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Regulation of the Early Growth Response Protein-1 in vascular smooth muscle cells

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Résumé

Une hyperactivation de la prolifération des cellules musculaires lisses vasculaires (CMLV) contribue à la pathogenèse des maladies des vaisseaux. Des travaux antérieurs suggèrent que l'augmentation de l'adénosine monophosphate cyclique (AMPc) inhibe la prolifération des CMLV. Provoquer une augmentation d'AMPc préviendrait aussi certaines maladies vasculaires qui sont associées à des altérations dans sa signalisation impliquant l'activité de la protéine kinase A (PKA). Des études ont démontré la contribution du facteur de transcription « *early growth response protein-1»* (Egr-1) dans la pathogenèse des maladies vasculaires et une surexpression d'Egr-1 a été rapportée dans des modèles d'athérosclérose et d'hyperplasie intimale. Divers agents vasoactifs contrôlent l'expression d'Egr-1 suivant des mécanismes qui ont fait l'objet de plusieurs études mais demeurent incomplètement élucidés. L'angiotensine-II (Ang-II) est l'un des principaux peptides vasoactifs impliqués dans la pathogenèse des maladies vasculaires. Une des voies de signalisation induite par l'Ang-II implique l'augmentation du calcium (Ca^{2+}) intracellulaire. Celle-ci se produit par l'activation de l'entrée de calcium opérée par la relâche des réserves (SOCE) de Ca^{2+} réticulaire suite à l'activation du récepteur à l'inositol-3-phosphate (IP_3R) et le recrutement ultérieur du complexe conducteur formé par la molécule d'interaction stromale 1 (STIM-1) et le canal Orai-1. Bien qu'il ait déjà été démontré que l'expression de l'Egr-1 est régulée par la signalisation calcique en réponse à plusieurs stimuli, l'implication du complexe STIM-1/Orai-1 dans l'expression d'Egr-1 dans la CMLV n'a jamais été étudiée. De même, la question de savoir si la signalisation induite par l'Ang-II conduisant à l'expression d'Egr-1 est modulée par l'AMPc n'a jamais été explorée. Par conséquent, les travaux menés dans cette thèse ont consisté à examiner le rôle de la signalisation du Ca^{2+} dans l'expression d'Egr-1 induite par l'Ang-II dans la CMLV avec une attention particulière portée sur le rôle joué par STIM-1 et Orai-1. En outre, nous avons examiné l'effet de l'augmentation de l'AMPc sur l'expression d'Egr-1 induite par l'Ang-II et étudié les voies de signalisation associées. Nos données montrent

que l'inhibition du récepteur IP_3R et du SOCE par le 2-aminoéthoxydiphénylborate atténue la libération de Ca^{2+} induite par l'Ang-II et ceci s'accompagne d'une baisse des niveaux d'expression de protéine et d'ARN messager de l'Egr-1. La stimulation de l'expression de l'Egr-1 a également été supprimée à la suite du blocage de la calmoduline et de la protéine kinase CaMKII. De plus, le blocage par interférence d'ARN de l'expression de STIM-1 et Orai-1 a atténué l'expression d'Egr-1 induite par l'Ang-II ainsi que la phosphorylation des protéines ERK et CREB. Par ailleurs, l'isoproterenol (ISO) et la forskoline (FSK), deux activateurs de l'adénylate cyclase ont atténué de manière dose-dépendante l'expression d'Egr-1 induite par l'Ang-II. Des réponses similaires ont été observées en utilisant des analogues non spécifique (dibutyryl-cAMP) et PKA-spécifique (Benzoyl-cAMP) de l'AMPc, ainsi qu'un inhibiteur à large spectre de l'activité phosphodiesterase intracellualaire (isobutylméthylxanthine). L'inhibition de l'expression d'Egr-1 induite par l'Ang-II s'accompagne d'une augmentation de l'activité de la PKA mesurée par la phosphorylation de la « phosphoprotéine activée par les vasodilatateurs (VASP) », et d'une diminution concomitante de la phosphorylation de la protéine ERK. Le blocage pharmacologique de la PKA a réduit la phosphorylation de VASP et restauré la phosphorylation de la protéine ERK ainsi que l'expression d'Egr-1 en présence de l'Ang-II.

En résumé, nos données démontrent que la voie STIM-1/Orai-1 /Ca^{2+} médie l'expression de l'Egr-1 induite par l'Ang-II dans la CMLV et suggèrent que la suppression de la réponse à l'Ang-II menant à l'expression de l'Egr-1 peut expliquer les effets vasoprotecteurs de l'AMPc. En outre, ces travaux montrent que les mécanismes moléculaires de régulation de l'expression d'Egr-1 en réponse aux signaux externes culminent vers la modulation des cascades de signalisation en aval de la protéine ERK dans les CMLV.

Mots-clés: Angiotensine-II, STIM-1, Orai-1, calcium, Egr-1, CREB, AMPc, PKA, CMLV.

Abstract

Aberrant vascular smooth muscle cell (VSMC) proliferative responses contribute to the development of neointimal lesions. Cyclic adenosine monophosphate (cAMP) is believed to inhibit VSMC proliferation, and vascular diseases are associated with impairments in cAMP-induced signalling responses involving protein kinase A (PKA) signaling. An enhanced expression of the early growth response protein-1 (Egr-1), a zinc finger transcription factor, has been reported in models of vascular diseases and, a crucial role of Egr-1 in regulating the expression of genes implicated in neointimal formation leading to atherogenesis has been suggested. Various vasoactive factors have been shown to modulate Egr-1 expression in VSMC via mechanisms which remain to be completely understood. Angiotensin-II (Ang-II) is one of the key vasoactive peptides implicated in the pathogenesis of vascular diseases. Ang-II elevates intracellular calcium (Ca^{2+}) through activation of voltage-gated calcium channels as well as store-operated calcium channels. The store-operated calcium entry (SOCE) involves an inositol-3-phosphate receptor (IP_3R)-coupled depletion of endoplasmic reticular Ca^{2+} and a subsequent activation of the stromal interaction molecule 1 (STIM-1) /Orai-1 complex. Although Egr-1 has been demonstrated to be upregulated in a Ca^{2+}-dependent fashion in response to several stimuli, the involvement of STIM-1/Orai-1-dependent signaling in Egr-1 expression in VSMC has never been addressed. Besides, whether Ang-II-induced signaling leading to Egr-1 expression is modulated by cAMP-dependent signaling pathway remains unexplored. Therefore, in the present studies, we have examined the role of Ca^{2+} signaling in Ang-II-induced Egr-1 expression in VSMC and investigated the contribution of STIM-1 or Orai-1. Additionnaly, we have examined the effect of cAMP on Ang-II-induced expression of Egr-1 and have investigated the associated signalling pathways. Pharmacological blockade of IP_3R and SOCE by 2-aminoethoxydiphenylborate (2-APB) decreased Ang-II-induced Ca^{2+} release and attenuated Ang-II-induced enhanced expression of Egr-1 protein and mRNA levels. Egr-1 upregulation was also suppressed following blockade of calmodulin and CaMKII. Furthermore, RNA interference-mediated depletion of STIM-1 or Orai-1

attenuated Ang-II-induced Egr-1 expression, as well as Ang-II-induced phosphorylation of ERK1/2 and CREB. Moreover, isoproterenol (ISO) and forskolin (FSK), two respective receptor and non-receptor activators of adenylate cyclase, attenuated Ang-II-induced Egr-1 expression in a dose-dependent fashion. Similar responses were observed using non-specific (dibutyryl-cAMP) and PKA-specific (Benzoyl-cAMP) analogs of cAMP, as well as a broad spectrum inhibitor of intracellular phosphodiesterase activity (isobutylmethylxanthine). The inhibition of Ang-II-induced Egr-1 expression was accompanied by an increase in serine 157 phosphorylation of the vasodilator-activated phosphoprotein (VASP), a marker of PKA activity, and this was associated with a concomitant decrease in ERK phosphorylation. Pharmacological blockade of PKA using H89 decreased VASP phosphorylation, restored Ang-II-induced ERK phosphorylation and abolished ISO- and FSK-mediated inhibition of Ang-II-induced Egr-1 expression.

In summary, our data demonstrate that STIM-1/Orai-1/Ca^{2+}-dependent signaling pathways mediate Ang-II-induced Egr-1 expression in A-10 VSMC and suggest that PKA-mediated suppression of Ang-II-induced Egr-1 expression and phosphorylation of ERK may be among the mechanisms by which cAMP exerts its vasoprotective effects. In addition, our data supports the notion that stimuli-induced regulation of Egr-1 expression involves the participation of signaling cascades downstream of ERK in VSMC.

Keywords: Angiotensin-II, STIM-1, Orai-1, calcium, Egr-1, CREB, cAMP, PKA, VSMC.

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List of Abbreviations

2-APB:	2-aminophenylborate
AC:	Adenylate cyclase
ACE:	Angiotensin-converting enzyme
Ang:	Angiotensin
Ang-II:	Angiotensin-II
APA:	Type A aminopeptidase
APN:	Type N aminopeptidase
AT1R:	Angiotensin-II type 1 receptor
AT2R:	Angiotensin-II type 2 receptor
AT3R:	Angiotensin-II type 3 receptor
AT4R:	Angiotensin-II type 4 receptor
ATP:	Adenylate triphosphate
ATPase:	Adenylate triphosphatase
ATRAP:	Angiotensin-II type 1 receptor-associated protein
BNZ-cAMP:	Benzoyl cyclic adenosine monophosphate
cAMP:	Cyclic adenosine monophosphate
Ca^{2+}:	Calcium
CAD:	CRAC-activation domain
CArD:	Carotid artery disease
CaMKII:	Calcium calmodulin-dependent protein kinase 2
CRAC:	Ca^{2+} release-activated Ca^{2+} channel
CRE:	Cyclic adenosine monophosphate response element
CREB:	Cyclic adenosine monophosphate response element binding protein
CVD:	Cardiovascular diseases
DAG:	Diacylglycerol
Db-cAMP:	Dibutyryl cyclic adenosine monophosphate
DBD:	DNA binding domain

DBP:	Diastolic blood pressure
EBS:	Early growth response protein-1 binding site
Elk-1:	Ets domain-containing protein
Egr-1:	Early growth response protein-1
eNOS:	Endothelial nitric oxide synthase
Epac:	Exchange channel activated by cAMP
ET-1:	Endothelin-1
Ets:	E-twenty-six
ER:	Endoplasmic reticulum
ERK:	Extracellular signal-regulated protein kinase
FSK:	Forskolin
FOXO/FKHR:	Forkhead transcription factor
GDP:	Guanosine diphosphate
GPCR:	G-protein coupled receptor
GRK:	G-protein receptor kinase
GSK:	Glycogen synthase kinase
GTP:	Guanosine triphosphate
IP_3:	Inositol-3-phosphate
IP_3R:	Inositol-3-phosphate receptor
JNK:	c-Jun NH2-terminal kinase
LPA:	Lysophosphatidic acid
LTCC:	L-type calcium channel
MAPK:	Mitogen-activated protein kinase
MEKK:	Mitogen extracellular signal-regulated kinase kinase
MSK:	Mitogen and stress-activated kinase
mTOR:	Mammalian target of rapamycin
NAB:	Nerve growth factor-induced-A-Binding proteins
NR-PTK:	Non-receptor protein tyrosine kinase
PDK:	Phosphoinositide- dependent kinases
PI3-K:	Phosphatidylinositol-3-kinase

PIP_2:	Phosphatidylinositol-4, 5-biphosphate
PIP_3:	Phosphatidylinositol-1, 4, 5-triphosphate
PKA:	Protein kinase A
PKAcat:	Protein kinase A catalytic subunit
PKAR:	Protein kinase A regulatory subunit
PKAI:	Protein kinase A type 1
PKAII:	Protein kinase A type 2
PKB:	Protein kinase B
PKC:	Protein kinase C
PLA:	Phospholipase A
PLC:	Phospholipase C
PLD:	Phospholipase D
PMCA:	Plasma membrane calcium adenylate triphosphatase
RAS:	Renin-angiotensin-aldosterone system
R-PTK:	Receptor protein tyrosine kinase
Sap:	Serum response factor-associated accessory protein
SAPK:	Stress- activated protein kinase
SBP:	Systolic blood pressure
SHR:	Spontaneously hypertensive rats
SERCA:	Sarco/endoplasmic reticulum calcium adenylate triphosphatase
SOAR:	STIM-1/Orai-1 activating region
SOCC:	Store-operated calcium channel
SOCE:	Store-operated calcium entry
SRE:	Serum-response element
SRF:	Serum-response factor
STIM:	Stromal interaction molecule
SMC:	Smooth muscle cell
TRPC:	Transient receptor-activated channel
VOCE:	Voltage-operated calcium entry
VSMC:	Vascular smooth muscle cell

WKY:	Wistar Kyoto rat

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Chapter 1: Literature review

1.1 VASCULAR DISEASES AND HYPERTENSION

The past decades have witnessed global transition of causes of death from historical nutrient deficiencies and acute infectious diseases to the modern day nutrient excesses associated with chronic diseases (1). One of the consequences of this transition is an increase in the prevalence of metabolic conditions including diabetes, hypertension, and related cardiovascular diseases (CVDs) (2). In diabetic patients, heightened levels of circulatory fatty acids and hyperglycemia are associated with an aberrant vascular function (3). As a result, the elevated mortality and morbidity observed in these patients are in large part attributed to cardiovascular complications (4, 5). However, the main underlying cause of congestive heart failure, myocardial infarction and other vascular diseases is hypertension (6, 7). For this reason, hypertension is now considered as the number one risk factor for premature death (8). In 2012, 80 million adults over 20 years old were diagnosed with hypertension in the United States (9) whereas in Canada, one in five adults has hypertension (10). Globally, it is estimated that between 1980 and 2008, the number of adults with hypertension worldwide increased from 605 to 978 million (11); this number is predicted to rise to 1.56 billion by 2025 (12, 13).

