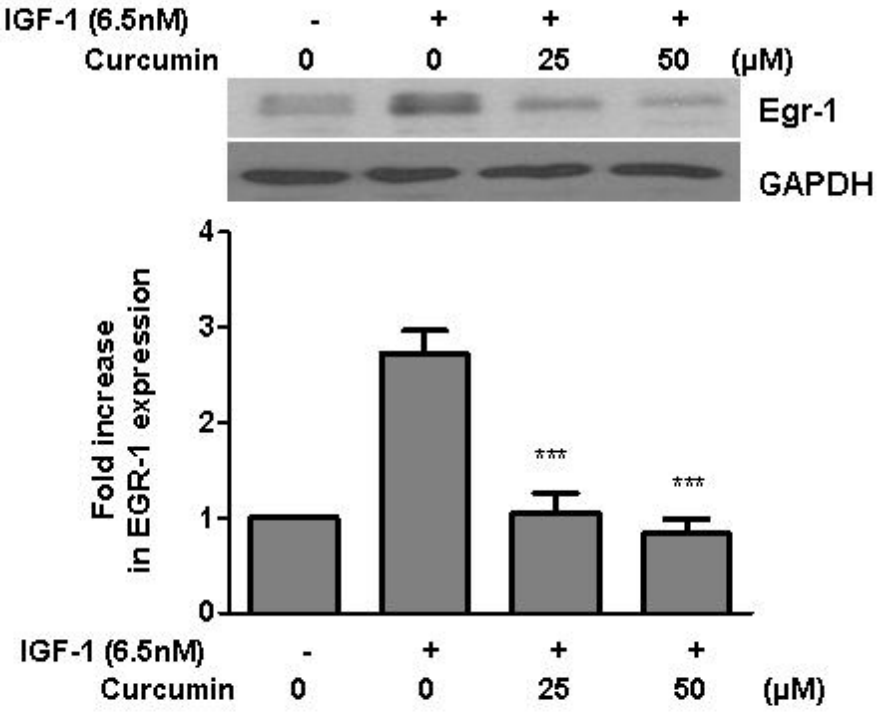
IGF-1 (6.5nM) - + + +
Curcumin 0 0 25 50 (μM)
Egr-1
GAPDH
Fold increase in EGR-1 expression
0
1
2
3
4
***
***
IGF-1 (6.5nM) - + + +
Curcumin 0 0 25 50 (μM)

Figure 6

Reference List

1. Bouallegue A, Daou GB, Srivastava AK. Endothelin-1-induced signaling pathways in vascular smooth muscle cells. Curr Vasc Pharmacol 2007;5:45-52.

2. Ivey ME, Osman N, Little PJ. Endothelin-1 signalling in vascular smooth muscle: pathways controlling cellular functions associated with atherosclerosis. Atherosclerosis 2008;199:237-47.

3. Reriani M, Raichlin E, Prasad A, Mathew V, Pumper GM, Nelson RE, et al. Long-term administration of endothelin receptor antagonist improves coronary endothelial function in patients with early atherosclerosis. Circulation 2010;122:958-66.

4. Touyz RM, Schiffrin EL. Role of endothelin in human hypertension. Can J Physiol Pharmacol 2003;81:533-41.

5. Motte S, McEntee K, Naeije R. Endothelin receptor antagonists. Pharmacol Ther 2006;110:386-414.

6. Jackson CL, Schwartz SM. Pharmacology of smooth muscle cell replication. Hypertension 1992;20:713-36.

7. Bouallegue A, Vardatsikos G, Srivastava AK. Role of insulin-like growth factor 1 receptor and c-Src in endothelin-1- and angiotensin II-induced PKB phosphorylation, and hypertrophic and proliferative responses in vascular smooth muscle cells. Can J Physiol Pharmacol 2009;87:1009-18.

8. Daou GB, Srivastava AK. Reactive oxygen species mediate Endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 signaling, as well as protein synthesis, in vascular smooth muscle cells. Free Radic Biol Med 2004;37:208-15.

9. Azar ZM, Mehdi MZ, Srivastava AK. Activation of insulin-like growth factor type-1 receptor is required for H2O2-induced PKB phosphorylation in vascular smooth muscle cells. Can J Physiol Pharmacol 2006;84:777-86.

10. Delafontaine P, Lou H, Alexander RW. Regulation of insulin-like growth factor I messenger RNA levels in vascular smooth muscle cells. Hypertension 1991;18:742-7.

11. King GL, Goodman AD, Buzney S, Moses A, Kahn CR. Receptors and growth-promoting effects of insulin and insulinlike growth factors on cells from bovine retinal capillaries and aorta. J Clin Invest 1985;75:1028-36.

12. Clemmons DR, Van Wyk JJ. Evidence for a functional role of endogenously produced somatomedinlike peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. J Clin Invest 1985;75:1914-8.

13. Duan C. The chemotactic and mitogenic responses of vascular smooth muscle cells to insulin-like growth factor-I require the activation of ERK1/2. Mol Cell Endocrinol 2003;206:75-83.

14. Duan C, Bauchat JR, Hsieh T. Phosphatidylinositol 3-kinase is required for insulin-like growth factor-I-induced vascular smooth muscle cell proliferation and migration. Circ Res 2000;86:15-23.

15. Epstein J, Sanderson IR, Macdonald TT. Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. Br J Nutr 2010;103:1545-57.

16. Soni KB, Kuttan R. Effect of oral curcumin administration on serum peroxides and cholesterol levels in human volunteers. Indian J Physiol Pharmacol 1992;36:273-5.

17. Ramirez BA, Soler A, Carrion-Gutierrez MA, Pamies MD, Pardo ZJ, Diaz-Alperi J, et al. An hydroalcoholic extract of Curcuma longa lowers the abnormally high values of human-plasma fibrinogen. Mech Ageing Dev 2000;114:207-10.

18. Ghosh SS, Salloum FN, Abbate A, Krieg R, Sica DA, Gehr TW, et al. Curcumin prevents cardiac remodeling secondary to chronic renal failure through deactivation of hypertrophic signaling in rats. Am J Physiol Heart Circ Physiol 2010;299:H975-H984.

19. Kang BY, Khan JA, Ryu S, Shekhar R, Seung KB, Mehta JL. Curcumin reduces angiotensin II-mediated cardiomyocyte growth via LOX-1 inhibition. J Cardiovasc Pharmacol 2010;55:176-83.

20. Morimoto T, Sunagawa Y, Kawamura T, Takaya T, Wada H, Nagasawa A, et al. The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats. J Clin Invest 2008;118:868-78.

21. Dikshit M, Rastogi L, Shukla R, Srimal RC. Prevention of ischaemia-induced biochemical changes by curcumin & quinidine in the cat heart. Indian J Med Res 1995;101:31-5.

22. Nirmala C, Puvanakrishnan R. Protective role of curcumin against isoproterenol induced myocardial infarction in rats. Mol Cell Biochem 1996;159:85-93.

23. Venkatesan N. Curcumin attenuation of acute adriamycin myocardial toxicity in rats. Br J Pharmacol 1998;124:425-7.

24. Zhou H, Beevers CS, Huang S. The Targets of Curcumin. Curr Drug Targets 2010.

25. Yang X, Thomas DP, Zhang X, Culver BW, Alexander BM, Murdoch WJ, et al. Curcumin inhibits platelet-derived growth factor-stimulated vascular smooth muscle cell function and injury-induced neointima formation. Arterioscler Thromb Vasc Biol 2006;26:85-90.

26. Huang HC, Jan TR, Yeh SF. Inhibitory effect of curcumin, an anti-inflammatory agent, on vascular smooth muscle cell proliferation. Eur J Pharmacol 1992;221:381-4.

27. Pae HO, Jeong GS, Jeong SO, Kim HS, Kim SA, Kim YC, et al. Roles of heme oxygenase-1 in curcumin-induced growth inhibition in rat smooth muscle cells. Exp Mol Med 2007;39:267-77.

28. Qin L, Yang YB, Tuo QH, Zhu BY, Chen LX, Zhang L, et al. Effects and underlying mechanisms of curcumin on the proliferation of vascular smooth muscle cells induced by Chol:MbetaCD. Biochem Biophys Res Commun 2009;379:277-82.

29. Chen HW, Huang HC. Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. Br J Pharmacol 1998;124:1029-40.

30. Ak T, Gulcin I. Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact 2008;174:27-37.

31. Reddy AC, Lokesh BR. Studies on the inhibitory effects of curcumin and eugenol on the formation of reactive oxygen species and the oxidation of ferrous iron. Mol Cell Biochem 1994;137:1-8.

32. Sreejayan, Rao MN. Curcuminoids as potent inhibitors of lipid peroxidation. J Pharm Pharmacol 1994;46:1013-6.

33. Khachigian LM. Early growth response-1 in cardiovascular pathobiology. Circ Res 2006;98:186-91.

34. Hashim S, Li Y, Anand-Srivastava MB. Small cytoplasmic domain peptides of natriuretic peptide receptor-C attenuate cell proliferation through Gialpha protein/MAP kinase/PI3-kinase/AKT pathways. Am J Physiol Heart Circ Physiol 2006;291:H3144-H3153.

35. Li Y, Hashim S, Anand-Srivastava MB. Intracellular peptides of natriuretic peptide receptor-C inhibit vascular hypertrophy via Gqalpha/MAP kinase signaling pathways. Cardiovasc Res 2006;72:464-72.

36. Khachigian LM, Lindner V, Williams AJ, Collins T. Egr-1-induced endothelial gene expression: a common theme in vascular injury. Science 1996;271:1427-31.

37. Nakmareong S, Kukongviriyapan U, Pakdeechote P, Donpunha W, Kukongviriyapan V, Kongyingyoes B, et al. Antioxidant and vascular protective effects of curcumin and tetrahydrocurcumin in rats with L-NAME-induced hypertension. Naunyn Schmiedebergs Arch Pharmacol 2011;383:519-29.

38. Liu Y, Dolence J, Ren J, Rao M, Sreejayan N. Inhibitory effect of dehydrozingerone on vascular smooth muscle cell function. J Cardiovasc Pharmacol 2008;52:422-9.

39. Li Y, Levesque LO, Anand-Srivastava MB. Epidermal growth factor receptor transactivation by endogenous vasoactive peptides contributes to hyperproliferation of vascular smooth muscle cells of SHR. Am J Physiol Heart Circ Physiol 2010;299:H1959-H1967.

40. Meng D, Lv DD, Fang J. Insulin-like growth factor-I induces reactive oxygen species production and cell migration through Nox4 and Rac1 in vascular smooth muscle cells. Cardiovasc Res 2008;80:299-308.