Hypertension is a multifactorial disease generally classified into two types: primary (essential) hypertension and secondary hypertension. With a proportion of nearly 95% among the population diagnosed with hypertension, essential hypertension, characterized by the absence of an identifiable cause, is the most prevalent type (14, 15). Some determinants, linked with either the genetic background or the environment, have been identified as possible risk factors for the development of this type of hypertension (16). Those related to familial history, ethnicity (17), gender and aging are classified among the non-modifiable risk factors. Several types of hypertension due to genetic causes are regrouped in Mendelian hypertension with identified gene polymorphisms that are positively associated with the increase in blood pressure (18, 19). With regard to the environmental factors, many lines of evidence from epidemiological studies have reported the positive association between the growing incidence of essential hypertension and

behaviors such as excessive food and alcohol consumption, a high salt diet, physical inactivity and cigarette smoking (14). Prolonged stress and a low potassium diet are also associated with a high risk of developing high blood pressure. Thus, choices related to lifestyle are increasingly being considered as major modifiable variables for the prevention of hypertension and related end-organ damage (20, 21).

In contrast to essential hypertension, secondary hypertension is diagnosed by the presence of an underlying condition that contributes to the increase in blood pressure (22). 10% of the population diagnosed with hypertension belong to this category (22). For example, hypertension associated with sleep apnea, one type of secondary hypertension, where respiratory disorders facilitate an aberrant stimulation of the central production of vasoconstrictive hormones (23). Gestional hypertension is another type where the first onset of high blood pressure occurs during pregnancy possibly due to remodeling of the vascular system observed in that condition (24). Secondary hypertension also comes from metabolic conditions like diabetes and obesity, as well as from kidney diseases including glomerular dysfunction, renovascular stenosis, and polycystic kidney disease. Aberrant hormonal conditions such as the Cushing syndrome, hyperaldosteronism, pheochromocytoma or dysfunctional thyroid, have also been described as underlying causes of secondary hypertension (25). Hormonal disorders are mostly due to the presence of a tumor that may either enhance the secretion of prohypertensive substances or facilitate aberrant growth in cardiovascular relevant organs (26). Secondary hypertension can also occur as a side effect of medications like corticosteroids, contraceptive pills and several antidepressive drugs and pain killers (27).

By definition, hypertension is the condition of persistent non physiologic elevated blood pressure (28). Blood pressure is generally assessed by two values. The systolic blood pressure (SBP) reflects the force exerted by the blood on the arterial wall when the heart beats whereas the diastolic blood pressure (DBP) is the measure of the pressure when the heart relaxes and refills at the end of a cardiac cycle (28). These values are expressed in millimeters of mercury (mmHg) and are considered normal when they are respectively lower than 120 mmHg and 80 mmHg at the resting state (28). Systemic arterial

hypertension is currently defined as a resting SBP at 140 mmHg or greater and a DBP at 90 mmHg or greater (29). Noteworthy, beginning at 115/75 mmHg, the risk for developing CVD doubles for each increment of 20/10 mmHg (30) suggesting that vascular homeostasis is highly influenced by blood pressure dynamics even at prehypertensive stages.

1.2 VASCULAR DAMAGE

Vascular damage is a hallmark feature in the pathophysiology of hypertension (31) and often precedes the increase in blood pressure as observed in some types of genetic hypertension (32). This is in part due to heightened activity of vasoconstrictive and mitogenic hormones like angiotensin-II (Ang-II) or endothelin-1 (ET-1) present at elevated systemic and local concentrations. Alone or in concert with other stimuli, an exaggerated activity of these vasoactive peptides can induce structural and functional changes within the vessel wall (33, 34). The consequences of these changes termed as vessel remodeling mainly manifest as lumen narrowing and not only occur under conditions of chronic hypertension, but may also happens in response to temporary elevations of blood pressure. Since it is tightly related to the diameter of small vessels such as small arteries and arterioles neighbouring the capillarie beds, vascular resistance is highly increased by vessel remodeling. An increased peripheral vascular resistance is therefore the hallmark of vascular damage and the major feature in the pathogenesis of hypertension (7, 35, 36). Advances in the description of the tissular components of the vasculature as well as understanding how their functional properties are modulated by physical and chemical clues have contributed in linking blood pressure variations and other determinants of cardiovascular risk to vascular damage. Below is a brief description of the structural features of the vessel wall.

1.2.1 Structure of the vessel wall and cellular basis of vascular damage

Similarly to other organs in the cardiovascular system, three main layers of tissue make up the wall of a vessel: an outer protective layer made of stromal tissue, a middle muscular layer that controls the tonus, and an innermost layer that consists of a single cell alignment directly in contact with the blood.

The outer layer (Figure 1) is the adventitia or *tunica adventitia*. It contains a mix of fibroblasts and smooth muscle cells (SMC) combined with an extracellular matrix rich in collagen (37). Small blood vessels that ensure oxygen-rich blood supply to the vessel wall can also be found in the adventitia of large vessels such as aorta and vena cava (38). Because of this structure, the adventitia plays a critical role in the maintenance of vessel integrity. In addition to the presence of connective tissue and differentiated SMC and fibroblasts, adventitia is also enriched in progenitor cells. As these cells can differentiate and give rise to cellular types that populate the other layers of the wall, their contribution in vascular damage is increasingly being considered (39-41) .

The medial layer (Figure 1) is the media or *tunica media* and essentially consists of a big population of vascular smooth muscle cells (VSMC) surrounded by elastin fibers organized into sheets that are intercalated by collagen fibers and proteoglycan. Contraction or relaxation of medial VSMC underlie the myogenic response to haemodynamic forces, this response is essential for the maintenance of a constant blood flow. In the capillaries, this role is played by the pericytes which replace VSMC in the wall of these small-diameter vessels and exhibit similar properties as well (42). In addition, based on specific cues, VSMC functional features can change from a contractile profile to a synthetic profile that allows them, among other actions, to synthesize and secrete the components of the extracellular matrix. This property is critical for vascular adaptation to external cues suggesting that VSMC functional integrity is a key determinant of vascular homeostasis (43). Similar to the progenitor cells found in the adventitia, multipotent stem cells are also

present in the medial layer and are able to differentiate into chondrogenic and SMC upon vascular injury (44).

The innermost layer (Figure 1), the *tunica intima* or intima, is bordered at the luminal side by endothelial cells directly in contact with the blood whereas at the peripheral side, a matrix of connective tissue combined with a lining of elastic fibres demarcates the intima from the media (37). Although the intima does not mechanically participate in the control of vessel conductance, endothelial cells are characterized by their secretory properties that enable them to recruit VSMC, immune and inflammatory cells during processes underlying vascular injury and neointima formation.

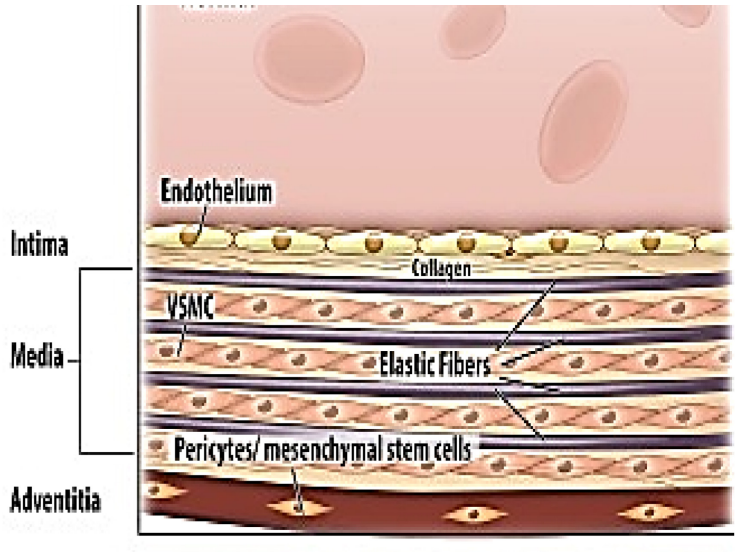

Figure 1: Cross sectional representation of the wall of an artery

(Modified from original figure in (45)).

Aberrant modifications in the physiology of vascular cells underlie persistent vessel remodeling which, along with prolonged vasoconstriction, represents the major contributor to the sustained vascular resistance associated with chronic hypertension (45, 46). Underlying processes are multicellular and converge towards changes in four main physiological responses within the vessel wall: cell growth in size or number, cell cycle

regulation (death or survival), cell motililty (migration, adhesion), and extracellular matrix turnover (secretion and degradation) (47). Endothelial dysfunction has been suggested to be the initial step that prompts susbsequent events in the progression of vascular dysfunction (48). In fact, the adluminal position of endothelial cells allows a direct contact with the bloodstream. This supports the idea that, hypertension and the other determinants of CVD such as hyperglycemia and dyslipidemia, by directly affecting endothelial cell physiology, are able to cause vascular damage (49). Deleterious consequences of chronic metabolic disturbances also involve aberrant expansion of inflammatory cells via hyperplasic and migratory responses. These inflammatory events participate in vascular lesion formation, an ultimate event in the atherogenic process (37). Because of the importance of the role played by inflammatory cells and determinants of innate immunity in vascular disease, it is also being considered as an immune disorder. This is supported by studies where animals bearing a deficiency in T or B lymphocytes (50), monocytes or macrophage (51), failed to develop vascular injury in disease promoting conditions. Finally, it is well documented that the pathophysiological responses exhibited by vascular smooth muscle cells (VSMC) underlie the structural changes observed in the vessel wall and further connect inflammatory responses and endothelial dysfunction to the development of vascular damage (52). Thus, together, individual properties beared by cells within the vasculature contribute to the pathogenesis of vascular damage. Accordingly, many vascular cell types in culture have been considered as in vitro models for the study of vascular disease. In the context of this thesis however, because of the central contribution of VSMC to vessel stiffness and reactivity, further description of the molecular basis of vessel remodeling will be focused on the signal transduction that control their physiology.

1.2.2 Pathophysiology of vessel remodeling

1.2.2.1 Determinants of vessel remodeling

The vasculature is sensitive to shear stress, the mechanical friction exerted by blood flow which results into an adaptative distension of the vessel (53). Additionally, it is

sensitive to blood internal pulsatility driven by blood pressure and which, despite being the key feature that ensures the continuity of the flow, also represents a stretch-promoting stimuli on the artery (53). Changes in blood volume or pressure are observed in some physiological states like in pregnancy, in response to transient vascular injury, or during transient metabolic disturbances accompanied with temporary changes in cardiac output or vascular resistance. Under such conditions, vascular homeostasis is maintained via either vasoconstrictive or vasodilatory responses resulting from stimulation by circulating and locally produced vasoactive peptides. This myogenic response is essential to accommodate transient flow-related disturbances while maintaining a stable vascular conductance (54). It requires a healthy functional cooperation between the cellular entities within the three layers of the vessel wall and the extracellular matrix. However, under circumstances of prolonged systemic hypertension or chronic metabolic disturbances as observed in obesity or type II diabetes, prolonged stimulation of vascular cells not only induces changes in the vasomotor tone, but also growth-promoting cascades. This is due to the mitogenic properties beared by vasoactive peptides that are able to trigger vascular hypertrophy, hyperplasia, as well as extracellular matrix formation. These events leading to structural modifications inside the wall define the process of vessel remodeling that is mostly reflected as aberrant thickening of the vessel wall and narrowing of the lumen. Increase in vascular resistance follows and mainly manifests as a decreased vessel capacity to appropriately adapt its size in response to environmental cues (46).