41. Blaschke F, Bruemmer D, Law RE. Egr-1 is a major vascular pathogenic transcription factor in atherosclerosis and restenosis. Rev Endocr Metab Disord 2004;5:249-54.

42. Houston P, Dickson MC, Ludbrook V, White B, Schwachtgen JL, McVey JH, et al. Fluid shear stress induction of the tissue factor promoter in vitro and in vivo is mediated by Egr-1. Arterioscler Thromb Vasc Biol 1999;19:281-9.

43. Day FL, Rafty LA, Chesterman CN, Khachigian LM. Angiotensin II (ATII)-inducible platelet-derived growth factor A-chain gene expression is p42/44 extracellular signal-regulated kinase-1/2 and Egr-1-dependent and mediated via the ATII type 1 but not type 2 receptor. Induction by ATII antagonized by nitric oxide. J Biol Chem 1999;274:23726-33.

44. McCaffrey TA, Fu C, Du B, Eksinar S, Kent KC, Bush H, Jr., et al. High-level expression of Egr-1 and Egr-1-inducible genes in mouse and human atherosclerosis. J Clin Invest 2000;105:653-62.

45. Harja E, Bucciarelli LG, Lu Y, Stern DM, Zou YS, Schmidt AM, et al. Early growth response-1 promotes atherogenesis: mice deficient in early growth response-1 and apolipoprotein E display decreased atherosclerosis and vascular inflammation. Circ Res 2004;94:333-9.

46. Morimoto M, Kume N, Miyamoto S, Ueno Y, Kataoka H, Minami M, et al. Lysophosphatidylcholine induces early growth response factor-1 expression and activates the core promoter of PDGF-A chain in vascular endothelial cells. Arterioscler Thromb Vasc Biol 2001;21:771-6.

47. Liu QF, Yu HW, Liu GN. Egr-1 upregulates OPN through direct binding to its promoter and OPN upregulates Egr-1 via the ERK pathway. Mol Cell Biochem 2009;332:77-84.

48. Fahmy RG, Khachigian LM. Suppression of growth factor expression and human vascular smooth muscle cell growth by small interfering RNA targeting EGR-1. J Cell Biochem 2007;100:1526-35.

49. Zhang YM, Shi GG, Tang Z, Zheng JH, Li WQ, Guo FX, et al. Effects of N-n-butyl haloperidol iodide on myocardial ischemia/reperfusion injury and Egr-1 expression in rat. Acta Biochim Biophys Sin (Shanghai) 2006;38:435-41.

50. Liu GN, Teng YX, Yan W. Transfected synthetic DNA enzyme gene specifically inhibits Egr-1 gene expression and reduces neointimal hyperplasia following balloon injury in rats. Int J Cardiol 2008;129:118-24.

51. Chen Y, Zheng J, Zhang Y, Wang J, Liu Q, Huang Z, et al. N-4-tert-butyl benzyl haloperidol chloride suppresses Ca2+-dependent Egr-1 expression and subsequently inhibits vascular smooth muscle cell proliferation induced by angiotensin II. Cell Physiol Biochem 2009;23:295-304.

52. Neyses L, Nouskas J, Vetter H. Inhibition of endothelin-1 induced myocardial protein synthesis by an antisense oligonucleotide against the early growth response gene-1. Biochem Biophys Res Commun 1991;181:22-7.

53. Sharma OP. Antioxidant activity of curcumin and related compounds. Biochem Pharmacol 1976;25:1811-2.

54. Levonen AL, Vahakangas E, Koponen JK, Yla-Herttuala S. Antioxidant gene therapy for cardiovascular disease: current status and future perspectives. Circulation 2008;117:2142-50.

55. Araujo CC, Leon LL. Biological activities of Curcuma longa L. Mem Inst Oswaldo Cruz 2001;96:723-8.

56. Shen L, Ji HF. Insights into the inhibition of xanthine oxidase by curcumin. Bioorg Med Chem Lett 2009;19:5990-3.

57. Pirvulescu MM, Gan AM, Stan D, Simion V, Calin M, Butoi E, et al. Curcumin and a Morus alba Extract Reduce Pro-Inflammatory Effects of Resistin in Human Endothelial Cells. Phytother Res 2011.

58. Yu YM, Lin HC. Curcumin prevents human aortic smooth muscle cells migration by inhibiting of MMP-9 expression. Nutr Metab Cardiovasc Dis 2010;20:125-32.

59. Azar ZM, Mehdi MZ, Srivastava AK. Insulin-like growth factor type-1 receptor transactivation in vasoactive peptide and oxidant-induced signaling pathways in vascular smooth muscle cells. Can J Physiol Pharmacol 2007;85:105-11.

CHAPITRE 3

DISCUSSION GÉNÉRALE

The discovery of endothelin (ET), the potent vasoconstrictor, surfaced only two decades ago and the endothelin peptide family has now evolved into an intricate system. Endothelin-1 (ET-1) is the most predominant isoform synthesized by the vasculature, mainly by the vascular endothelium and smooth muscle cells, and is accountable for the majority of the pathobiological effects exerted by the endothelin peptide family. In the vascular system, ET-1 mainly exerts a basal vascular tone regulation. A long term upregulated ET-1 system has been suggested to contribute to the deterioration of vascular function that leads to the development of vascular diseases. ET-1 exerts its action through the activation of its endothelin receptors, ET_A and ET_B, but ET_A is mainly responsible in the contribution of ET-1 to the pathogenesis of vascular abnormalities, such as hypertension, atherosclerosis, restenosis, and other cardiovascular diseases. Based on recent research, ET-1 receptor antagonists exhibit inhibition of ET-1-induced functional and structural alterations in the vasculature. Nevertheless, ET-1 mediates its pleiotropic actions through the activation of several signaling pathways and further investigation is required to provide a better knowledge of these ET-1 signal transducing pathways for designing specific therapeutic agents directed against critical components of the signaling systems implicated in pathological contribution of ET-1-induced vascular remodeling.

ET-1 elicits its biological effects in a paracrine-autocrine manner through the activation of its G-protein-coupled receptors (GPCR). This leads to the activation of multiple signaling pathways including mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3-K/PKB) pathways that promote cellular growth, proliferation, hypertrophy and survival, and these are all key processes involved in vascular cell remodeling often encountered in ET-1 triggered pathophysiological states. Once ET-1 activates its receptor, the latter recruits Ras, a small G-protein that leads to the activation of MAPK, including

extracellular signal-regulated kinase 1/2 (ERK1/2), c-jun-NH_2-terminal kinase (JNK), and p38mapk (276). In parallel, ET-1 also activates PI3-K (131) and several downstream targets of PI3-K have also been documented, the most studied being protein kinase B (PKB), also know as Akt (a product of akt proto-oncogene) (5). Activation of receptor and non receptor protein tyrosine kinases (PTK) has been proposed to be implicated in transducing ET-1-induced signaling events. Epidermal growth factor receptor (EGFR) (277, 278), c-Src (278, 279), and Ca^{2+} dependent PYK2 (278-280) are amongst the PTK that are activated by ET-1. We have recently shown a requirement of insulin-like growth factor type 1 receptor (IGF-1R) in ET-1-induced PKB activation in VSMC (151). However, the precise molecular mechanism responsible for this transactivation still remains unresolved. A possible contribution to this mechanism might be mediated through ROS generation. Several studies have demonstrated that ROS play a key role in propagating growth factor and vasoactive peptide signaling. The high levels of ET-1 observed in hypertension and atherosclerosis lead to excessive production generation of ROS and in a decrease in the antioxidant status (159). At physiological concentrations, ROS generated within the vasculature are important signaling molecules. However, an excessive synthesis of ROS can overcome antioxidant mechanisms and generate deleterious effects often seen in cardiovascular disorders (11). The involvement of ROS in ET-1-induced activation of MAPKs including JNK, p38mapk and ERK1/2 has been demonstrated in cardiac fibroblasts (125). Moreover, a role of ROS in ET-1-induced activation of the redox-sensitive ERK1/2 and PKB signaling pathways has been demonstrated in VSMC (5). In this context, several studies have investigated the possible vascular protective effect of antioxidants. A surge of interest has recently erupted around the use of naturally-occurring compounds with antioxidant capacity. Medicinal plants are rich in phytochemicals that possess several therapeutic effects, including antioxidative properties. Curcumin, the main constituent of the spice turmeric that is extracted

from the rhizomes of the *Curcuma Longa* L. herb, is an ancient known phytochemical that exhibits a vast spectrum of biological properties, amongst them, antioxidant and anti-inflammatory properties. It has mainly been investigated for its effects against cancer. Recent studies though have demonstrated that curcumin also exhibits cardiovascular protective effects and its actions are pleiotropic and appear to involve the regulation of transcription factors, growth factors, cytokines, protein kinases and others (185). Curcumin has been shown to suppress ROS generation (174, 202, 203) and to inhibit several of the mediators involved in the ET-1 signaling pathway in several cellular lines. However, the therapeutic efficacy of curcumin in ET-1-induced signaling has yet to be reported. Therefore, these studies were undertaken to investigate the effect of curcumin on ET-1-induced signaling of the redox-sensitive ERK1/2, PKB pathways which are believed to be key players in ET-1-induced proliferative and hypertrophic responses. In an attempt to understand the mechanism, we studied the effect of curcumin on ET-1-stimulated IGF-1R, PKB, c-Raf and ERK1/2 signaling in A-10 VSMC.

Our results are the first to report an inhibitory effect of curcumin on ET-1-stimulated hypertrophic and proliferative signaling in VSMC. In the first part of this study, we observed a dose-dependent inhibitory effect of curcumin on ET-1 stimulation of IGF-1R, PKB, c-Raf and ERK1/2 phosphorylation in A-10 VSMC. Given that ET-1 requires IGF-1R transactivation, we further investigated the effect of curcumin on IGF-1-induced signaling in VSMC. A similar dose-dependent inhibitory effect was observed on the IGF-1-induced IGF-1R, PKB, c-Raf and ERK1/2 phosphorylation in A-10 VSMC. Moreover, we tested if transcription factor early growth response-1 (Egr-1), a downstream component of ERK1/2 that is a key player in multiple cardiovascular pathological processes (281), is a target of curcumin in response to ET-1 as well as IGF-1. Curcumin attenuated ET-1 and IGF-1-induced expression of Egr-1 in VSMC.

Taken together, we demonstrate that curcumin inhibits ET-1 and IGF-1-stimulated increase in the phosphorylation state of IGF-1R, PKB, c-Raf and ERK1/2, as well as Egr-1 expression in A-10 VSMC. Since ERK1/2, PKB and Egr-1 play a crucial role in mediating VSMC growth and hypertrophy, it may be suggested that the ability of curcumin to attenuate these pathways may serve as a potential mechanism by which curcumin counteracts the biological responses of ET-1 and thus exerts a cardiovascular protective effect.

CHAPITRE 4

CONCLUSION

The results presented here demonstrate for the first time a negative modulatory effect of curcumin on ET-1-stimulated signaling components: PKB, c-Raf, ERK1/2 and IGF-1R, in A-10 VSMC. Given the fact the curcumin exhibits a significant inhibitory effect on ET-1-induced IGF-1R phosphorylation, we expanded our studies to investigate its effect on IGF-1-induced signaling and highlighted an inhibitory effect of curcumin on IGF-1-induced phosphorylation of PKB, c-Raf, ERK1/2 and IGF-1R, in A-10 VSMC. Our work also demonstrates that curcumin inhibits the expression of ET-1 and IGF-1-induced transcription factor Egr-1 in VSMC (Figure 4.5).

Thus, the findings in the present study have uncovered curcumin as a potent antagonist of ET-1 action in VSMC. Provided that curcumin exhibits such profound inhibitory effects on ET-1-induced signaling events that have been shown to promote mitogenic and hypertrophic actions in the vasculature, it may be suggested that curcumin may be beneficial in attenuating vascular remodeling associated with several cardiovascular diseases. However, due to its vast array of biological properties, curcumin is able to interfere with numerous signaling pathways and it is suggested that a single site of action is possibly not enough to represent all the effects of this substance.

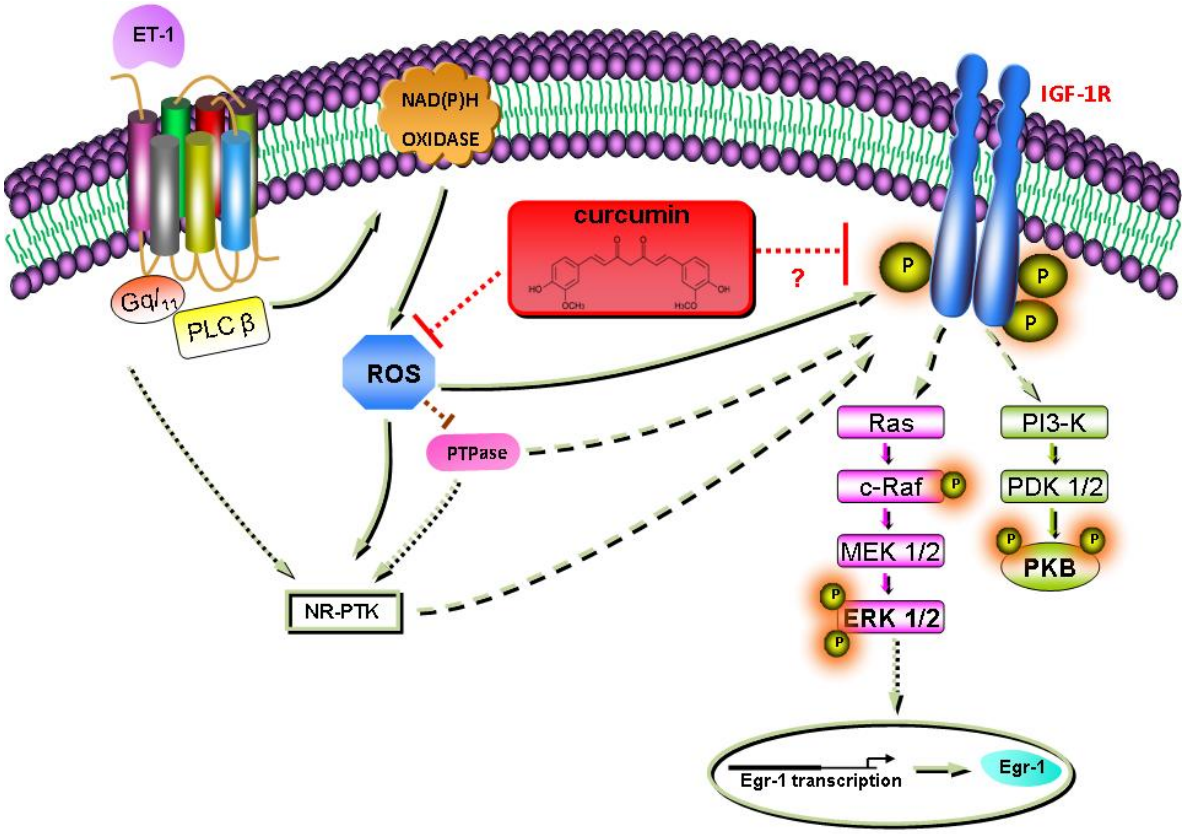

Figure 4.5 Schematic hypothetical model summarizing the potential interaction of curcumin with ET-1 signaling pathways in VSMC.

The ability of curcumin to inhibit ET-1-induced IGF-1Rβ phosphorylation and downstream phosphorylation of PKB, c-Raf and ERK1/2, along with the expression of Egr-1 suggests that curcumin might act upstream of IGF-1R in mediating this inhibitory effect. Curcumin may also inhibit oxidative stress generation thereby turn off ET-1-induced signaling in VSMC.

RÉFÉRENCES

Reference List

1. Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988;332:411-5.

2. Agapitov AV, Haynes WG. Role of endothelin in cardiovascular disease. J Renin Angiotensin Aldosterone Syst 2002;3:1-15.

3. Taddei S, Virdis A, Ghiadoni L, Salvetti A. Vascular effects of endothelin-1 in essential hypertension: relationship with cyclooxygenase-derived endothelium-dependent contracting factors and nitric oxide. J Cardiovasc Pharmacol 2000;35:S37-S40.

4. El-Shafey EM, El-Nagar GF, Selim MF, El-Sorogy HA, Sabry AA. Is there a role for endothelin-1 in the hemodynamic changes during hemodialysis? Clin Exp Nephrol 2008;12:370-5.

5. Daou GB, Srivastava AK. Reactive oxygen species mediate Endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 signaling, as well as protein synthesis, in vascular smooth muscle cells. Free Radic Biol Med 2004;37:208-15.

6. Inoue A, Yanagisawa M, Kimura S, Kasuya Y, Miyauchi T, Goto K, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A 1989;86:2863-7.

7. Bloch KD, Hong CC, Eddy RL, Shows TB, Quertermous T. cDNA cloning and chromosomal assignment of the endothelin 2 gene: vasoactive intestinal contractor peptide is rat endothelin 2. Genomics 1991;10:236-42.

8. Kloog Y, Ambar I, Sokolovsky M, Kochva E, Wollberg Z, Bdolah A. Sarafotoxin, a novel vasoconstrictor peptide: phosphoinositide hydrolysis in rat heart and brain. Science 1988;242:268-70.

9. Sawamura T, Kimura S, Shinmi O, Sugita Y, Yanagisawa M, Masaki T. Analysis of endothelin related peptides in culture supernatant of porcine aortic endothelial cells: evidence for biosynthetic pathway of endothelin-1. Biochem Biophys Res Commun 1989;162:1287-94.

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

11. Piechota A, Polanczyk A, Goraca A. Role of endothelin-1 receptor blockers on hemodynamic parameters and oxidative stress. Pharmacol Rep 2010;62:28-34.

12. Inoue A, Yanagisawa M, Takuwa Y, Mitsui Y, Kobayashi M, Masaki T. The human preproendothelin-1 gene. Complete nucleotide sequence and regulation of expression. J Biol Chem 1989;264:14954-9.

13. Lee ME, Bloch KD, Clifford JA, Quertermous T. Functional analysis of the endothelin-1 gene promoter. Evidence for an endothelial cell-specific cis-acting sequence. J Biol Chem 1990;265:10446-50.

14. Emori T, Hirata Y, Ohta K, Kanno K, Eguchi S, Imai T, et al. Cellular mechanism of endothelin-1 release by angiotensin and vasopressin. Hypertension 1991;18:165-70.

15. Haynes WG, Webb DJ. Endothelin as a regulator of cardiovascular function in health and disease. J Hypertens 1998;16:1081-98.

16. Gray GA. Generation of endothelin. In: Gray G, Webb D, eds. Molecular biology and pharmacology of the endothelins: Austin: RG Landes, 1995:13-32.

17. Goraca A. New views on the role of endothelin (minireview). Endocr Regul 2002;36:161-7.

18. Alonso D, Radomski MW. The nitric oxide-endothelin-1 connection. Heart Fail Rev 2003;8:107-15.

19. Rubanyi GM, Polokoff MA. Endothelins: molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. Pharmacol Rev 1994;46:325-415.

20. Turner AJ, Murphy LJ. Molecular pharmacology of endothelin converting enzymes. Biochem Pharmacol 1996;51:91-102.

21. Wagner OF, Christ G, Wojta J, Vierhapper H, Parzer S, Nowotny PJ, et al. Polar secretion of endothelin-1 by cultured endothelial cells. J Biol Chem 1992;267:16066-8.

22. Plumpton C, Haynes WG, Webb DJ, Davenport AP. Phosphoramidon inhibition of the in vivo conversion of big endothelin-1 to endothelin-1 in the human forearm. Br J Pharmacol 1995;116:1821-8.

23. Sirvio ML, Metsarinne K, Saijonmaa O, Fyhrquist F. Tissue distribution and half-life of 125I-endothelin in the rat: importance of pulmonary clearance. Biochem Biophys Res Commun 1990;167:1191-5.

24. Attina T, Camidge R, Newby DE, Webb DJ. Endothelin antagonism in pulmonary hypertension, heart failure, and beyond. Heart 2005;91:825-31.

25. Johnstrom P, Fryer TD, Richards HK, Harris NG, Barret O, Clark JC, et al. Positron emission tomography using 18F-labelled endothelin-1 reveals prevention of binding to cardiac receptors owing to tissue-specific clearance by ET B receptors in vivo. Br J Pharmacol 2005;144:115-22.

26. Waggoner WG, Genova SL, Rash VA. Kinetic analyses demonstrate that the equilibrium assumption does not apply to [125I]endothelin-1 binding data. Life Sci 1992;51:1869-76.

27. Vierhapper H, Wagner O, Nowotny P, Waldhausl W. Effect of endothelin-1 in man. Circulation 1990;81:1415-8.

28. Chun M, Lin HY, Henis YI, Lodish HF. Endothelin-induced endocytosis of cell surface ETA receptors. Endothelin remains intact and bound to the ETA receptor. J Biol Chem 1995;270:10855-60.