1.2.2.2 Types of vessel remodeling

Vessel remodeling is qualified as inward or outward based on whether the lumen diameter of the remodeled artery is smaller or bigger compared to the initial state (Figure 2). Due to prolonged wall tension and vasoconstriction, inward remodeling is very common in peripheral circulation during hypertension and contributes to the deleterious increase in peripheral vascular resistance (55). Outward remodeling usually accompanies the progression of atherosclerotic disease defined as the progressive accumulation of lipid and inflammatory cells toward plaque formation within the vessel lumen. In this situation,

the reduction in the lumen space due to atherogenic lesion is balanced by the outward remodeling resulting in a lumen dilation that serves as a counter mechanism to maintain a suitable blood flow (45). In addition to the size, the amount of material inside the vessel wall of the newly remodeled vessel with regard to the original state is also a parameter used to make a distinction between vessel remodeling events (Figure 2). Noteworthy, this criterion is closely linked to changes in mechanisms governing growth and survival of medial VSMC as well as extracellular matrix turn over. In this view, hypertension-induced inward remodeling can be eutrophic with no changes in the vessel wall mass. It can also be hypertrophic with an increase in the amount of cellular and non-cellular material in the vessel wall leading to increased stiffness. Neointima formation caused by aberrant hyperplasia and migration of VSMC toward the intima is the underlying mechanism of hypertrophic inward remodeling (56). It is moslty observed in hypertensive vascular stenosis or in restenosis following vascular interventions (57). In contrast, a decreased wall thickness due to matrix proteolysis and loss of medial cellular content defines outward hypotrophic remodeling. This process is at the basis of local aneurysm formation. Because of elevated mechanical forces exerted by high blood pressure, hypertension can cause aneurysm formation and rupture leading to deleterious consequences depending on the location of the organ (58).

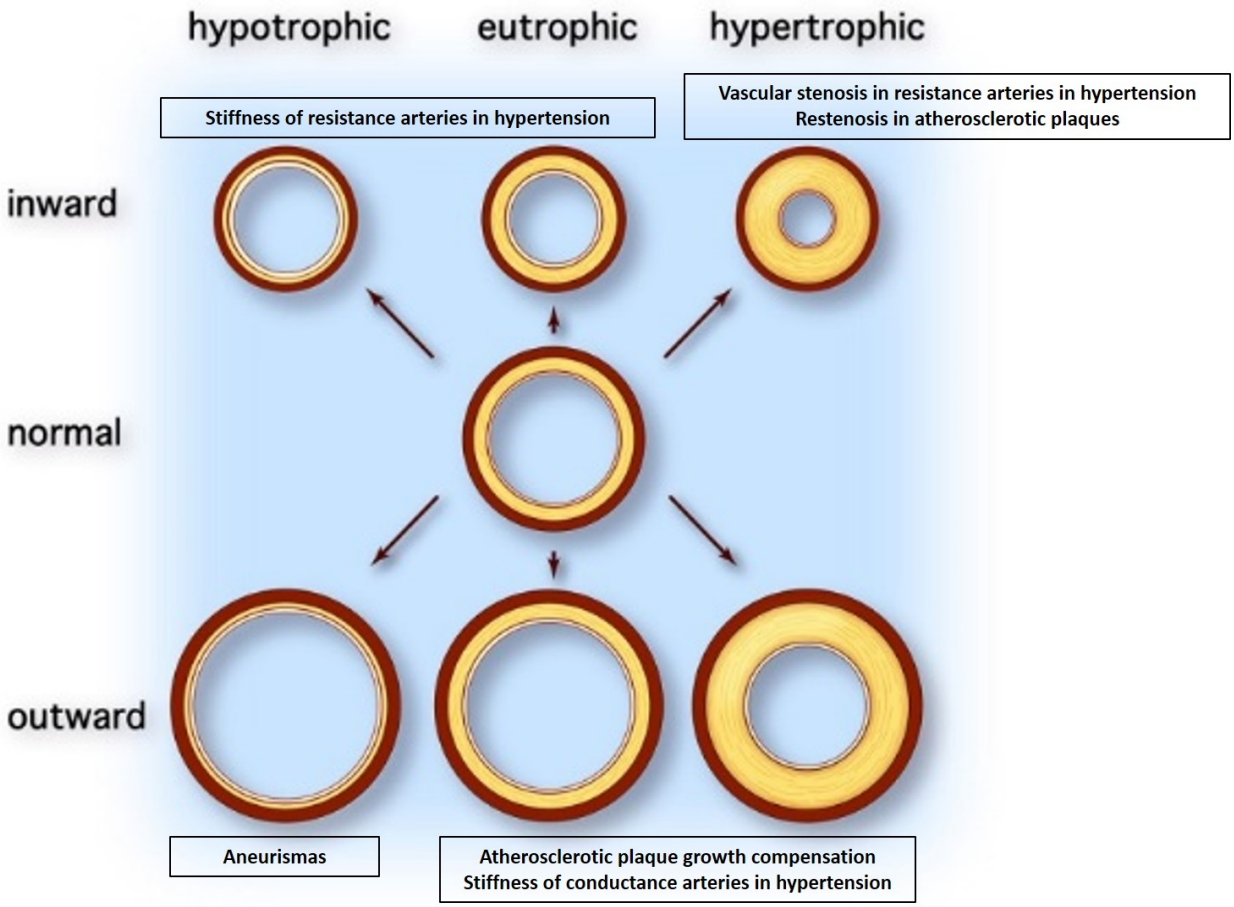

Figure 2: Types of vessel remodeling and associated pathologies

How the structural changes affect the wall thickness or the lumen diameter depends on the function of the affected vessel and the underlying pathology. Large conductance arteries like aorta are prone to outward eutrophic or hypertrophic remodeling under circumstances of constantly elevated blood flow whereas small resistance arteries at the periphery are prone to inward remodeling. Atherosclerosis is accompanied by outward eutrophic remodeling later on followed by hypertrophic remodeling leading to vessel stenosis (Adapted from (55) and (45)).

1.2.2.3 Clinical manifestations of vessel remodeling

Vessel remodeling is a hallmark of two major damages to the vascular system, arteriosclerosis and atherosclerosis. Arteriosclerosis affects the vasculature in a generalized fashion and is mainly associated with hypertension and/or other related determinants of CVD such as aging, obesity, diabetes, and inflammatory disorders. The main characteristic of atherosclerosis is that vessel remodeling accompanies the local formation of a lipid plaque leading to progressive vessel narrowing and eventual thrombus-mediated occlusion (37). Several hypertensive vascular diseases are defined based on the function of the damaged artery.

In coronary circulation: vessel remodeling within large coronary arteries alters the blood flow to the heart and clinically manifests as *coronary heart disease*. Angina and heart attack may occur under this condition where vessel narrowing or eventual occlusion leads to cardiac ischemia (59, 60). In some other cases of coronary disease, structural impairments in the wall of coronary ramifications can result in aberrant coronary vasomotor tone underlying ischemic symptoms (61). This other condition is known as *microvascular coronary dysfunction.*

In cerebral circulation**:** arteriosclerosis within the cerebral circulation mainly targets carotid arteries responsible for blood delivery to the brain through the neck. In this case, aberrant vessel remodeling manifests as *cerebrovascular disease* or *carotid artery disease (CrAD)*(62). Concomitant with atherosclerotic plaque formation, CrAD can cause disruption of blood flow to the brain causing stroke.

In peripheral circulation: *Peripheral vascular disease (PVD)* is the condition of remodeling within the wall of arteries that supply blood to legs, arms, pelvis, kidneys, and lungs. *Pulmonary artery disease (PAD)* and *chronic kidney disease* are types of (PVD) that are caused by similar risk factors and are associated with hypertension and chronic metabolic disorders.

Another major clinical manifestation of aberrant vessel remodeling is restenosis following intravascular angioplasty. This is an intervention that consists of artificial distension of an arteriosclerotic artery using a stent inserted within the narrowed region. This approach has shown positive outcomes in the treatment of coronary heart disease (57). Following this surgery however, vessel re-narrowing usually occurs as a result of friction-induced inward wall restructuring in the surroundings of the stent. In such circumstances, exaggerated growth-promoting processes in VSMC underlie the formation of a neointima that elevates the risk of in-stent thrombotic complications (57) (Figure 3).

Figure 3: Inward eutrophic remodeling in restenosis

Schematic representation of aberrant VSMC hyperplasia during in-stent neointima formation. (Image from (57)).

1.2.2.4 Features of vascular smooth muscle cell physiology

Based on their capacity to contract and relax, the main function of VSMC within the vessel wall is to enable accurate vasomotor responses to haemodynamic stimuli. Maintenance of a contractile profile is essential for VSMC to fit this purpose. Determinants of VSMC profile have been the centre of intensive investigations since it is well demonstrated that depending on the nature of the vessel or even in the same vessel, VSMC are present in a diversity of profiles described as specific phenotypes (reviewed in (63)). This multiplicity can be explained by two factors: the diversity of embryonic sources of VSMC precursors (64, 65) and their capacity to adapt their physiological properties in response to specific conditions (56). This capacity designated as VSMC phenotypic

plasticity underlies the occurrence of VSMC in a spectrum of phenotypes that range from a normal quiescent contractile profile to a disease-prone synthetic profile. Proliferative, migratory, secretory or osteogenic phenotypes are intermediary profiles whose occurrence depends on the physiopathological condition. Phenotypic plasticity of VSMC represents a vital attribute for the vascular system as it enables the adaptative remodeling of the vessels in conditions of temporary modifications of vascular needs as observed during pregnancy where remarkable outward remodeling take place in the uteroplacental circulation (66). Also, adaptative remodeling occurs during vascular repair following injury, as well as in response to increased needs due to exercise training (reviewed in (67)).

Due to this phenotypic plasticity however, arterial wall exposed to hypertension and associated endo/paracrine stimulation is enriched with synthetic VSMC which, in contrast to contractile VSMC, exhibit exaggerated proliferative rate as well as increased migratory and secretory capacities (56). This is well demonstrated by early studies that showed that VSMC isolated from aorta of adult spontaneously hypertensive rat (SHR), which is a well-established rat model for essential hypertension (68), proliferate significantly more rapidly than those from normotensive Wistar Kyoto (WKY) rats (69). In several experimental models of vascular disease, phenotypic switch of VSMC toward a hyperproliferative profile forms the basis of neointima formation (43). In addition to exacerbated growth and motility responses, alterations in VSMC survival cycles also represent hallmarks of synthetic phenotype and result from major perturbations in intracellular signal transduction cascades triggered in a large part by vasoactive peptides (52). Several vasoactive peptides including angiotensin II (Ang-II) are well known inducers of VSMC hypertrophy and proliferation. In hypertensive conditions, Ang-II is present at heightened plasmatic levels and its concentrations near the cells could be much more elevated due tissular production (70). This elevation correlates with an augmentation in the level of molecular markers of remodeling and proliferation (68). Based on data showing that in addition to modulating vessel tone, Ang-II also exhibits growth promoting properties (71-73), its contribution to the pathophysiology of vessel remodeling has been widely explored making this peptide a relevant tool in investigating the intracellular

mechanisms involved in aberrant VSMC physiology. Noteworthy, Ang-II is the leading endocrine factor of the renin angiotensin aldosterone system (RAS) and its action on the vessel tone is an ultimate response in the regulation of cardiovascular homeostasis jointly controlled by the RAS and the sympathetic nervous system. Before an in-depth description of the molecular cascades induced by Ang-II in the control of VSMC physiology, here follows an overview of the components of the RAS.

1.2.3 Angiotensin-II in the renin angiotensin aldosterone system

The RAS represents the main system involved in sodium and body fluid regulation and thus, the principal regulator of blood pressure.

Renin is an aspartyl protease originally synthesized in an inactive form called pre-pro-renin. Conversions of pre-pro-renin into pro-renin and subsequent cleavage of pro-renin are the sequential steps resulting in the production of active renin. It has been demonstrated that pro-renin can be converted into renin through either enzymatic or receptor-mediated reactions respectively involving catalytic cleavage of the prosegment or pro-renin receptor-mediated conformational change (74).

Renin is released in response to three main physiological signals. First, a decrease in blood pressure within the kidney afferent arteriole is sensed by mechanoreceptors of juxtaglomerular pericytes lining the wall. Signal transduction from these cells leads to a paracrine stimulation of adjacent granular cells responsible for renin synthesis, storage and release. Secondly, a drop in blood pressure within the afferent arteriole induces a lowering of the glomerular filtration rate resulting in a drop in sodium concentration in the kidney distal convoluted tube. This is sensed by a third group of juxtaglomerular cells condensed as the macula densa. Upon sensibilization, these cells are also able to stimulate the granular cells to release renin. Thirdly, endocrine stimulation by cathecholamines released from the sympathetic nervous system is proposed to be the signal that triggers juxtaglomerular recruitment and subsequent increase in the number of renin-secreting cells arising from cellular differentiation resulting in an increase in renin production.