29. Arai H, Hori S, Aramori I, Ohkubo H, Nakanishi S. Cloning and expression of a cDNA encoding an endothelin receptor. Nature 1990;348:730-2.

30. Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, et al. Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 1990;348:732-5.

31. Elshourbagy NA, Korman DR, Wu HL, Sylvester DR, Lee JA, Nuthalaganti P, et al. Molecular characterization and regulation of the human endothelin receptors. J Biol Chem 1993;268:3873-9.

32. Arai H, Nakao K, Takaya K, Hosoda K, Ogawa Y, Nakanishi S, et al. The human endothelin-B receptor gene. Structural organization and chromosomal assignment. J Biol Chem 1993;268:3463-70.

33. Endoh M, Fujita S, Yang HT, Talukder MA, Maruya J, Norota I. Endothelin: receptor subtypes, signal transduction, regulation of Ca2+ transients and contractility in rabbit ventricular myocardium. Life Sci 1998;62:1485-9.

34. Perez d, V, Garcia Alonso CJ, Feldstein CA, Juncos LA, Romero JC. Role of endothelin in the pathogenesis of hypertension. Mayo Clin Proc 2005;80:84-96.

35. Clozel M, Clozel JP. Effects of endothelin on regional blood flows in squirrel monkeys. J Pharmacol Exp Ther 1989;250:1125-31.

36. Pernow J, Ahlborg G, Lundberg JM, Kaijser L. Long-lasting coronary vasoconstrictor effects and myocardial uptake of endothelin-1 in humans. Acta Physiol Scand 1997;159:147-53.

37. Wagner OF, Vierhapper H, Gasic S, Nowotny P, Waldhausl W. Regional effects and clearance of endothelin-1 across pulmonary and splanchnic circulation. Eur J Clin Invest 1992;22:277-82.

38. Rabelink TJ, Kaasjager KA, Boer P, Stroes EG, Braam B, Koomans HA. Effects of endothelin-1 on renal function in humans: implications for physiology and pathophysiology. Kidney Int 1994;46:376-81.

39. Mortensen LH, Fink GD. Hemodynamic effect of human and rat endothelin administration into conscious rats. Am J Physiol 1990;258:H362-H368.

40. Weitzberg E, Ahlborg G, Lundberg JM. Differences in vascular effects and removal of endothelin-1 in human lung, brain, and skeletal muscle. Clin Physiol 1993;13:653-62.

41. Huang H, Yanagisawa M, Kisanuki YY, Jelicks LA, Chandra M, Factor SM, et al. Role of cardiac myocyte-derived endothelin-1 in chagasic cardiomyopathy: molecular genetic evidence. Clin Sci (Lond) 2002;103 Suppl 48:263S-6S.

42. Sugden PH. Signalling pathways in cardiac myocyte hypertrophy. Ann Med 2001;33:611-22.

43. Masaki T. Historical review: Endothelin. Trends Pharmacol Sci 2004;25:219-24.

44. Ezra D, Goldstein RE, Czaja JF, Feuerstein GZ. Lethal ischemia due to intracoronary endothelin in pigs. Am J Physiol 1989;257:H339-H343.

45. Pieske B, Beyermann B, Breu V, Loffler BM, Schlotthauer K, Maier LS, et al. Functional effects of endothelin and regulation of endothelin receptors in isolated human nonfailing and failing myocardium. Circulation 1999;99:1802-9.

46. Yorikane R, Sakai S, Miyauchi T, Sakurai T, Sugishita Y, Goto K. Increased production of endothelin-1 in the hypertrophied rat heart due to pressure overload. FEBS Lett 1993;332:31-4.

47. Brunner F, Bras-Silva C, Cerdeira AS, Leite-Moreira AF. Cardiovascular endothelins: essential regulators of cardiovascular homeostasis. Pharmacol Ther 2006;111:508-31.

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

49. Davie N, Haleen SJ, Upton PD, Polak JM, Yacoub MH, Morrell NW, et al. ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. Am J Respir Crit Care Med 2002;165:398-405.

50. Chua BH, Krebs CJ, Chua CC, Diglio CA. Endothelin stimulates protein synthesis in smooth muscle cells. Am J Physiol 1992;262:E412-E416.

51. Hofman FM, Chen P, Jeyaseelan R, Incardona F, Fisher M, Zidovetzki R. Endothelin-1 induces production of the neutrophil chemotactic factor interleukin-8 by human brain-derived endothelial cells. Blood 1998;92:3064-72.

52. Pedram A, Razandi M, Hu RM, Levin ER. Vasoactive peptides modulate vascular endothelial cell growth factor production and endothelial cell proliferation and invasion. J Biol Chem 1997;272:17097-103.

53. Hafizi S, Allen SP, Goodwin AT, Chester AH, Yacoub MH. Endothelin-1 stimulates proliferation of human coronary smooth muscle cells via the ET(A) receptor and is co-mitogenic with growth factors. Atherosclerosis 1999;146:351-9.

54. Schiffrin EL. Endothelin: potential role in hypertension and vascular hypertrophy. Hypertension 1995;25:1135-43.

55. Schiffrin EL. State-of-the-Art lecture. Role of endothelin-1 in hypertension. Hypertension 1999;34:876-81.

56. Callera GE, Touyz RM, Teixeira SA, Muscara MN, Carvalho MH, Fortes ZB, et al. ETA receptor blockade decreases vascular superoxide generation in DOCA-salt hypertension. Hypertension 2003;42:811-7.

57. Li L, Watts SW, Banes AK, Galligan JJ, Fink GD, Chen AF. NADPH oxidase-derived superoxide augments endothelin-1-induced venoconstriction in mineralocorticoid hypertension. Hypertension 2003;42:316-21.

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

59. Pu Q, Neves MF, Virdis A, Touyz RM, Schiffrin EL. Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. Hypertension 2003;42:49-55.

60. Tostes RC, Touyz RM, He G, Ammarguellat F, Schiffrin EL. Endothelin A receptor blockade decreases expression of growth factors and collagen and improves matrix metalloproteinase-2 activity in kidneys from stroke-prone spontaneously hypertensive rats. J Cardiovasc Pharmacol 2002;39:892-900.

61. Sedeek MH, Llinas MT, Drummond H, Fortepiani L, Abram SR, Alexander BT, et al. Role of reactive oxygen species in endothelin-induced hypertension. Hypertension 2003;42:806-10.

62. Ammarguellat F, Larouche I, Schiffrin EL. Myocardial fibrosis in DOCA-salt hypertensive rats: effect of endothelin ET(A) receptor antagonism. Circulation 2001;103:319-24.

63. Muller DN, Mervaala EM, Schmidt F, Park JK, Dechend R, Genersch E, et al. Effect of bosentan on NF-kappaB, inflammation, and tissue factor in angiotensin II-induced end-organ damage. Hypertension 2000;36:282-90.

64. Muller DN, Mullally A, Dechend R, Park JK, Fiebeler A, Pilz B, et al. Endothelin-converting enzyme inhibition ameliorates angiotensin II-induced cardiac damage. Hypertension 2002;40:840-6.

65. Vaneckova I, Kramer HJ, Backer A, Schejbalova S, Vernerova Z, Eis V, et al. Early-onset endothelin receptor blockade in hypertensive heterozygous Ren-2 rats. Vascul Pharmacol 2006;45:163-70.

66. Schiffrin EL. Reactivity of small blood vessels in hypertension: relation with structural changes. State of the art lecture. Hypertension 1992;19:II1-II9.

67. Mulvany MJ. Small Artery Remodelling in Hypertension. Basic Clin Pharmacol Toxicol 2011.

68. Hirai Y, Adachi H, Fujiura Y, Hiratsuka A, Enomoto M, Imaizumi T. Plasma endothelin-1 level is related to renal function and smoking status but not to blood pressure: an epidemiological study. J Hypertens 2004;22:713-8.

69. Ergul A, Tackett RL, Puett D. Distribution of endothelin receptors in saphenous veins of African Americans: implications of racial differences. J Cardiovasc Pharmacol 1999;34:327-32.

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

71. Rossi GP, Colonna S, Pavan E, Albertin G, Della RF, Gerosa G, et al. Endothelin-1 and its mRNA in the wall layers of human arteries ex vivo. Circulation 1999;99:1147-55.

72. Cardillo C, Kilcoyne CM, Waclawiw M, Cannon RO, III, Panza JA. Role of endothelin in the increased vascular tone of patients with essential hypertension. Hypertension 1999;33:753-8.

73. Cardillo C, Campia U, Kilcoyne CM, Bryant MB, Panza JA. Improved endothelium-dependent vasodilation after blockade of endothelin receptors in patients with essential hypertension. Circulation 2002;105:452-6.

74. Krum H, Viskoper RJ, Lacourciere Y, Budde M, Charlon V. The effect of an endothelin-receptor antagonist, bosentan, on blood pressure in patients with essential hypertension. Bosentan Hypertension Investigators. N Engl J Med 1998;338:784-90.

75. Nakov R, Pfarr E, Eberle S. Darusentan: an effective endothelinA receptor antagonist for treatment of hypertension. Am J Hypertens 2002;15:583-9.

76. Nguyen A, Thorin-Trescases N, Thorin E. Working under pressure: Coronary arteries and the endothelin system. Am J Physiol Regul Integr Comp Physiol 2010.

77. Lerman A, Webster MW, Chesebro JH, Edwards WD, Wei CM, Fuster V, et al. Circulating and tissue endothelin immunoreactivity in hypercholesterolemic pigs. Circulation 1993;88:2923-8.

78. Kobayashi T, Miyauchi T, Iwasa S, Sakai S, Fan J, Nagata M, et al. Corresponding distributions of increased endothelin-B receptor expression and increased endothelin-1 expression in the aorta of apolipoprotein E-deficient mice with advanced atherosclerosis. Pathol Int 2000;50:929-36.

79. Zeiher AM, Ihling C, Pistorius K, Schachinger V, Schaefer HE. Increased tissue endothelin immunoreactivity in atherosclerotic lesions associated with acute coronary syndromes. Lancet 1994;344:1405-6.

80. Minamino T, Kurihara H, Takahashi M, Shimada K, Maemura K, Oda H, et al. Endothelin-converting enzyme expression in the rat vascular injury model and human coronary atherosclerosis. Circulation 1997;95:221-30.

81. Kowala MC, Rose PM, Stein PD, Goller N, Recce R, Beyer S, et al. Selective blockade of the endothelin subtype A receptor decreases early atherosclerosis in hamsters fed cholesterol. Am J Pathol 1995;146:819-26.