Although recent studies have reported alternative biological actions of pro-renin and renin, the principal role of renin is to convert the α-glycoprotein angiotensinogen into a decapeptide called angiotensin-1 (Ang-I) (Figure 4). Enzymatic cleavages of Ang-1 by two types of dipeptidyl carboxypeptidases named angiotensin-1 converting enzymes (ACE) give rise to the effectors of the RAS among which Ang-II (also called Ang-(1-8)) and Ang-(1-7) are the most studied and exert opposite activities (75, 76) (Figure 4). Additionally, ACE-independent reactions involving the participation of endopeptidases such as chymase, thimet oligopeptidase, endopeptidase, represent alternative pathways for the transformation of Ang-1 into specific angiotensins (77). Because angiotensinogen is constantly synthesized and since ACE synthesis by endothelial cells is ubiquitous, production of renin has been for a long time considered as the rate-limiting step that determines the function of the RAS. However, the discovery of an intracellular angiotensinogen-derived product named Ang-(1-12) has challenged this consideration since this 12 amino-acid product can be converted into Ang-1 following a renin-independent reaction (78). The strict endogenous occurrence of Ang-(1-12) and its capacity to give rise to angiotensins have generated a lot of questions regarding the enzymatic processes underlying its production from angiotensinogen and its conversion into Ang-1 (75, 79).

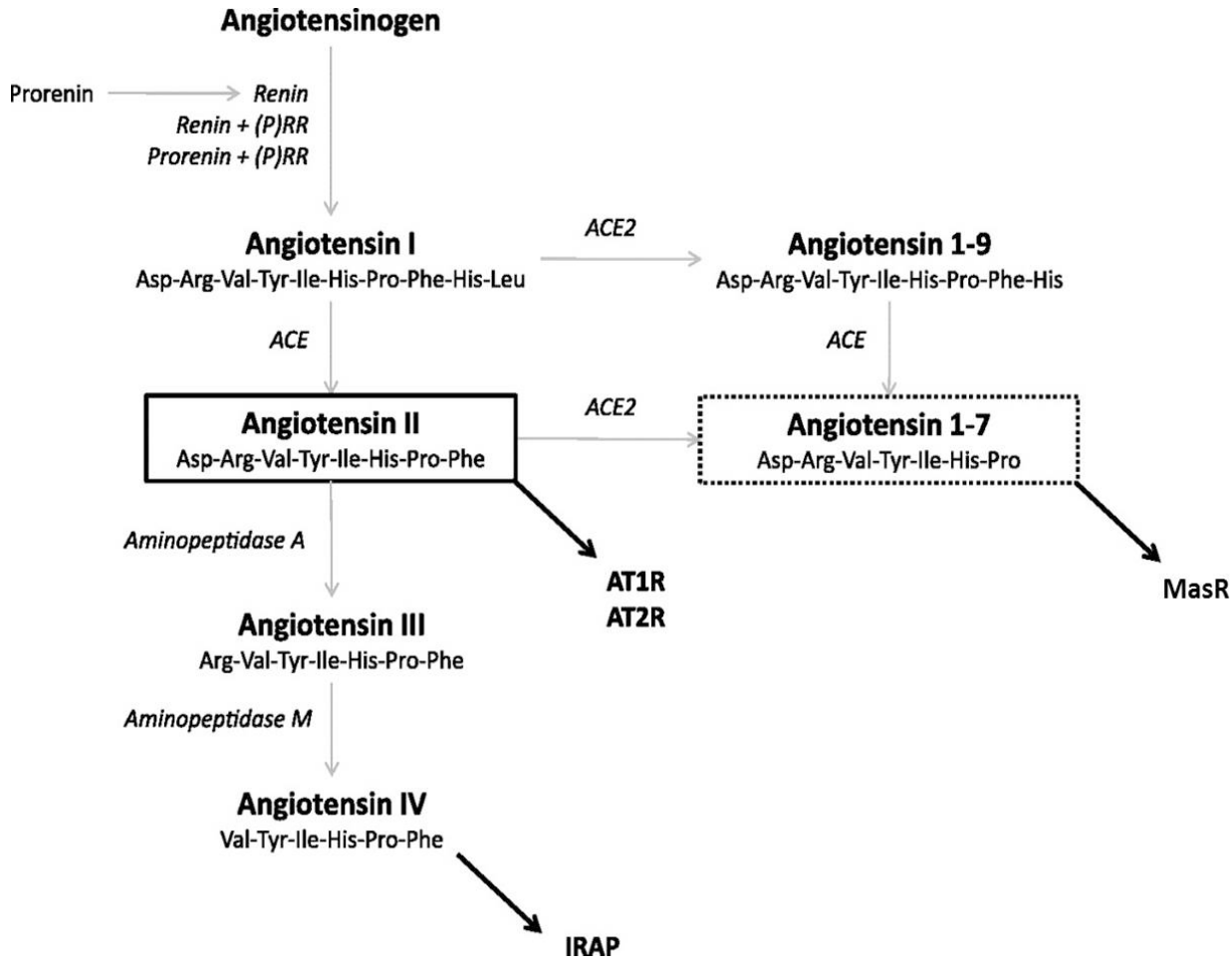

Figure 4: Components of the renin-angiotensin system (RAS)

The precursor peptide, angiotensinogen, is cleaved by renin to form the decapeptide angiotensin I. The catalytic activity of renin increases when bound to the (pro)renin receptor [(P)RR], and furthermore, the otherwise inactive prorenin can become catalytically active when bound to the (P)RR. The dipeptidase angiotensin-converting enzyme (ACE) cleaves angiotensin I to form the octapeptide angiotensin II (ANG II), the central active component of this system. ANG II can be catabolized by angiotensin-converting enzyme 2 (ACE2) into angiotensin-(1–7) [ANG-(1–7)], another active peptide of this system which typically opposes the actions of ANG II. ANG II can also be cleaved into smaller fragments, such as angiotensin III and angiotensin IV by aminopeptidases A and M, respectively. Most effects of ANG II are mediated by the angiotensin type 1 receptor (AT1R); however, ANG II can also bind to the angiotensin type 2 receptor (AT2R), which generally exhibits opposing effects to those at the AT1R. ANG-(1–7) acts via the Mas receptor and angiotensin IV can bind to the insulin-regulated aminopeptidase receptors (IRAP). (Figure and legend from (80)).

1.2.3.1 Structure of angiotensin-II and receptor signaling

Ang-II is an octapeptide with two free N-terminal and C-terminal groups that enable further cleavage to form other types of angiotensins named on the basis of the number of remaining amino acids (Figure 4). The most biologically relevant angiotensin that can be made from Ang-II is Ang-(1-7) which is obtained from the ACE type 2 (ACE2)-catalyzed removal of the carboxyterminal amino-acid. The fact that Ang-(1-7) shows opposite physiological effects suggests a self regulatory mechanism within the RAS. Additionnally, from Ang-(1-8), Ang-III (Ang-(2-8)) is formed by the removal of the N-terminal aspartate; the reaction is catalyzed by a type A aminopeptidase (APA). Ang-III exhibits a biological activity close to that of Ang-II in terms of vasoactivity and aldosterone stimulation. Ang-III can further be a substrate for the generation of Ang-IV (Ang-(3-8)) following another aminopeptidase-catalyzed reaction (Type N, APN). Ang-IV acts mostly in the central nervous system where it is implicated in the control of synaptic plasticity and long term potention at the basis of learning and memory formation.

The characteristic of tissues that respond to Ang-II is the presence, at their cell surface, of two types of receptors, Ang-II type 1 and type 2 receptors (AT1R and AT2R), both of which have been cloned and characterized. AT3R and AT4R subtypes have also been described, yet these subtypes have not been fully characterized and do not account for the main vasoactive effects of Ang-II. The AT1R is widely distributed throughout the cardiovascular system and is also abundantly found in the renal, endocrine and nervous systems in humans. In the vasculature, VSMC exhibit high levels of AT1R (81, 82), while a certain amount is also found in the endothelium (33) and in the adventitia (83). Most of the vascular effects of Ang-II are mediated by the AT1R (84, 85). In terms of vascular damage however, eventhough AT1R-mediated responses of Ang-II are well demonstrated by the vasculo-protective consequences of whole body loss of function of AT1R, a cellular specificity of AT1R-mediated response remains a subject of controversy. Whether Ang-II-induced vascular damage is mediated by the AT1R present in VSMC, endothelial cells or fibroblasts is still unclear (86-88).

AT2R is mainly expressed in fetal mesenchyme, uterine smooth muscle, brain, ovary, adrenal medulla and heart, and plays an important modulatory role during embryonic development. AT2R expression decreases rapidly, however, after birth (89). In adults, this receptor is expressed mainly in pancreas, heart, kidney, adrenal brain and vascular tissues. AT2R can also bind Ang-(1-7) and similar to MasR-mediated signaling, transduction through AT2R generally antagonizes several of the Ang-II-induced AT1R activated events.

1.2.3.2 Angiotensin-II-mediated biological responses in cardiovascular relevant tissues

As depicted in Figure 5, Ang-II regulates cardiovascular homeostasis mainly by acting on the vasculature, the heart, the kidneys and adrenals, as well as in the central nervous system.

1.2.3.2.1 In the vasculature

Data revealing the distinct expression of the precursors of the RAS including renin (90), angiotensinogen and ACE (91) in the vasculature, where both AT1R and AT2R are expressed, have provided evidence of a strong local activity of Ang-II (92). The role of Ang-II in the modulation of vessel tone and thereby in cardiovascular homeostasis seems to be very critical since the respective activation of these two receptors produces opposite consequences. Ang-II induces vasoconstriction through a direct AT1R-mediated contraction of VSMC. Eventhough AT2R are also stimulated by Ang-II and mediate vasodilatory actions, AT1R-mediated effect dominates resulting in vasoconstriction as the overall net response to Ang-II. However, vasorelaxation could happen as a net response to Ang-II following AT1R blockade as observed in studies where a chronic administration of losartan to SHR unmasked Ang-II-induced activation of AT2R (93). Ang-II can exert its vasoconctrictive actions via its ability to either induce the endothelial secretion of the pressor peptides endothelins (94, 95) or reduce the release of endothelial vasodilatory molecules, such as nitric oxide, in a ROS-dependent fashion (96, 97). Furthermore, data

demonstrating an inhibition of the sympathetic activity following the use of AT1R have supported the notion that an additional mode of action of Ang-II in the modulation of vascular tone is via the adrenergic function (98). Ang-II stimulates vasoconstriction by improving VSMC sensitivity to norepinephrine whose release from vascular nerve endings is also facilitated by Ang-II (99). AT1R has indeed been shown to mediate norepinephrine release in mesenteric arteries from rat (100), rabbit (101), and guinea pig (102), as well as in thoracic aorta (103). Further vascular effects of Ang-II in cellular growth and fibrotic responses are mostly relevant to vessel remodeling and reviewed in details through section 1.2.3.3 of this thesis. Overall, prolonged Ang-II-induced contraction in the resistance vessels promotes the increase in systemic blood pressure (Figure 5).

1.2.3.2.2 In the cardiac tissue

Due to AT1R internalization by cardiac cells and the presence of a local RAS in the heart, cardiac Ang-II levels are found to be almost five times higher than plasmatic levels (104). Early studies reported that in SHR, left ventricular hypertrophy is accompanied by elevated levels of AT1R protein expression in cardiac tissue suggesting an increase in Ang-II-induced signaling responses in the pathogenesis of heart failure (105). Indeed, aberrant growth (106) and apoptotic (107) features observed in cardiomyocytes exposed to Ang-II indicate its implication in cardiac fibrosis and aberrant cardiac tissue remodeling in a failing heart. Additionally, Ang-II modulates the cellular events linked with calcium handling and excitation-contraction coupling in the heart (108). Heightened levels of Ang-II cause alterations in the function of intracellular calcium pumps and impairs diastolic relaxation (109). Ang-II exerts an influence on the conduction of action potential. This is underlied by the ability of Ang-II to alter junctional communication between cardiac cells (110, 111). In accordance with this, aberrant AT1R activation has been shown to cause arrhythmias (112) while ACE inhibition was earlier reported to ameliorate the electrophysiological responses in a failing heart (113).

1.2.3.2.3 In the central nervous system

In response to Ang-II, the release of vasopressin from the supraoptic nucleus is among the indirect actions that contribute to Ang-II-induced systemic vasoconstriction (114). Despite its inability to cross the blood brain barrier, circulating Ang-II is able to induce changes in brain function by acting on the circumventricular organs to regulate drinking behavior and sympathetic activity (115). In addition, similar to the heart and vascular tissues, the brain exhibits a local RAS and increasing body of evidence has revealed local Ang-II-induced pleiotropic actions in the brain. Ang-II is implicated in the brain response to stress where it regulates the release of the corticotrophin-releasing factor for cortisol production (116, 117) and the central sympathetic outflow towards the production of norepinephrine (118). A decrease in norepinephrine production following local brain AT1R blockade was associated with an attenuation of blood pressure and cardiac remodeling in SHR (119). Ang-II is additionally involved in the baroreflex deregulation (120), in brain response to inflammation (121) as well as in the control of cerebrovascular flow (122).

1.2.3.2.4 In the kidney and adrenals

Upon stimulation of the kidney AT1R, a contribution of intrarenal vasoconstriction has been demonstrated to account for a considerable proportion of the systemic increase in blood pressure (123). Additionally, actions of Ang-II on the kidney are critical for the maintenance of body fluids and electrolyte balance. Ang-II-induced contraction of the afferent arterioles initializes a sequence of events that result in an increase in tubular sodium and water reabsorption as well as a decrease in urinary volume, both phenomenons leading to a rise in blood pressure (124). While AT2R-mediated signaling has been suggested to reduce the synthesis of renin (125), Ang-II-AT1R-mediated signaling potentiates the release of kidney renin as well as it stimulates cortisol and aldosterone release from the adrenals (Reviewed by (126)).

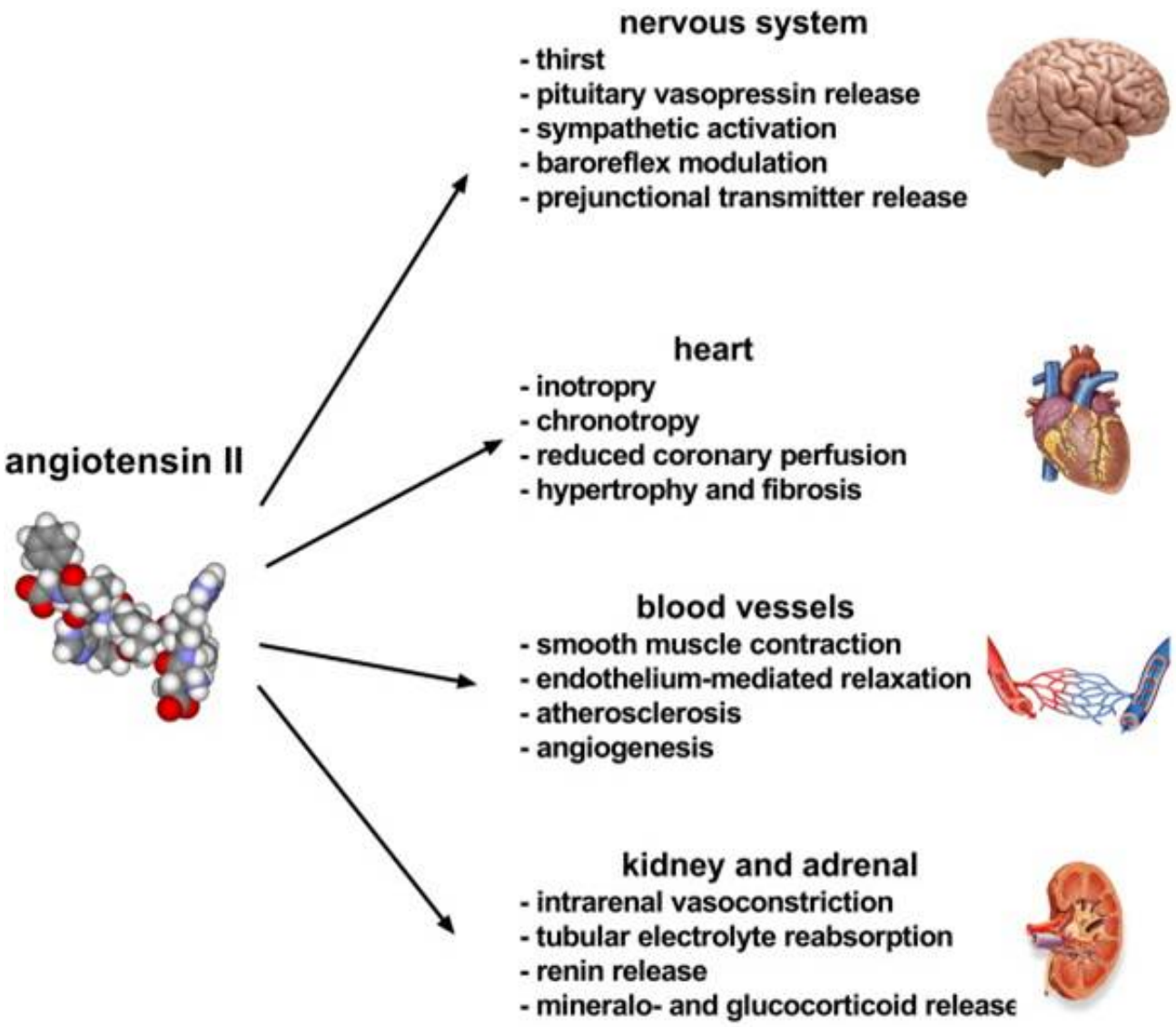

Figure 5: Biological actions of Ang-II in the control of cardiovascular homeostasis

(Figure from (127))

1.2.3.3 Angiotensin-II in vessel remodeling

Current therapeutical approaches involving the routine administration of anti RAS reagents along with other classes of drugs have shown positive outcomes in the regression of vessel remodeling in hypertensive patients (36, 128). The role of Ang-II in inflammatory responses, cellular hyperplasia, hypertrophy and adhesion, as well as in collagen and matrix deposition, has been extensively reported (129) indicating that Ang-II is an important molecular determinant for the vessel wall rearrangements observed under chronic disturbances. Specific regulatory actions of Ang-II in this context have been documented in reports from studies that used strategies aiming at RAS inhibition, targeting in particular the Ang-I/Ang-II/AT1R-mediated axis, in various models of vascular diseases. Since vascular remodeling manifests differently depending on the underlying pathology, these regulatory actions are discussed below distinctly based on whether the prevailing condition is atherosclerosis, hypertension, or neointimal thickening.

Administration of RAS antagonists to patients suffering from CAD reduces aberrant cellular adhesion and vessel stiffness as well as ischemic symptoms providing evidence of the pro-atherogenic action of Ang-II in these patients (130, 131). In animal models of atherosclerosis and hypertension, pharmacological blockade or depletion of AT1R inhibits the processes leading to increased vessel thickness, lesion progression and plaque rupture. In apolipoprotein-E knockout ($ApoE^{-/-}$) mice for example, loss of function of AT1R attenuates the signaling pathways underlying lipid accumulation, macrophage infiltration or low-density lipoprotein oxidization (132, 133). In the same model, concomitant administration of Ang-II with valsartan reduced the risk of thrombosis by attenuating the levels of the extracellular matrix metalloproteinase inducer, a protein that promote plaque rupture (133). These suggest a prominent role played by Ang-II in facilitating atherosclerotic plaque formation rupture. Indeed, a recent report assimilated the beneficial atheroprotective effects of AT1R antagonism to those of exercise training (134).

In arsenic-induced hypertensive rats, pharmacological blockade of AT1R blunted mitogenic signaling and thereby restored vessel wall integrity as exhibited by a regression

of intimal hyperplasia (135). Ang-II/AT1R-mediated signaling responses participate in the events linked with the incidence of restenosis following angioplasty. Indeed, in human, non-human primates and rodents that have undergone intracoronary stenting, AT1R blockade was able to prevent neointima formation via mechanisms involving attenuation of inflammatory and oxidative signaling (136, 137). Under conditions of vascular injury, Ang-II via AT1R stimulates the mechanisms at the basis of vessel wall restructuring such as collagen and elastin deposition together with neointima formation (138). By stimulating aberrant growth in VSMC, signaling pathways induced by Ang-II have been the center of several investigations in experimental models of neointima formation.

1.2.4 Molecular basis of vessel remodeling: cascades related to angiotensin-II-induced signaling in vascular smooth muscle cells

1.2.4.1 Angiotensin-II receptor signaling

As previously mentioned, Ang-II receptors are members of a large family of the seven transmembrane domains G-protein coupled receptors (GPCR). Classical G proteins possess three subunits α, β, and γ that form together an inactive complex maintained by the presence of a guanosine diphosphate (GDP) group bound to the α subunit (139). Upon binding of Ang-II to its receptor, a ligand-induced conformational change occurs and enables the transformation of GDP into GTP resulting in the release of α subunit (Gα) from the βγ complex (Gβγ). The GTP-bound α subunit (GTP-Gα) initializes the intracellular signal transduction by phosphorylating downstream enzymes or other effectors. This signal transduction is arrested by upstream mechanisms involving the phosphorylation of Ang-II receptor by particular proteins called G-protein receptor kinases (GRK) and the subsequent recruitment of β-arrestins to process the endocytosis of the phosphorylated receptor (140). In addition, AT1R is subject to endogenous regulation by a specific 18kDa protein called AT1R-associated protein (ATRAP) that colocalizes with the receptor in VSMC and has been shown to attenuate vascular remodeling by negatively regulating Ang-II-induced

VSMC proliferation, senescence and gene expression (141-143). The mode of action of ATRAP is based on a spontaneous binding with the intracytoplasmic portion of AT1R leading to its internalization and a subsequent decrease in the signal transduction (142, 144).

Depending on the nature of the ligand, the effector and the second messenger required for the signal transduction upon formation of GTP-Gα, G proteins are divided into four groups: Gi, Gs, Gq/11, and G12/13 (139). AT1R is coupled to Gαq/11 activity which is involved in the regulation of vascular tone and has been widely demonstrated to play a role in VSMC differentiation and vessel remodeling (145, 146). Activation of Gαq/11 prompts phospholipase activation and involves intracellular calcium as a second messenger. Gs/Gi proteins modulate the activity of adenylate cyclase (AC) and thereby the production of another second messenger, the cyclic-adenosine monophosphate (cAMP). Increased levels of cAMP as observed upon activation of Gαs protein and subsequent simulation of adenylate cyclase activity have been associated with a contractile phenotype in VSMC (147). In contrast to AT1R, AT2R activation was reported to be coupled with Gαi activity which exerts an inhibitory action on adenylate cyclase activity leading to a negative regulation of the formation of cAMP (148) (Figure 6). Similarly, the effects of the vasoactive peptide apelin-13, known to induce aberrant migration of VSMC, were recently reported to be mediated by Gi protein activation (149). Evidence additionally demonstrates that in hypertension, heightened activity of Ang-II induces an enhanced expression of Gi (150) suggesting an involvement of AC-dependent cAMP fluctuations in hypertensive vessel remodelling.

1.2.4.2 Adenylate cyclase and cyclic AMP-dependent pathway

AC is an effector for Gi and Gs proteins that respectively exert inhibitory and stimulatory actions. Therefore an increase in Gi protein expression as observed under conditions of exaggerated activity of Ang-II results in the attenuation of AC activity in VSMC. The principal role of AC is to convert molecules of intracellular adenosine triphosphate (ATP) into cAMP whose signal is transduced by downstream effector proteins

including protein kinase A (PKA) and the exchange protein-activated by cAMP (EPAC)). PKA is a tetrameric protein made of catalytic (PKAcat) and regulatory (PKAR) subunits each consisting of two α and β subunits. Although the PKAcat is constant, PKAI and PKAII isozymes were identified based on the differences between their regulatory subunits. Inactive PKA is characterized by the binding of all four subunits together into a stable holomere (151). This binding is made possible by the PKAcat-induced phosphorylation of the PKAR on serine 96 (151). Upon binding of cAMP to the four cAMP binding sites found on PKAR, this phosphorylation is inhibited enabling the detachment of the PKAcat from the holoenzyme (152-154). Released PKAcat is active and phosphorylates downstream kinases. PKA-dependent signaling attenuates VSMC proliferative responses and induces VSMC relaxation and vasodilation (155, 156). Together with cAMP, PKA is also able to stimulate the activity of intracellular phosphodiesterases leading to signal termination (157). PKA becomes inactive following degradation of cAMP that increases PKAR affinity for PKAcat, enhances serine 96 phosphorylation and subsequent holoenzyme formation (151, 154). This represents a negative feedback loop for cAMP-mediated pathway. While early studies showed that Ang-II-activated PKA activity induces AT1R downregulation (158), reports have demonstrated that aberrant VSMC proliferation (159) and senescence (160) induced by Ang-II can be significantly reduced by the activation of cAMP-PKA signaling.