82. Barton M, Haudenschild CC, d'Uscio LV, Shaw S, Munter K, Luscher TF. Endothelin ETA receptor blockade restores NO-mediated endothelial function and inhibits atherosclerosis in apolipoprotein E-deficient mice. Proc Natl Acad Sci U S A 1998;95:14367-72.

83. Marano G, Palazzesi S, Bernucci P, Grigioni M, Formigari R, Ballerini L. ET(A)/ET(B) receptor antagonist bosentan inhibits neointimal development in collared carotid arteries of rabbits. Life Sci 1998;63:L259-L266.

84. Okada K, Nishida Y, Murakami H, Sugimoto I, Kosaka H, Morita H, et al. Role of endogenous endothelin in the development of graft arteriosclerosis in rat cardiac

allografts: antiproliferative effects of bosentan, a nonselective endothelin receptor antagonist. Circulation 1998;97:2346-51.

85. Bohm F, Ahlborg G, Pernow J. Endothelin-1 inhibits endothelium-dependent vasodilatation in the human forearm: reversal by ETA receptor blockade in patients with atherosclerosis. Clin Sci (Lond) 2002;102:321-7.

86. Komuro I, Kurihara H, Sugiyama T, Yoshizumi M, Takaku F, Yazaki Y. Endothelin stimulates c-fos and c-myc expression and proliferation of vascular smooth muscle cells. FEBS Lett 1988;238:249-52.

87. Kohno M, Yokokawa K, Yasunari K, Kano H, Minami M, Yoshikawa J. Effect of the endothelin family of peptides on human coronary artery smooth-muscle cell migration. J Cardiovasc Pharmacol 1998;31 Suppl 1:S84-S89.

88. Ishida N, Tsujioka K, Tomoi M, Saida K, Mitsui Y. Differential activities of two distinct endothelin family peptides on ileum and coronary artery. FEBS Lett 1989;247:337-40.

89. Naito S, Shimizu S, Maeda S, Wang J, Paul R, Fagin JA. Ets-1 is an early response gene activated by ET-1 and PDGF-BB in vascular smooth muscle cells. Am J Physiol 1998;274:C472-C480.

90. Rodriguez-Vita J, Ruiz-Ortega M, Ruperez M, Esteban V, Sanchez-Lopez E, Plaza JJ, et al. Endothelin-1, via ETA receptor and independently of transforming growth factor-beta, increases the connective tissue growth factor in vascular smooth muscle cells. Circ Res 2005;97:125-34.

91. Hahn AW, Resink TJ, Bernhardt J, Ferracin F, Buhler FR. Stimulation of autocrine platelet--derived growth factor AA-homodimer and transforming growth factor beta in vascular smooth muscle cells. Biochem Biophys Res Commun 1991;178:1451-8.

92. Margulies KB, Hildebrand FL, Jr., Lerman A, Perrella MA, Burnett JC, Jr. Increased endothelin in experimental heart failure. Circulation 1990;82:2226-30.

93. Rodeheffer RJ, Lerman A, Heublein DM, Burnett JC, Jr. Increased plasma concentrations of endothelin in congestive heart failure in humans. Mayo Clin Proc 1992;67:719-24.

94. Kirkby NS, Hadoke PW, Bagnall AJ, Webb DJ. The endothelin system as a therapeutic target in cardiovascular disease: great expectations or bleak house? Br J Pharmacol 2008;153:1105-19.

95. Ergul A, Grubbs AL, Zhang Y, Spinale FG. Selective upregulation of endothelin converting enzyme-1a in the human failing heart. J Card Fail 2000;6:314-20.

96. Denver R, Tzanidis A, Martin P, Krum H. Salivary endothelin concentrations in the assessment of chronic heart failure. Lancet 2000;355:468-9.

97. Wei CM, Lerman A, Rodeheffer RJ, McGregor CG, Brandt RR, Wright S, et al. Endothelin in human congestive heart failure. Circulation 1994;89:1580-6.

98. Yamauchi-Kohno R, Miyauchi T, Hoshino T, Kobayashi T, Aihara H, Sakai S, et al. Role of endothelin in deterioration of heart failure due to cardiomyopathy in hamsters: increase in endothelin-1 production in the heart and beneficial effect of endothelin-A receptor antagonist on survival and cardiac function. Circulation 1999;99:2171-6.

99. Sakai S, Miyauchi T, Kobayashi T, Yamaguchi I, Goto K, Sugishita Y. Altered expression of isoforms of myosin heavy chain mRNA in the failing rat heart is ameliorated by chronic treatment with an endothelin receptor antagonist. J Cardiovasc Pharmacol 1998;31 Suppl 1:S302-S305.

100. Choussat R, Hittinger L, Barbe F, Maistre G, Carayon A, Crozatier B, et al. Acute effects of an endothelin-1 receptor antagonist bosentan at different stages of heart failure in conscious dogs. Cardiovasc Res 1998;39:580-8.

101. Ohnishi M, Wada A, Tsutamoto T, Fukai D, Kinoshita M. Comparison of the acute effects of a selective endothelin ETA and a mixed ETA/ETB receptor antagonist in heart failure. Cardiovasc Res 1998;39:617-24.

102. New RB, Sampson AC, King MK, Hendrick JW, Clair MJ, McElmurray JH, III, et al. Effects of combined angiotensin II and endothelin receptor blockade with developing heart failure: effects on left ventricular performance. Circulation 2000;102:1447-53.

103. Rufanova VA, Pozdnev VF, Kalenikova EI, Postnikov AB, Storozhilova AN, Masenko VP, et al. Endothelin-converting enzyme inhibition in the rat model of acute heart failure: heart function and neurohormonal activation. Exp Biol Med (Maywood) 2009;234:1201-11.

104. Kiowski W, Sutsch G, Oechslin E, Bertel O. Hemodynamic effects of bosentan in patients with chronic heart failure. Heart Fail Rev 2001;6:325-34.

105. Sutsch G, Kiowski W, Yan XW, Hunziker P, Christen S, Strobel W, et al. Short-term oral endothelin-receptor antagonist therapy in conventionally treated patients with symptomatic severe chronic heart failure. Circulation 1998;98:2262-8.

106. Spieker LE, Mitrovic V, Noll G, Pacher R, Schulze MR, Muntwyler J, et al. Acute hemodynamic and neurohumoral effects of selective ET(A) receptor blockade in patients with congestive heart failure. ET 003 Investigators. J Am Coll Cardiol 2000;35:1745-52.

107. Torre-Amione G, Young JB, Colucci WS, Lewis BS, Pratt C, Cotter G, et al. Hemodynamic and clinical effects of tezosentan, an intravenous dual endothelin receptor antagonist, in patients hospitalized for acute decompensated heart failure. J Am Coll Cardiol 2003;42:140-7.

108. Anand I, McMurray J, Cohn JN, Konstam MA, Notter T, Quitzau K, et al. Long-term effects of darusentan on left-ventricular remodelling and clinical outcomes in the EndothelinA Receptor Antagonist Trial in Heart Failure (EARTH): randomised, double-blind, placebo-controlled trial. Lancet 2004;364:347-54.

109. Kelland NF, Webb DJ. Clinical trials of endothelin antagonists in heart failure: a question of dose? Exp Biol Med (Maywood) 2006;231:696-9.

110. Bouallegue A, Daou GB, Srivastava AK. Endothelin-1-induced signaling pathways in vascular smooth muscle cells. Curr Vasc Pharmacol 2007;5:45-52.

111. Pierce KL, Premont RT, Lefkowitz RJ. Seven-transmembrane receptors. Nat Rev Mol Cell Biol 2002;3:639-50.

112. Rhee SG. Regulation of phosphoinositide-specific phospholipase C. Annu Rev Biochem 2001;70:281-312.

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

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

115. Rosen B, Barg J, Zimlichman R. The effects of angiotensin II, endothelin-1, and protein kinase C inhibitor on DNA synthesis and intracellular calcium mobilization in vascular smooth muscle cells from young normotensive and spontaneously hypertensive rats. Am J Hypertens 1999;12:1243-51.

116. Idris I, Gray S, Donnelly R. Protein kinase C activation: isozyme-specific effects on metabolism and cardiovascular complications in diabetes. Diabetologia 2001;44:659-73.

117. Chua BH, Krebs CJ, Chua CC, Diglio CA. Endothelin stimulates protein synthesis in smooth muscle cells. Am J Physiol 1992;262:E412-E416.

118. Assender JW, Irenius E, Fredholm BB. Endothelin-1 causes a prolonged protein kinase C activation and acts as a co-mitogen in vascular smooth muscle cells. Acta Physiol Scand 1996;157:451-60.

119. McNair LL, Salamanca DA, Khalil RA. Endothelin-1 promotes Ca2+ antagonist-insensitive coronary smooth muscle contraction via activation of epsilon-protein kinase C. Hypertension 2004;43:897-904.

120. Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev 2001;22:153-83.

121. Luo G, Jamali R, Cao YX, Edvinsson L, Xu CB. Vascular endothelin ET(B) receptor-mediated contraction requires phosphorylation of ERK1/2 proteins. Eur J Pharmacol 2006;538:124-31.

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

123. Chen QW, Edvinsson L, Xu CB. Role of ERK/MAPK in endothelin receptor signaling in human aortic smooth muscle cells. BMC Cell Biol 2009;10:52.

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

125. Cheng CM, Hong HJ, Liu JC, Shih NL, Juan SH, Loh SH, et al. Crucial role of extracellular signal-regulated kinase pathway in reactive oxygen species-mediated endothelin-1 gene expression induced by endothelin-1 in rat cardiac fibroblasts. Mol Pharmacol 2003;63:1002-11.

126. Sorokin A, Foschi M, Dunn MJ. Endothelin signalling and regulation of protein kinases in glomerular mesangial cells. Clin Sci (Lond) 2002;103 Suppl 48:132S-6S.

127. Hashim S, Li Y, Anand-Srivastava MB. Small cytoplasmic domain peptides of natriuretic peptide receptor-C attenuate cell proliferation through Gialpha protein/MAP kinase/PI3-kinase/AKT pathways. Am J Physiol Heart Circ Physiol 2006;291:H3144-H3153.

128. Li Y, Hashim S, Anand-Srivastava MB. Intracellular peptides of natriuretic peptide receptor-C inhibit vascular hypertrophy via Gqalpha/MAP kinase signaling pathways. Cardiovasc Res 2006;72:464-72.