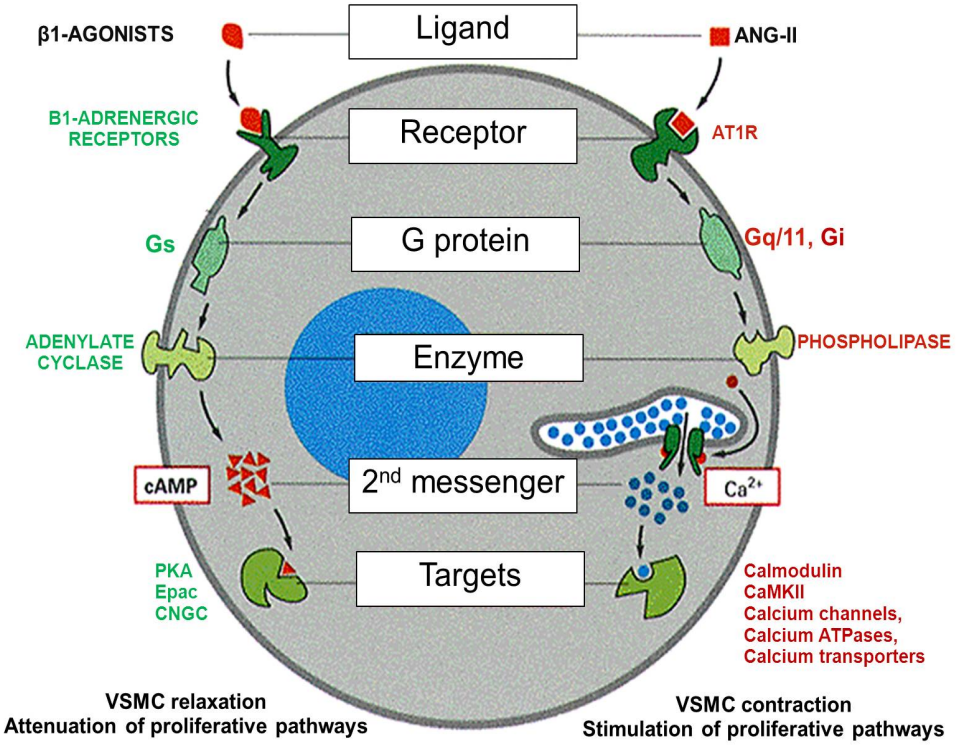

Figure 6: Schematic illustration of differential induction of G protein-mediated responses in VSMC

Angiotensin-II type 1 receptor (AT1R) and β-adrenergic receptor respectively activate Gαq11, Gαi and Gαs activities in VSMC. While Gi and Gs modulate the activity of adenylate cyclase and the production of the second messenger cyclic adenosine monophosphate (cAMP), Gq11 stimulates phospholipase activity toward the elevation of intracellular calcium levels. cAMP-dependent signalling involves the participation of protein kinase A (PKA), exchange protein activated by cAMP (Epac) and the cyclic nucleotide gated channels (CNGC). Calcium handling involves the participation of calmodulin, calcium/calmodulin- dependent protein kinase (CaMKII) calcium channels, transporters and pumps (ATPases). β-adrenergic receptor and AT1R exert opposite actions on VSMC physiology.

1.2.4.3 Phospholipase and calcium-dependent pathway

Several types of phospholipases have been identified and characterized depending on their site of action during phospholipids hydrolysis. Although activation of the types A, C and D (PLA, PLD, PLC) has been reported in VSMC stimulated with Ang-II (161), typical AT1R-GTP-Gα activation is coupled with the activation of PLC (PLC-β) that catalyzes the cleavage of membrane inositol phospholipid into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) (161). Both IP_3 and DAG-mediated activities lead to an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) by regulating two distinct pathways (162). DAG is additionally known to bind to and activate isoforms of protein kinase C (PKC) which plays a prominent role in transducing Ang-II-induced proliferative responses in VSMC (163). IP_3 activity mediates an increase in $[Ca^{2+}]_i$ via its binding to specific receptors (IP_3R) distributed inside the membranes of intracellular organelles that act as internal Ca^{2+} stores (164). These include the endoplasmic reticulum (ER) from where an efflux of Ca^{2+} is produced upon IP_3R activation. Early studies in VSMC reported a rapid increase in $[Ca^{2+}]_i$ in response to Ang-II (165). This increase in cytosolic $[Ca^{2+}]$ is handled by a wide family of proteins including calmodulin and calcium/calmodulin-dependent protein kinases (CaMK) involved in contractile and growth responses in VSMC (166). The role of CaMKII in VSMC signaling and physiology has been addressed and evidences demonstrate that, under conditions of vascular injury, CaMKII expression and activity are upregulated (reviewed in (166)). CaMKII depletion has further been demonstrated to protect against experimentally induced hypertension, vessel wall thickening, and hypertrophic and proliferative responses of VSMC in *in vivo* and *in vitro* models of disease (167, 168). Additionally, an IP_3R-mediated increase in cytosolic $[Ca^{2+}]$ triggers an influx of external Ca^{2+} named store-operated calcium entry (SOCE) (169). The SOCE results from the activation a group of store–operated calcium channels (SOCC), also known as Ca^{2+} release-activated Ca^{2+} channels (CRAC), defined as plasma membrane voltage-independent calcium channels whose gating depends on the amount of Ca^{2+} inside the intracellular stores (169-171). SOCE provides a sustained Ca^{2+} signal important to trigger several physiological responses in VSMC (172). In this regard, proliferation, migration and

transcriptional responses in VSMC have been shown to be controlled by SOCE (170, 173, 174) and an upregulation of the molecular components (see 1.2.4.3.1) of SOCE has been associated with SMC switch toward a synthetic profile (174, 175). The intracellular clearance of Ca^2 arising from SOCE is achieved by the activity of the ER pump sarco/endoplasmic Ca^{2+} ATPase (SERCA) or the efflux from the Ca^{2+} plasma membrane ATPase (PMCA) both expressed at high concentrations in hypertensive animals (176-178). SOCE thus participates in the maintenance of stable amounts of Ca^{2+} in the internal stores reinforcing its contribution in determining VSMC phenotype and in vessel remodelling (179, 180). Data on the function of SOCE in VSMC pathophysiology come from studies that addressed the molecular identities of SOCC and the mechanisms underlying their activation.

1.2.4.3.1 Molecular basis of SOCE

Two coordinated functions are required to mediate SOCE: calcium sensing and calcium conduction. Calcium sensing is achieved by the stromal interaction molecules (STIM) found inside the ER membrane (181, 182). Although two types of STIM (STIM-1 and STIM-2) exhibiting similar structures and functions have been identified, STIM-2 has been shown to be constitutively activated due to its higher Ca^{2+} affinity at ER resting states (183) whereas STIM-1 was shown to be essential to trigger SOCE in several types of cells (184-187). At the resting state, STIM-1 consists of a transmembrane protein with a luminal N-terminal portion that exhibits a Ca^{2+} binding domain with typical EF hands where binding prevents STIM-1 activation (Figure 7). In addition, sterile alpha motives (SAM) also found in the luminal portion enable spatial reorganization of STIM-1 monomeres into aggregates for further interactions with plasma membrane channels (188). The intraER domain of STIM-1 is followed by a single transmembrane portion terminated at the cytoplasmic side by another portion divided into several functional domains including a STIM-1/Orai1-activating region (SOAR) whose binding with Orai channels leads to their activation (184, 189, 190). The C-terminal portion completes this intra-cytoplasmic region and is exhibited as a lysine-rich domain that plays a role in cluster formation with Orai

channels. Calcium conduction is achieved with Orai channels found inside the plasma membrane. Although three types of Orai proteins are expressed in VSMC and induce similar effects when overexpressed (191), Orai-1 has been suggested to be the major endogenous type implicated in SOCE (192). Orai-1 is made of four transmembrane domains bound together as tetramers (193) or hexamers (194) with intracytoplasmic N and C termini (Figure 7). Orai function was partially uncovered during experiments where a single point mutation in Orai resulted in a suppression of current induced by SOCE (I_{CRAC}) in T-lymphocytes in immune deficiency patients (195). Interestingly, the requirement of STIM-1 for Orai function was clearly demonstrated in studies where overexpressing Orai-1 alone attenuated SOCE due to irregular stoichiometry between STIM-1 and Orai-1 (196). Additionally, reports have proposed the implication of a third group of transmembrane non-selective cation channels named transient receptor potential cation channels (TRPC). TRPC has been shown to play a role in Ang-II-induced VSMC hypertrophy (197). TRPC channels have long time been considered as receptor-operated calcium channels since they are activated by stimuli-induced synthesis of DAG as well as by membrane depolarization (105). However, increasing evidence demonstrates that in VSMC, TRPC coregulates SOCE by interacting with Orai-STIM complex (105). Nevertheless, recent studies addressing the role of the functional components of SOCE in VSMC physiology and vascular diseases have focused on the implication of newly discovered STIM-1 and Orai-1 (106-108).

The SOCE process starts with STIM-1 being activated by detachment of Ca^{2+} from the EF hand following IP_3R-mediated depletion of ER Ca^{2+} from its resting value of ~400 μM up to a threshold of 35% to 40% of reduction (198, 199). Luik and his collaborators have quantified this decrease and they showed that half-maximal activation of the I_{CRAC} occurs when the ER Ca^{2+} reaches ~200 μM (198). STIM-1 activation induces the release of lysine-rich tail that frees the CRAC-activation domain (CAD) and enables the assembling of several single STIM into multimers that facilitate their relocalization at specific junctions between the ER and the plasma membrane (198). At these sites, CADs interact with Orai channels to form aggregates that trigger the opening of Orai-1 and

subsequent Ca^{2+} influx (188, 200) (Figure 8). While SOCE mostly involves Orai-1 channels (195), Orai-3 has also been proposed to complex with Orai-1 to mediate a store-independent Ca^{2+} influx (193, 201).

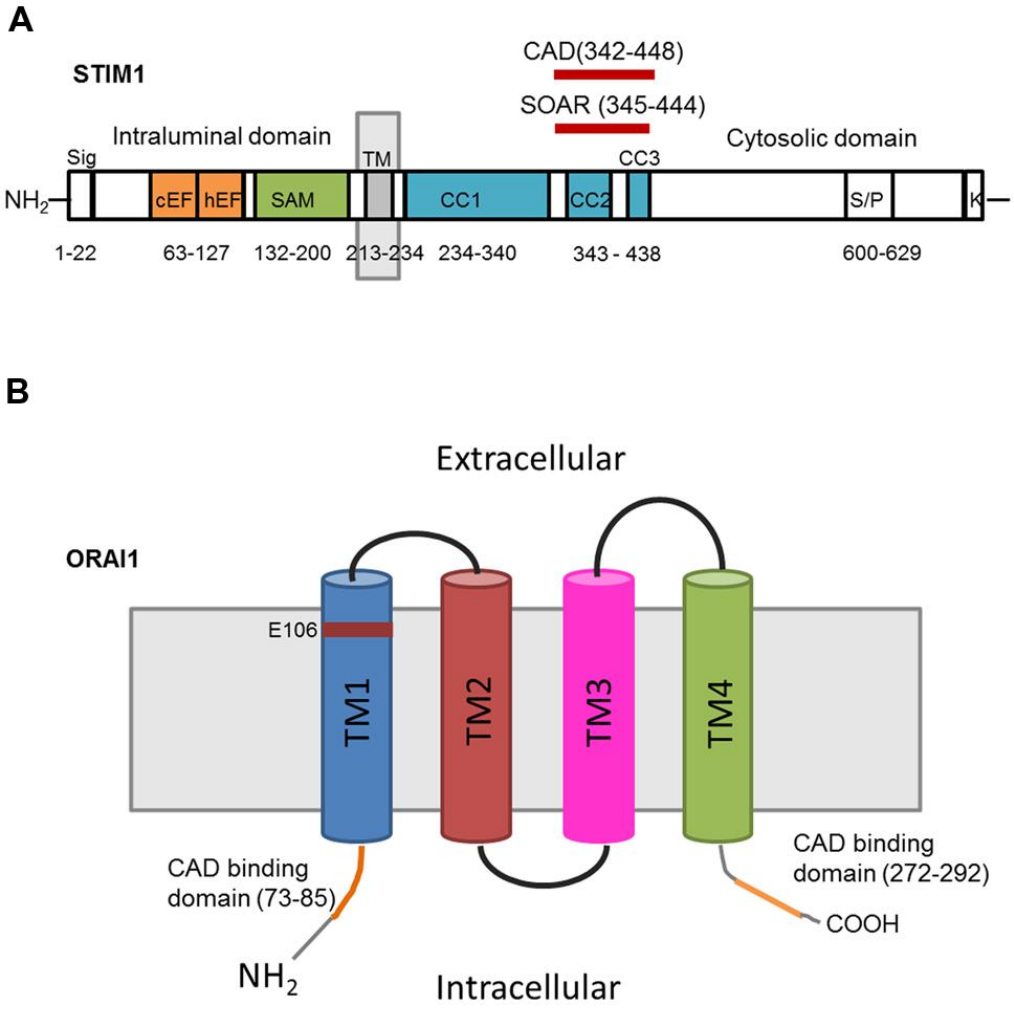

Figure 7: Topology and predicted domains of STIM-1 and Orai-1

(A) STIM-1 comprises a signal peptide (Sig), a canonical EF-hand (cEF) domain, a hidden EF (hEF) domain, a sterile alpha motif (SAM), a transmembrane domain (TM), three coiled-coil domains (CC1, CC2, CC3), CAD, SOAR, serine/proline-rich domain (S/P), and lysine-rich domain (K-rich). (B) Each Orai-1 monomer consists of four transmembrane domains (TM1TM4) and presents CAD binding domains in the cytosolic NH2 and COOH termini. E106 is the residue crucial for conferring Ca^{2+}-selectivity to the channel pore. (Figure and legend from (202))

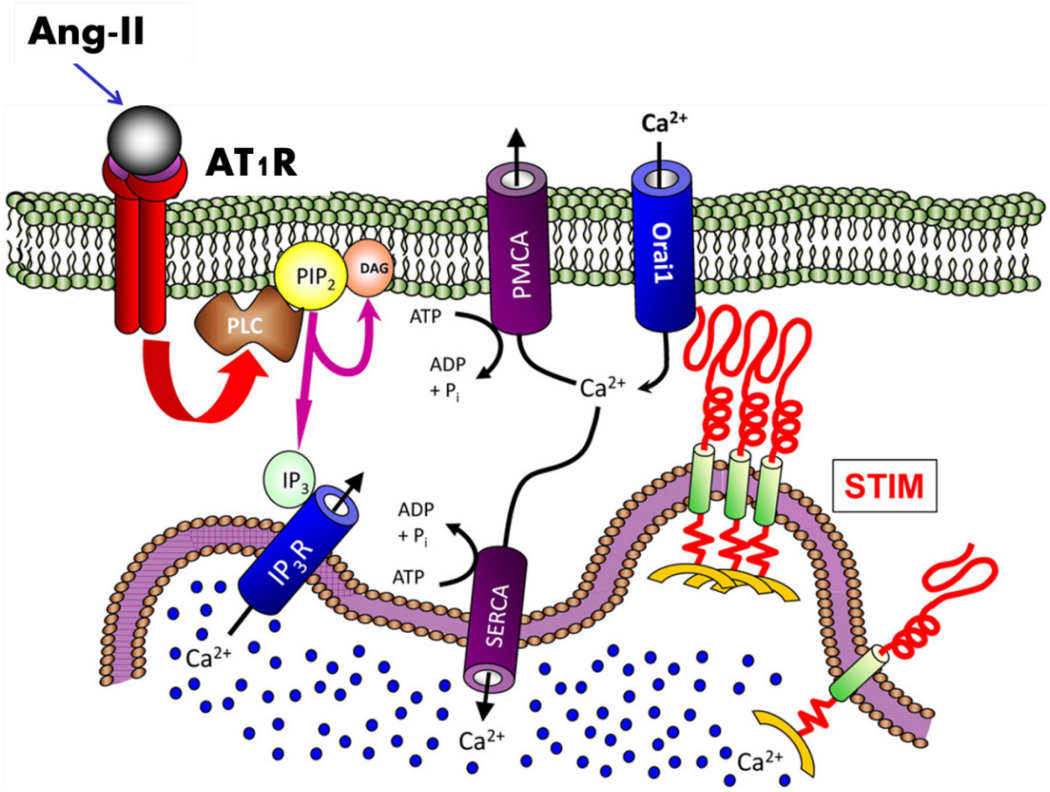

Figure 8: Model depicting Angiotensin-II-induced STIM-1/Orai-1-mediated Ca^{2+} entry and clearance by ATPases

Ang-II type ` receptor (AT1R)-activated phospholipase C (PLC) initiates a signaling cascade where in newly generated inositol 1,4,5-triphosphate (IP_3) binds to and activates its receptor (IP_3R) located on the endoplasmic reticulum (ER) membrane. Once activated, the IP_3R releases ER Ca^{2+}content into the cytoplasm. This resultant ER Ca^{2+}depletion is sensed by STIM proteins, which aggregate near the plasma membrane (PM) where it interacts with Orai1, causing store-operated Ca^{2+} entry. Following downstream activation, Ca^{2+}/ATPases located on both the ER (SERCA, sarco/endoplasmic calcium ATPase) and PM (PMCA, plasma membrane calcium ATPase) rapidly remove cytosolic Ca^{2+}, resulting in recovery of both ER and cytosolic Ca^{2+} concentration. (Figure and legend modified from (203)

1.2.4.4 The Mitogen-Activated Protein Kinase-dependent pathway

Downstream of PLC/Ca^{2+} signaling pathway, the activation of mitogen-activated protein kinases (MAPKs) is another key signaling event induced by Ang-II in VSMC. MAPKs are serine/threonine protein kinases, which are activated in response to a variety of external stimuli, vasoactive peptides, growth factors, hormones and stress. MAPKs have been classified into several subfamilies: ERK (extracellular signal-regulated kinases 1 and 2), p38mapk, JNK/SAPK (c-Jun NH2-terminal kinase/stress- activated protein kinase), ERK 3/4 and ERK 5. MAPKs are activated by dual phosphorylation on both tyrosine and threonine residues by dual specificity protein kinases known as MAPKK or MEK (mitogen extracellular signal-regulated kinase kinase). The sequential upstream signaling molecules to MEK are MEK kinases (MEKKs), a serine/threonine kinase, and p21ras. p21ras belongs to family of low molecular weight GTP binding proteins which cycles between an active GTP-bound conformation and an inactive GDP- bound form. Activation of p21ras and other low molecular weight GTP-binding protein such as Rho, leads to sequential activation of several serine/threonine protein kinases which are involved in the activation of MAP kinase families. MAPKs phosphorylate downstream cytosolic regulatory proteins, such as P90rsk, and many nuclear transcription factors, such as c-Jun, Elk-1, CREB, and MEF-2. P90rsk phosphorylates ribosomal proteins and participates in protein synthesis, whereas the phosphorylation of transcription factors by MAPKs leads to activation of several genes involved in growth and differentiation. Thus, activation of the MAPK pathway can potentially result in increased growth, hypertrophy, gene expression and proliferation of VSMC in response to vasoactive peptides. Aberrant activation of MAPK has been observed in several models of vascular diseases (84, 204-206). In fact, Ang-II and ET-1 have been shown to activate several members of the MAPK family in VSMC and other cell types. In addition, in SHR-derived VSMC, external stimuli, including Ang-II, were shown to cause a much higher activation of ERK-1/2 as compared to those from WKY (207-209). However, the regulatory mechanisms that modulate MAPK and the nature of downstream molecular events that contribute to the vessel remodeling in synthetic SHR VSMC are not fully uncovered.

1.2.4.5 The phosphatidylinositol 3-kinase/protein kinase B dependent pathway

Additionally, vascular functions are also reported to be modulated by the PI3K/PKB(Akt) signaling pathway. PI3K is a heterodimeric lipid kinase, composed of an 85-kDa (p85) regulatory subunit and a 110-kDa (p110) catalytic subunit. The p85 subunit contains the src homology-2 (SH-2) domain and interacts with phosphorylated tyrosine residues on receptor or other docking proteins, leading to stimulation of its 110-kDa catalytic subunit. P110 catalyzes the generation of PI-3, 4, 5-triphosphate (PIP_3). Binding of PIP_3 to its downstream substrate PKB (Akt) recruits it to the plasma membrane for phosphorylation by phosphoinositide- dependent kinases 1 (PDK-1) and mammalian target of rapamycin (mTOR) complex 2 previously known as PDK-2 (210) PDK-1 phosphorylates PKB at Thr308 in the catalytic domain while the putative PDK-2 phosphorylates it at Serine 473 (Ser473) in the C-terminal regulatory domain of PKB. Activated PKB phosphorylates several downstream substances, such as glycogen synthase kinase-3β (GSK-3β), Forkhead transcription factor (FKHR, also termed FOXO), Bcl-2/Bcl-X_L antagonist causing cell death (BAD), Caspase 9, mammalian target of rapamycin (mTOR), nuclear factor-κB (NF- κB) and endothelial nitric oxide synthase (eNOS). Phosphorylated forms of these substrates regulate diverse cellular functions, such as glucose transport, cell growth, gene expression, cell survival and death as well as protein synthesis. An involvement of PKB in vascular disease was suggested from studies in which an enhanced PKB activity was associated with Ang-II-induced hypertension in New Zealand White rats (211). Furthermore, a role of PKB in regulating growth factor-induced proliferation and hypertrophy of VSMCs has been reported (212-214) suggesting that together with MAPK, PKB (Akt)-mediated signaling is a key molecular events in the pathophysiology of vascular diseases (215).

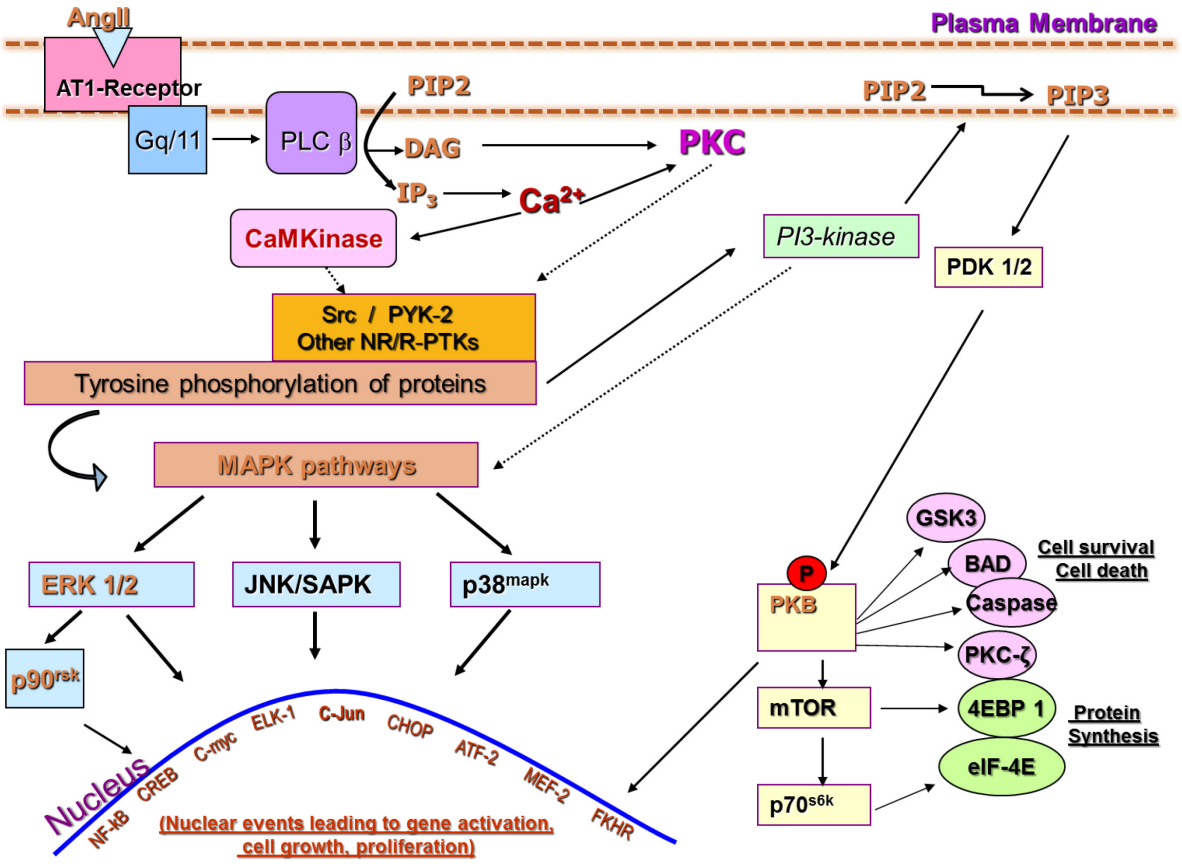

Figure 9: Ang-II-induced signalling

Ang-II type 1 receptor (AT1-Receptor)-mediated activation of phospholipase C (PLC)β converts PIP2 into IP_3 and diacylglycerol (DAG). IP_3 elevates the concentration of intracellular calcium and participates in muscle contraction. DAG activates PKC. PKC and/or Ca^{2+}/Calmodulin (CaM)-dependent protein kinase (CaMKinase) activate receptor and non-receptor tyrosine kinases such as Src and Pyk2. Activation of these components signals the stimulation of Ras/Raf/ MEK /ERK1/2, p38mapk and JNK. The MAPK family members are translocated to nucleus and regulate nuclear events by activating transcription factors through phosphorylation. It can also contribute to the activation of PI3-K. PI3-K is composed of a catalytic subunit, p110 and regulatory subunit, p101, and its activation leads to the production of PI(3,4,5)P3 from PI (4,5) which results in recruitment and activation of PKB. PKB has several effectors such as glycogen synthase kinase 3 (GSK-3), Bcl2 associated death promoter (BAD), caspases, mammalian target of rapamycin (mTOR), 70 KDa ribosomal protein S6 kinase (P70S6K) which regulate cell survival, protein synthesis and cell growth. mTOR phosphorylates eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) which, in basal state is complexed with eIF4E. This phosphorylation dissociates and liberates eIF4E which then binds to other translation initiation factors leading to protein synthesis.