129. Leevers SJ, Vanhaesebroeck B, Waterfield MD. Signalling through phosphoinositide 3-kinases: the lipids take centre stage. Curr Opin Cell Biol 1999;11:219-25.

130. Deleris P, Gayral S, Breton-Douillon M. Nuclear Ptdlns(3,4,5)P3 signaling: an ongoing story. J Cell Biochem 2006;98:469-85.

131. Goruppi S, Bonventre JV, Kyriakis JM. Signaling pathways and late-onset gene induction associated with renal mesangial cell hypertrophy. EMBO J 2002;21:5427-36.

132. Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp Cell Res 1999;253:210-29.

133. Toker A. Protein kinases as mediators of phosphoinositide 3-kinase signaling. Mol Pharmacol 2000;57:652-8.

134. Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. Genes Dev 1999;13:2905-27.

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

136. Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, et al. Regulation of cell death protease caspase-9 by phosphorylation. Science 1998;282:1318-21.

137. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 1995;378:785-9.

138. Proud CG. Ras, PI3-kinase and mTOR signaling in cardiac hypertrophy. Cardiovasc Res 2004;63:403-13.

139. Pham FH, Cole SM, Clerk A. Regulation of cardiac myocyte protein synthesis through phosphatidylinositol 3' kinase and protein kinase B. Adv Enzyme Regul 2001;41:73-86.

140. Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, et al. Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts. Mol Biol Cell 2004;15:2707-19.

141. Dong F, Zhang X, Wold LE, Ren Q, Zhang Z, Ren J. Endothelin-1 enhances oxidative stress, cell proliferation and reduces apoptosis in human umbilical vein endothelial

cells: role of ETB receptor, NADPH oxidase and caveolin-1. Br J Pharmacol 2005;145:323-33.

142. Eguchi S, Iwasaki H, Inagami T, Numaguchi K, Yamakawa T, Motley ED, et al. Involvement of PYK2 in angiotensin II signaling of vascular smooth muscle cells. Hypertension 1999;33:201-6.

143. Iwasaki H, Eguchi S, Marumo F, Hirata Y. Endothelin-1 stimulates DNA synthesis of vascular smooth-muscle cells through transactivation of epidermal growth factor receptor. J Cardiovasc Pharmacol 1998;31 Suppl 1:S182-S184.

144. Kelly DJ, Cox AJ, Gow RM, Zhang Y, Kemp BE, Gilbert RE. Platelet-derived growth factor receptor transactivation mediates the trophic effects of angiotensin II in vivo. Hypertension 2004;44:195-202.

145. Delafontaine P. Insulin-like growth factor I and its binding proteins in the cardiovascular system. Cardiovasc Res 1995;30:825-34.

146. Laviola L, Natalicchio A, Giorgino F. The IGF-I signaling pathway. Curr Pharm Des 2007;13:663-9.

147. LeRoith D, Werner H, Beitner-Johnson D, Roberts CT, Jr. Molecular and cellular aspects of the insulin-like growth factor I receptor. Endocr Rev 1995;16:143-63.

148. Rozakis-Adcock M, McGlade J, Mbamalu G, Pelicci G, Daly R, Li W, et al. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature 1992;360:689-92.

149. Giorgetti S, Pelicci PG, Pelicci G, Van OE. Involvement of Src-homology/collagen (SHC) proteins in signaling through the insulin receptor and the insulin-like-growth-factor-I-receptor. Eur J Biochem 1994;223:195-202.

150. Vardatsikos G, Sahu A, Srivastava AK. The insulin-like growth factor family: molecular mechanisms, redox regulation, and clinical implications. Antioxid Redox Signal 2009;11:1165-90.

151. Bouallegue A, Vardatsikos G, Srivastava AK. Role of insulin-like growth factor 1 receptor and c-Src in endothelin-1- and angiotensin II-induced PKB phosphorylation, and hypertrophic and proliferative responses in vascular smooth muscle cells. Can J Physiol Pharmacol 2009;87:1009-18.

152. Azar ZM, Mehdi MZ, Srivastava AK. Activation of insulin-like growth factor type-1 receptor is required for H2O2-induced PKB phosphorylation in vascular smooth muscle cells. Can J Physiol Pharmacol 2006;84:777-86.

153. Bouallegue A, Vardatsikos G, Srivastava AK. Involvement of insulin-like growth factor 1 receptor transactivation in endothelin-1-induced signaling in vascular smooth muscle cells. Can J Physiol Pharmacol 2010;88:501-9.

154. Kahler J, Mendel S, Weckmuller J, Orzechowski HD, Mittmann C, Koster R, et al. Oxidative stress increases synthesis of big endothelin-1 by activation of the endothelin-1 promoter. J Mol Cell Cardiol 2000;32:1429-37.

155. Kahler J, Ewert A, Weckmuller J, Stobbe S, Mittmann C, Koster R, et al. Oxidative stress increases endothelin-1 synthesis in human coronary artery smooth muscle cells. J Cardiovasc Pharmacol 2001;38:49-57.

156. Fei J, Viedt C, Soto U, Elsing C, Jahn L, Kreuzer J. Endothelin-1 and smooth muscle cells: induction of jun amino-terminal kinase through an oxygen radical-sensitive mechanism. Arterioscler Thromb Vasc Biol 2000;20:1244-9.

157. Bayorh MA, Ganafa AA, Socci RR, Silvestrov N, Abukhalaf IK. The role of oxidative stress in salt-induced hypertension. Am J Hypertens 2004;17:31-6.

158. Levonen AL, Vahakangas E, Koponen JK, Yla-Herttuala S. Antioxidant gene therapy for cardiovascular disease: current status and future perspectives. Circulation 2008;117:2142-50.

159. Skalska AB, Pietrzycka A, Stepniewski M. Correlation of endothelin 1 plasma levels with plasma antioxidant capacity in elderly patients treated for hypertension. Clin Biochem 2009;42:358-64.

160. Ivey ME, Osman N, Little PJ. Endothelin-1 signalling in vascular smooth muscle: pathways controlling cellular functions associated with atherosclerosis. Atherosclerosis 2008;199:237-47.

161. Hynynen MM, Khalil RA. The vascular endothelin system in hypertension--recent patents and discoveries. Recent Pat Cardiovasc Drug Discov 2006;1:95-108.

162. Craig W, Beck L. Phytochemicals: Health Protective Effects. Can J Diet Pract Res 1999;60:78-84.

163. Nakmareong S, Kukongviriyapan U, Pakdeechote P, Donpunha W, Kukongviriyapan V, Kongyingyoes B, et al. Antioxidant and vascular protective effects of curcumin and tetrahydrocurcumin in rats with L-NAME-induced hypertension. Naunyn Schmiedebergs Arch Pharmacol 2011;383:519-29.

164. Abe Y, Hashimoto S, Horie T. Curcumin inhibition of inflammatory cytokine production by human peripheral blood monocytes and alveolar macrophages. Pharmacol Res 1999;39:41-7.

165. Wilken R, Veena MS, Wang MB, Srivatsan ES. Curcumin: A review of anti-cancer properties and therapeutic activity in head and neck squamous cell carcinoma. Mol Cancer 2011;10:12.

166. Anand P, Thomas SG, Kunnumakkara AB, Sundaram C, Harikumar KB, Sung B, et al. Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. Biochem Pharmacol 2008;76:1590-611.

167. Aggarwal BB, Sundaram C, Malani N, Ichikawa H. Curcumin: the Indian solid gold. Adv Exp Med Biol 2007;595:1-75.

168. Eigner D, Scholz D. Ferula asa-foetida and Curcuma longa in traditional medical treatment and diet in Nepal. J Ethnopharmacol 1999;67:1-6.

169. Goel A, Kunnumakkara AB, Aggarwal BB. Curcumin as "Curecumin": from kitchen to clinic. Biochem Pharmacol 2008;75:787-809.

170. Araujo CC, Leon LL. Biological activities of Curcuma longa L. Mem Inst Oswaldo Cruz 2001;96:723-8.

171. Ammon HP, Anazodo MI, Safayhi H, Dhawan BN, Srimal RC. Curcumin: a potent inhibitor of leukotriene B4 formation in rat peritoneal polymorphonuclear neutrophils (PMNL). Planta Med 1992;58:226.

172. Epstein J, Sanderson IR, Macdonald TT. Curcumin as a therapeutic agent: the evidence from in vitro, animal and human studies. Br J Nutr 2010;103:1545-57.

173. Payton F, Sandusky P, Alworth WL. NMR study of the solution structure of curcumin. J Nat Prod 2007;70:143-6.

174. Ak T, Gulcin I. Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact 2008;174:27-37.

175. Kunnumakkara AB, Anand P, Aggarwal BB. Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. Cancer Lett 2008;269:199-225.

176. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004;79:727-47.

177. Kidd PM. Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. Altern Med Rev 2009;14:226-46.

178. Jurenka JS. Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research. Altern Med Rev 2009;14:141-53.

179. Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, et al. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res 2001;21:2895-900.

180. Vareed SK, Kakarala M, Ruffin MT, Crowell JA, Normolle DP, Djuric Z, et al. Pharmacokinetics of curcumin conjugate metabolites in healthy human subjects. Cancer Epidemiol Biomarkers Prev 2008;17:1411-7.

181. Shankar TN, Shantha NV, Ramesh HP, Murthy IA, Murthy VS. Toxicity studies on turmeric (Curcuma longa): acute toxicity studies in rats, guineapigs & monkeys. Indian J Exp Biol 1980;18:73-5.

182. Sharma RA, Euden SA, Platton SL, Cooke DN, Shafayat A, Hewitt HR, et al. Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. Clin Cancer Res 2004;10:6847-54.

183. Somasundaram S, Edmund NA, Moore DT, Small GW, Shi YY, Orlowski RZ. Dietary curcumin inhibits chemotherapy-induced apoptosis in models of human breast cancer. Cancer Res 2002;62:3868-75.

184. Sandur SK, Ichikawa H, Pandey MK, Kunnumakkara AB, Sung B, Sethi G, et al. Role of pro-oxidants and antioxidants in the anti-inflammatory and apoptotic effects of curcumin (diferuloylmethane). Free Radic Biol Med 2007;43:568-80.

185. Zhou H, Beevers CS, Huang S. The Targets of Curcumin. Curr Drug Targets 2010.

186. Aggarwal BB, Harikumar KB. Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. Int J Biochem Cell Biol 2009;41:40-59.

187. Basnet P, Skalko-Basnet N. Curcumin: an anti-inflammatory molecule from a curry spice on the path to cancer treatment. Molecules 2011;16:4567-98.

188. Maskrey BH, Megson IL, Whitfield PD, Rossi AG. Mechanisms of resolution of inflammation: a focus on cardiovascular disease. Arterioscler Thromb Vasc Biol 2011;31:1001-6.

189. Venkatesan N, Punithavathi D, Babu M. Protection from acute and chronic lung diseases by curcumin. Adv Exp Med Biol 2007;595:379-405.

190. Aggarwal BB, Sung B. Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. Trends Pharmacol Sci 2009;30:85-94.

191. Menon VP, Sudheer AR. Antioxidant and anti-inflammatory properties of curcumin. Adv Exp Med Biol 2007;595:105-25.

192. Singh S, Aggarwal BB. Activation of transcription factor NF-kappa B is suppressed by curcumin (diferuloylmethane) [corrected]. J Biol Chem 1995;270:24995-5000.

193. Hong J, Bose M, Ju J, Ryu JH, Chen X, Sang S, et al. Modulation of arachidonic acid metabolism by curcumin and related beta-diketone derivatives: effects on cytosolic phospholipase A(2), cyclooxygenases and 5-lipoxygenase. Carcinogenesis 2004;25:1671-9.

194. Shishodia S, Singh T, Chaturvedi MM. Modulation of transcription factors by curcumin. Adv Exp Med Biol 2007;595:127-48.

195. Pendurthi UR, Rao LV. Suppression of transcription factor Egr-1 by curcumin. Thromb Res 2000;97:179-89.

196. Giri RK, Rajagopal V, Kalra VK. Curcumin, the active constituent of turmeric, inhibits amyloid peptide-induced cytochemokine gene expression and CCR5-mediated chemotaxis of THP-1 monocytes by modulating early growth response-1 transcription factor. J Neurochem 2004;91:1199-210.

197. Kim YS, Ahn Y, Hong MH, Joo SY, Kim KH, Sohn IS, et al. Curcumin attenuates inflammatory responses of TNF-alpha-stimulated human endothelial cells. J Cardiovasc Pharmacol 2007;50:41-9.

198. Collett GP, Campbell FC. Curcumin induces c-jun N-terminal kinase-dependent apoptosis in HCT116 human colon cancer cells. Carcinogenesis 2004;25:2183-9.

199. Hu M, Du Q, Vancurova I, Lin X, Miller EJ, Simms HH, et al. Proapoptotic effect of curcumin on human neutrophils: activation of the p38 mitogen-activated protein kinase pathway. Crit Care Med 2005;33:2571-8.

200. Virani SS, Polsani VR, Nambi V. Novel markers of inflammation in atherosclerosis. Curr Atheroscler Rep 2008;10:164-70.

201. Sharma OP. Antioxidant activity of curcumin and related compounds. Biochem Pharmacol 1976;25:1811-2.

202. Reddy AC, Lokesh BR. Studies on the inhibitory effects of curcumin and eugenol on the formation of reactive oxygen species and the oxidation of ferrous iron. Mol Cell Biochem 1994;137:1-8.

203. Sreejayan, Rao MN. Curcuminoids as potent inhibitors of lipid peroxidation. J Pharm Pharmacol 1994;46:1013-6.

204. Shen L, Ji HF. Insights into the inhibition of xanthine oxidase by curcumin. Bioorg Med Chem Lett 2009;19:5990-3.

205. Sreejayan N, Rao MN. Free radical scavenging activity of curcuminoids. Arzneimittelforschung 1996;46:169-71.

206. Motterlini R, Foresti R, Bassi R, Green CJ. Curcumin, an antioxidant and anti-inflammatory agent, induces heme oxygenase-1 and protects endothelial cells against oxidative stress. Free Radic Biol Med 2000;28:1303-12.

207. Wongcharoen W, Phrommintikul A. The protective role of curcumin in cardiovascular diseases. Int J Cardiol 2009;133:145-51.

208. Subramanian M, Sreejayan, Rao MN, Devasagayam TP, Singh BB. Diminution of singlet oxygen-induced DNA damage by curcumin and related antioxidants. Mutat Res 1994;311:249-55.

209. Ruby AJ, Kuttan G, Babu KD, Rajasekharan KN, Kuttan R. Anti-tumour and antioxidant activity of natural curcuminoids. Cancer Lett 1995;94:79-83.

210. Negre-Salvayre A, Auge N, Ayala V, Basaga H, Boada J, Brenke R, et al. Pathological aspects of lipid peroxidation. Free Radic Res 2010;44:1125-71.

211. Ramirez-Tortosa MC, Mesa MD, Aguilera MC, Quiles JL, Baro L, Ramirez-Tortosa CL, et al. Oral administration of a turmeric extract inhibits LDL oxidation and has hypocholesterolemic effects in rabbits with experimental atherosclerosis. Atherosclerosis 1999;147:371-8.

212. Thephinlap C, Phisalaphong C, Lailerd N, Chattipakorn N, Winichagoon P, Vadolus J, et al. Reversal of cardiac iron loading and dysfunction in thalassemic mice by curcuminoids. Med Chem 2011;7:62-9.

213. Rai B, Kaur J, Jacobs R, Singh J. Possible action mechanism for curcumin in pre-cancerous lesions based on serum and salivary markers of oxidative stress. J Oral Sci 2010;52:251-6.

214. Manikandan P, Sumitra M, Aishwarya S, Manohar BM, Lokanadam B, Puvanakrishnan R. Curcumin modulates free radical quenching in myocardial ischaemia in rats. Int J Biochem Cell Biol 2004;36:1967-80.

215. Aggarwal BB, Kumar A, Bharti AC. Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res 2003;23:363-98.

216. Shankar S, Chen Q, Sarva K, Siddiqui I, Srivastava RK. Curcumin enhances the apoptosis-inducing potential of TRAIL in prostate cancer cells: molecular mechanisms of apoptosis, migration and angiogenesis. J Mol Signal 2007;2:10.

217. Shankar S, Srivastava RK. Involvement of Bcl-2 family members, phosphatidylinositol 3'-kinase/AKT and mitochondrial p53 in curcumin (diferulolylmethane)-induced apoptosis in prostate cancer. Int J Oncol 2007;30:905-18.

218. Dorai T, Gehani N, Katz A. Therapeutic potential of curcumin in human prostate cancer. II. Curcumin inhibits tyrosine kinase activity of epidermal growth factor receptor and depletes the protein. Mol Urol 2000;4:1-6.

219. Chen A, Xu J, Johnson AC. Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor through reducing the activity of the transcription factor Egr-1. Oncogene 2006;25:278-87.

220. Xia Y, Jin L, Zhang B, Xue H, Li Q, Xu Y. The potentiation of curcumin on insulin-like growth factor-1 action in MCF-7 human breast carcinoma cells. Life Sci 2007;80:2161-9.

221. Schwer CI, Guerrero AM, Humar M, Roesslein M, Goebel U, Stoll P, et al. Heme oxygenase-1 inhibits the proliferation of pancreatic stellate cells by repression of the extracellular signal-regulated kinase1/2 pathway. J Pharmacol Exp Ther 2008;327:863-71.

222. Dhandapani KM, Mahesh VB, Brann DW. Curcumin suppresses growth and chemoresistance of human glioblastoma cells via AP-1 and NFkappaB transcription factors. J Neurochem 2007;102:522-38.

223. Woo MS, Jung SH, Kim SY, Hyun JW, Ko KH, Kim WK, et al. Curcumin suppresses phorbol ester-induced matrix metalloproteinase-9 expression by inhibiting the PKC to MAPK signaling pathways in human astroglioma cells. Biochem Biophys Res Commun 2005;335:1017-25.

224. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685-95.

225. Srivastava R, Dikshit M, Srimal RC, Dhawan BN. Anti-thrombotic effect of curcumin. Thromb Res 1985;40:413-7.

226. Quiles JL, Mesa MD, Ramirez-Tortosa CL, Aguilera CM, Battino M, Gil A, et al. Curcuma longa extract supplementation reduces oxidative stress and attenuates aortic fatty streak development in rabbits. Arterioscler Thromb Vasc Biol 2002;22:1225-31.

227. Olszanecki R, Jawien J, Gajda M, Mateuszuk L, Gebska A, Korabiowska M, et al. Effect of curcumin on atherosclerosis in apoE/LDLR-double knockout mice. J Physiol Pharmacol 2005;56:627-35.

228. Yuan HY, Kuang SY, Zheng X, Ling HY, Yang YB, Yan PK, et al. Curcumin inhibits cellular cholesterol accumulation by regulating SREBP-1/caveolin-1 signaling pathway in vascular smooth muscle cells. Acta Pharmacol Sin 2008;29:555-63.

229. Mahfouz MM, Zhou SQ, Kummerow FA. Curcumin prevents the oxidation and lipid modification of LDL and its inhibition of prostacyclin generation by endothelial cells in culture. Prostaglandins Other Lipid Mediat 2009;90:13-20.

230. Soni KB, Kuttan R. Effect of oral curcumin administration on serum peroxides and cholesterol levels in human volunteers. Indian J Physiol Pharmacol 1992;36:273-5.

231. Alwi I, Santoso T, Suyono S, Sutrisna B, Suyatna FD, Kresno SB, et al. The effect of curcumin on lipid level in patients with acute coronary syndrome. Acta Med Indones 2008;40:201-10.

232. Ramirez-Bosca A, Soler A, Carrion MA, Diaz-Alperi J, Bernd A, Quintanilla C, et al. An hydroalcoholic extract of curcuma longa lowers the apo B/apo A ratio. Implications for atherogenesis prevention. Mech Ageing Dev 2000;119:41-7.

233. Ramirez BA, Soler A, Carrion-Gutierrez MA, Pamies MD, Pardo ZJ, Diaz-Alperi J, et al. An hydroalcoholic extract of Curcuma longa lowers the abnormally high values of human-plasma fibrinogen. Mech Ageing Dev 2000;114:207-10.

234. Huang HC, Jan TR, Yeh SF. Inhibitory effect of curcumin, an anti-inflammatory agent, on vascular smooth muscle cell proliferation. Eur J Pharmacol 1992;221:381-4.

235. Yang X, Thomas DP, Zhang X, Culver BW, Alexander BM, Murdoch WJ, et al. Curcumin inhibits platelet-derived growth factor-stimulated vascular smooth muscle cell function and injury-induced neointima formation. Arterioscler Thromb Vasc Biol 2006;26:85-90.

236. Qin L, Yang YB, Tuo QH, Zhu BY, Chen LX, Zhang L, et al. Effects and underlying mechanisms of curcumin on the proliferation of vascular smooth muscle cells induced by Chol:MbetaCD. Biochem Biophys Res Commun 2009;379:277-82.

237. Pae HO, Jeong GS, Jeong SO, Kim HS, Kim SA, Kim YC, et al. Roles of heme oxygenase-1 in curcumin-induced growth inhibition in rat smooth muscle cells. Exp Mol Med 2007;39:267-77.

238. Yu YM, Lin HC. Curcumin prevents human aortic smooth muscle cells migration by inhibiting of MMP-9 expression. Nutr Metab Cardiovasc Dis 2010;20:125-32.

239. Liu Y, Dolence J, Ren J, Rao M, Sreejayan N. Inhibitory effect of dehydrozingerone on vascular smooth muscle cell function. J Cardiovasc Pharmacol 2008;52:422-9.

240. Selvendiran K, Kuppusamy ML, Bratasz A, Tong L, Rivera BK, Rink C, et al. Inhibition of vascular smooth-muscle cell proliferation and arterial restenosis by HO-3867, a novel synthetic curcuminoid, through up-regulation of PTEN expression. J Pharmacol Exp Ther 2009;329:959-66.

241. Shim JS, Kim DH, Jung HJ, Kim JH, Lim D, Lee SK, et al. Hydrazinocurcumin, a novel synthetic curcumin derivative, is a potent inhibitor of endothelial cell proliferation. Bioorg Med Chem 2002;10:2439-44.

242. Frey N, Olson EN. Cardiac hypertrophy: the good, the bad, and the ugly. Annu Rev Physiol 2003;65:45-79.

243. Backs J, Olson EN. Control of cardiac growth by histone acetylation/deacetylation. Circ Res 2006;98:15-24.

244. Dai YS, Markham BE. p300 Functions as a coactivator of transcription factor GATA-4. J Biol Chem 2001;276:37178-85.

245. Kakita T, Hasegawa K, Morimoto T, Kaburagi S, Wada H, Sasayama S. p300 protein as a coactivator of GATA-5 in the transcription of cardiac-restricted atrial natriuretic factor gene. J Biol Chem 1999;274:34096-102.

246. Yanazume T, Hasegawa K, Morimoto T, Kawamura T, Wada H, Matsumori A, et al. Cardiac p300 is involved in myocyte growth with decompensated heart failure. Mol Cell Biol 2003;23:3593-606.

247. Morimoto T, Sunagawa Y, Kawamura T, Takaya T, Wada H, Nagasawa A, et al. The dietary compound curcumin inhibits p300 histone acetyltransferase activity and prevents heart failure in rats. J Clin Invest 2008;118:868-78.

248. Conraads VM, Bosmans JM, Vrints CJ. Chronic heart failure: an example of a systemic chronic inflammatory disease resulting in cachexia. Int J Cardiol 2002;85:33-49.

249. Bradham WS, Moe G, Wendt KA, Scott AA, Konig A, Romanova M, et al. TNF-alpha and myocardial matrix metalloproteinases in heart failure: relationship to LV remodeling. Am J Physiol Heart Circ Physiol 2002;282:H1288-H1295.

250. Yao QH, Wang DQ, Cui CC, Yuan ZY, Chen SB, Yao XW, et al. Curcumin ameliorates left ventricular function in rabbits with pressure overload: inhibition of the remodeling of the left ventricular collagen network associated with suppression of myocardial tumor necrosis factor-alpha and matrix metalloproteinase-2 expression. Biol Pharm Bull 2004;27:198-202.

251. Nazam AM, Bhandari U, Pillai KK. Protective role of curcumin in myocardial oxidative damage induced by isoproterenol in rats. Hum Exp Toxicol 2007;26:933-8.

252. Venkatesan N. Curcumin attenuation of acute adriamycin myocardial toxicity in rats. Br J Pharmacol 1998;124:425-7.

253. Kang BY, Khan JA, Ryu S, Shekhar R, Seung KB, Mehta JL. Curcumin reduces angiotensin II-mediated cardiomyocyte growth via LOX-1 inhibition. J Cardiovasc Pharmacol 2010;55:176-83.

254. Ainscough JF, Drinkhill MJ, Sedo A, Turner NA, Brooke DA, Balmforth AJ, et al. Angiotensin II type-1 receptor activation in the adult heart causes blood pressure-independent hypertrophy and cardiac dysfunction. Cardiovasc Res 2009;81:592-600.

255. Wu S, Gao J, Ohlemeyer C, Roos D, Niessen H, Kottgen E, et al. Activation of AP-1 through reactive oxygen species by angiotensin II in rat cardiomyocytes. Free Radic Biol Med 2005;39:1601-10.

256. Hu C, Dandapat A, Sun L, Chen J, Marwali MR, Romeo F, et al. LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet. Cardiovasc Res 2008;79:287-93.

257. Freund C, Schmidt-Ullrich R, Baurand A, Dunger S, Schneider W, Loser P, et al. Requirement of nuclear factor-kappaB in angiotensin II- and isoproterenol-induced cardiac hypertrophy in vivo. Circulation 2005;111:2319-25.

258. Xu PH, Long Y, Dai F, Liu ZL. The relaxant effect of curcumin on porcine coronary arterial ring segments. Vascul Pharmacol 2007;47:25-30.

259. Rungseesantivanon S, Thenchaisri N, Ruangvejvorachai P, Patumraj S. Curcumin supplementation could improve diabetes-induced endothelial dysfunction associated with decreased vascular superoxide production and PKC inhibition. BMC Complement Altern Med 2010;10:57.

260. Khajehdehi P, Zanjaninejad B, Aflaki E, Nazarinia M, Azad F, Malekmakan L, et al. Oral Supplementation of Turmeric Decreases Proteinuria, Hematuria, and Systolic Blood Pressure in Patients Suffering from Relapsing or Refractory Lupus Nephritis: A Randomized and Placebo-controlled Study. J Ren Nutr 2011.

261. Srinivasan M. Effect of curcumin on blood sugar as seen in a diabetic subject. Indian J Med Sci 1972;26:269-70.

262. Farhangkhoee H, Khan ZA, Kaur H, Xin X, Chen S, Chakrabarti S. Vascular endothelial dysfunction in diabetic cardiomyopathy: pathogenesis and potential treatment targets. Pharmacol Ther 2006;111:384-99.

263. Stadler K, Jenei V, von BG, Somogyi A, Jakus J. Increased nitric oxide levels as an early sign of premature aging in diabetes. Free Radic Biol Med 2003;35:1240-51.

264. Farhangkhoee H, Khan ZA, Mukherjee S, Cukiernik M, Barbin YP, Karmazyn M, et al. Heme oxygenase in diabetes-induced oxidative stress in the heart. J Mol Cell Cardiol 2003;35:1439-48.

265. Farhangkhoee H, Khan ZA, Chen S, Chakrabarti S. Differential effects of curcumin on vasoactive factors in the diabetic rat heart. Nutr Metab (Lond) 2006;3:27.

266. Feng B, Chen S, Chiu J, George B, Chakrabarti S. Regulation of cardiomyocyte hypertrophy in diabetes at the transcriptional level. Am J Physiol Endocrinol Metab 2008;294:E1119-E1126.

267. Chan MM. Inhibition of tumor necrosis factor by curcumin, a phytochemical. Biochem Pharmacol 1995;49:1551-6.

268. Jang MK, Sohn DH, Ryu JH. A curcuminoid and sesquiterpenes as inhibitors of macrophage TNF-alpha release from Curcuma zedoaria. Planta Med 2001;67:550-2.

269. Matsuda H, Tewtrakul S, Morikawa T, Nakamura A, Yoshikawa M. Anti-allergic principles from Thai zedoary: structural requirements of curcuminoids for inhibition of degranulation and effect on the release of TNF-alpha and IL-4 in RBL-2H3 cells. Bioorg Med Chem 2004;12:5891-8.

270. Gulcubuk A, Altunatmaz K, Sonmez K, Haktanir-Yatkin D, Uzun H, Gurel A, et al. Effects of curcumin on tumour necrosis factor-alpha and interleukin-6 in the late

phase of experimental acute pancreatitis. J Vet Med A Physiol Pathol Clin Med 2006;53:49-54.

271. Jang EM, Choi MS, Jung UJ, Kim MJ, Kim HJ, Jeon SM, et al. Beneficial effects of curcumin on hyperlipidemia and insulin resistance in high-fat-fed hamsters. Metabolism 2008;57:1576-83.

272. Seo KI, Choi MS, Jung UJ, Kim HJ, Yeo J, Jeon SM, et al. Effect of curcumin supplementation on blood glucose, plasma insulin, and glucose homeostasis related enzyme activities in diabetic db/db mice. Mol Nutr Food Res 2008;52:995-1004.

273. El-Moselhy MA, Taye A, Sharkawi SS, El-Sisi SF, Ahmed AF. The antihyperglycemic effect of curcumin in high fat diet fed rats. Role of TNF-alpha and free fatty acids. Food Chem Toxicol 2011;49:1129-40.

274. Usharani P, Mateen AA, Naidu MU, Raju YS, Chandra N. Effect of NCB-02, atorvastatin and placebo on endothelial function, oxidative stress and inflammatory markers in patients with type 2 diabetes mellitus: a randomized, parallel-group, placebo-controlled, 8-week study. Drugs R D 2008;9:243-50.

275. Chen HW, Huang HC. Effect of curcumin on cell cycle progression and apoptosis in vascular smooth muscle cells. Br J Pharmacol 1998;124:1029-40.

276. Touyz RM, Yao G, Viel E, Amiri F, Schiffrin EL. Angiotensin II and endothelin-1 regulate MAP kinases through different redox-dependent mechanisms in human vascular smooth muscle cells. J Hypertens 2004;22:1141-9.

277. Flamant M, Tharaux PL, Placier S, Henrion D, Coffman T, Chatziantoniou C, et al. Epidermal growth factor receptor trans-activation mediates the tonic and fibrogenic effects of endothelin in the aortic wall of transgenic mice. FASEB J 2003;17:327-9.

278. Kodama H, Fukuda K, Takahashi T, Sano M, Kato T, Tahara S, et al. Role of EGF Receptor and Pyk2 in endothelin-1-induced ERK activation in rat cardiomyocytes. J Mol Cell Cardiol 2002;34:139-50.

279. Kodama H, Fukuda K, Takahashi E, Tahara S, Tomita Y, Ieda M, et al. Selective involvement of p130Cas/Crk/Pyk2/c-Src in endothelin-1-induced JNK activation. Hypertension 2003;41:1372-9.

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

281. Khachigian LM. Early growth response-1 in cardiovascular pathobiology. Circ Res 2006;98:186-91.