1.3 THE EARLY GROWTH RESPONSE PROTEIN-1

As described above, Ca^{2+} mediates the activity of a range of transcription factors that translate the initial extracellular signals into specific physiological responses via the modulation of protein and gene expression (216). With regard to vessel remodeling in the pathogenesis of vascular disease, these responses may involve the expression of numerous pro-inflammatory mediators and various other molecular factors able to trigger or amplify growth promoting cascades toward intimal lesion, neointima formation, and progressive vessel narrowing. Thus, a further description of the signalling cascades involved in vascular pathogenesis requires the characterization of the molecular targets that act as key effectors for vasoactive stimuli-induced activity of second messengers like $[Ca^{2+}]_i$ in vascular tissues. In this regard, several reports have identified immediate early genes products including Nur77 (217), c-fos (217, 218), c-jun (217) and Egr-1 (219) as Ca^{2+}-dependent transcription factors that participate in the molecular events linked with vessel remodeling under circumstances of elevated activity of vasoactive agents. The orphan receptor Nur77 also named Nerve growth factor 1-B has recently been shown to be upregulated by Ang-II in VSMC where, by attenuating the migratory and proliferative responses, it reduces Ang-II-induced vascular remodeling (220). Among these early genes, much interest has been accorded to Egr-1 ever since it has been demonstrated to be involved in stimuli-induced growth responses in vascular physiology ((221-225) Reviewed in (166)). Indeed, Egr-1 is expressed in the vasculature where, although barely detectable in quiescent state, it was found to be rapidly and transiently induced by a multitude of stimuli including oxidative stress (226), vascular injury (227), growth factors and fatty acids (228).

1.3.1 Egr family

Egr-1 is a member of a family of four zinc finger transcription factors (Egr-1 to Egr-4) that share at least 84% homology. Transcriptional repression has been attributed to Egr-4 which exhibits different DNA–binding patterns (229). Even though a coexpression

of elements of the EGR family is frequently observed in tissues, distinct roles have been attributed to each member following studies using targeted gene deletion or loss of function. In general, EGR-1 knockout female mice exhibit infertility due to attenuated expression of luteinizing hormone (230), whereas EGR-4 deletion leads to male infertility as a result of heightened germ cell death and aberrant spermiogenesis (231). Mice subject to EGR-2 deletion exhibit impairment in central nervous system myelination due to lack of myelin-rich cholesterol formation (232) and EGR-3 knockout mice suffer from gait ataxia due to a failure in the development of muscle spindles (233). Notably, an upregulation of Egr-2 and Egr-4 has been observed in transgenic animal deficient in Egr-1 suggesting a coregulated expression of Egr family members (234).

1.3.2 Historical background and structural properties of Egr-1

EGR-1 was first designated in 1987 as a nerve growth factor inducible gene in a pheochromocytoma-derived cell line (PC-12) stimulated with the neuronal growth factor (235). The gene product was consequently named Nerve Growth Factor Inducible A (NGFI-A), a transcription factor that belongs to the family of the Cys2-His2-zinc-finger DNA-binding proteins. Northern analysis of NGF1-A cDNA probe revealed the rapid nature of EGR-1 induction (15 minutes) following treatment with NGF, phorbol myristate and the ionophore A23187. Egr-1 induction and function were studied in subsequent investigations using other cell types where it was distinctly designated as KROX-24 (236), zif268 (237), and TIS8 (238). The name EGR-1 was adopted by studies depicting the immediate and transient nature of its mitogen-dependent induction potentiated by protein synthesis inhibitors, as well as the short half-life of both Egr-1 mRNA and protein (239). However, detection of Egr-1 in different tissues like bones and cartilage in mouse fetus suggested that, in addition to function like an immediate early gene transducing external mitogenic signals into long term physiological responses, Egr-1 plays a role in the expression of tissue-specific genes during murine development (240). Before 1993, Egr-1 DNA binding properties including the zinc dependency and the affinity with GC-rich promoter sequences were discovered through assays with DNA sequences and Egr-1

protein translated in vitro or recombinated from Escherichia Coli (241). DNA-binding features of murine and human cellular Egr-1 were first characterized using mobility shift assay with nuclear extracts from NIH3T3 cells (242). It was found that Sp1 transcription factor binds to a consensus DNA sequence that partially overlaps with Egr-1 binding sequence suggesting that Egr-1 and Sp1 may both activate transcription through Egr-1 binding sites (EBS) present in the structure of several murine and human target gene promoter sequences (242). Noteworthy, EBS is also present on Egr-1 gene promoter suggesting an autoregulatory capacity. Sequencing have revealed the three zinc finger motives lined up between amino acids 332-419 of the mature Egr-1 protein form the central DNA-binding domain (DBD) (242). Together, two of the three DBD zinc fingers (361-419) and a 15 amino acids sequence adjacent to the N-terminal region of the DBD make up the classis nuclear localization sequence of Egr-1 protein (243). Recent reports however have described another sequence, C-terminal serine-proline-serine domain, which participates, together with importin-7, in Egr-1 protein nuclear import (244). Structural studies have determined an N-terminal strong activation domain stretching from amino acids 3-281 and a weak activation domain at the C-terminal. Furthermore, a repressor domain consisting of a region of 34 amino acids residues has been described between the activation and DNA-binding domains and act as a binding region for the family of transcriptional co-repressor proteins Nerve growth factor-induced-A-Binding proteins (NAB) (245).

1.3.3 Transcriptional regulation of Egr-1

Egr-1 plays an important role in many cellular processes such as growth, apoptosis and differentiation. Therefore, positive and negative regulatory mechanisms that direct the signaling cascades towards stimulation or suppression of Egr-1 are essential to achieve an adequately timed transcriptional response. This regulation depends on the type of tissue, the primary stimuli, and involves a variety of molecular mediators essentially located in the nucleus. Molecular mechanisms controlling Egr-1 transcriptional activity are governed by the functional features present on Egr-1 promoter as reviewed below.

1.3.3.1 Functional features of Egr-1 promoter

Typically, in addition to the EBS domain, Egr-1 promoter contains two binding sites for Sp1 and activator protein (AP1) (Figure 10). Five serum response elements (SRE) are present between the TATA box and the EBS (241). Rather than upregulating Egr-1 transcription, the binding of Egr-1 to the EBS on the promoter leads to Egr-1 repression and consequently represents a self-regulatory mechanism of Egr-1 expression (242). This could be a reason of the rapid but transient induction of Egr-1 upon stimulation. Furthermore, five E-twenty six (Ets) family transcription factor binding sites, each corresponding to one SRE, are located close to SREs (Figure 10). Their phosphorylation enhances their binding to adjacent SREs. Two transcriptional factors mediate the activity of SRE: the serum response factor (SRF) and the ternary complex factor containing the Ets domain-containing protein (Elk1) and SRF accessory protein-1 or 2 (Sap1 or Sap2 proteins) (246). Elk1 protein is phosphorylated by the MAPK JNK and ERK1/2, leading to increased DNA binding, ternary complex formation as well as SRE-mediated transcriptional activation. Additionally, two cAMP response elements (CRE) located in the regulatory region of the human Egr-1 promoter have a proven involvement in modulating Egr-1 gene transcription through the activation of p38/stress-activated protein kinase 2 (SAPK2) signaling cascade and in response to lysophosphatidic acid (LPA) in VSMC (247). In addition to transcriptional regulation, Egr-1 expression can also be controlled by post-translational modifications. Acetylation, for instance, is one of the Egr-1 post-translational modifications resulting in decreased Egr-1 activity whereas phosphorylation and sumoylation are reported to modulate its expression.

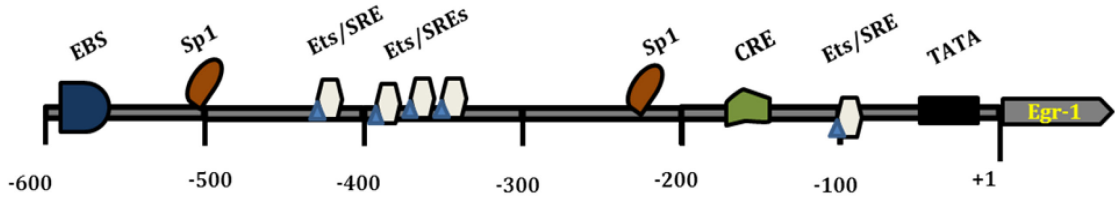

Figure 10: Structure of Egr-1 promoter present on target genes

Egr-1 promoter contains gene-specific activator protein 1 binding sites (Sp1), one cAMP response element (CRE) and five serum response elements (SRE) between the TATA box and the EGR-1 binding site (EBS). Functional EBS allows Egr-1 autoregulation. The phosphorylation of Ets transcriptional factors enhances their binding to adjacent SREs. (Figure and legend from (248)).

1.3.3.2 NAB-dependent regulation of Egr-1

Interaction with NAB proteins negatively regulates Egr-1-mediated transcriptional responses and deletion of the repressor binding site on Egr-1 induces a significant increase in Egr-1 DNA-binding activity. NAB1 is ubiquitously expressed at low levels in human cell lines (249), whereas the expression of NAB2 is more tissue-selective. Several EBS have been characterized on NAB2 promoter suggesting its induction by Egr-1 (250). Vascular injury (251) and other signals reported to upregulate Egr-1 expression also induced NAB2 expression on a later time point (252) revealing a negative feedback loop controlling Egr-1 activity. The function of NAB2 therefore enables Egr-1 to regulate its own biological activity avoiding over-transactivation of target genes (253). Further data on NAB-dependent regulation of Egr-1 were reported in studies where overexpression of NAB1 resulted in its complete blockade (249). Mechanistically, although there is a possibility of a direct fusion of NAB proteins with Gal4 DNA-binding region of some genes, NAB-mediated repression of EGR-1 involves the recruitment of NAB/EGR-1 complex, formed by association between EGR-1 and the NAB N-terminal domain, to the promoter (254). Subsequently, with the contribution of the inhibitory nucleosomal remodeling and deacetylation complex, histone deacetylases 1 and 2 (HDAC1/2) are also recruited to Egr-1 promoter and participate in the gene repression process (255).

1.3.3.3 Signaling pathways upstream of Egr-1 expression

Several studies have addressed the molecular cascades involved in the regulation of Egr-1 expression in response to a variety of stimuli in the pathogenesis of vascular diseases (248). Evidence supports that MEK/ERK pathway transduces the responses of vasoactive peptides including Ang-II and ET-1, both reported to induce Egr-1 expression in VSMC and rat carotid artery (256, 257) through mechanisms that are yet to be fully uncovered (258). Apelin-13 has also been shown to trigger VSMC migration via an ERK-mediated upregulation of Egr-1 expression (222). Another study reported that blockade of Gi protein, PI3K/Akt and PKC resulted in a suppression of apelin-13-induced Egr-1 expression (149). Yet in these same studies, blockade of JNK did not attenuate Egr-1 expression suggesting that depending on the external stimuli, MAPK are differentially required for Egr-1 induction. Indeed, activation of JNK signaling by the receptor for advanced-glycation end products (RAGE) was required to mediate hypoxia-induced Egr-1 expression (259). Together, these reports, in addition to studies from our laboratory (260), support the involvement of MAPK pathways as well as PKC and PI3K/PKB pathways in the upstream regulation of Egr-1 expression in response to vasoactive peptides (Figure 11). Evidence supports a similar involvement of MAPK signaling in response to atherosclerotic inflammatory stimuli. Notably JNK and ERK1/2 were shown to mediate the effect of LPA in inducing Egr-1 expression (261). The epidermal growth factor receptor (EGFR) which exhibits a tyrosine kinase activity (RTK) was demonstrated to play a critical role in mediating the effect of the pro-inflammatory interleukin-1β in inducing Egr-1 expression (262). Although in these studies EGFR required the activity of metalloproteinase as well as a disintegrin and a metalloproteinase (ADAM) to mediate Egr-1 expression (262), RTKs have been widely demonstrated to activate MAPK in response to GPCR-mediated transactivation in VSMC (263-266). Moreover, the fact that Egr-1 is a Ca^{2+}-dependent transcription factor may imply the contribution of the multifunctional calcium/calmodulin-dependent protein kinase II (CaMKII) in the control of its expression. Hence, CaMKII was demonstrated to mediate MEK/ERK1/2 activation (166, 256) and both blockade of CaMKII activity and MEK/ERK1/2 pathway resulted in an attenuation of ET-1-induced

upregulation of Egr-1 in VSMC (256). Furthermore, studies have investigated the downstream effectors of MAPK in transducing extracellular signals into Egr-1 expression. For instance, nicotine-induced response was shown to require ERK1/2-dependent Ets-like gene 1 activation to induce Egr-1 expression (267). Transcriptional mechanisms involving the participation of cAMP- and serum- response elements were recently demonstrated to control the expression of Egr-1 in VSMC (247). LPA upregulated Egr-1 expression with an involvement of CRE and SRE, implying the respective activation of CREB and the Elk-1 downstream of MAPK activation (247). Elk-1 is a transcriptional co-activator and its involvement in Egr-1 transcriptional regulation has been suggested. Elk-1 plays a role in the recruitment of ERK and its downstream kinase mitogen and stress-activated kinase (MSK) to Egr-1 promoter and thereby, enables stimuli-induced transcription of Egr-1 gene (268). Taken together, these studies demonstrate an involvement of transcriptional co-activators downstream of MAPK-induced cascades in Egr-1 induction (Figure 11).

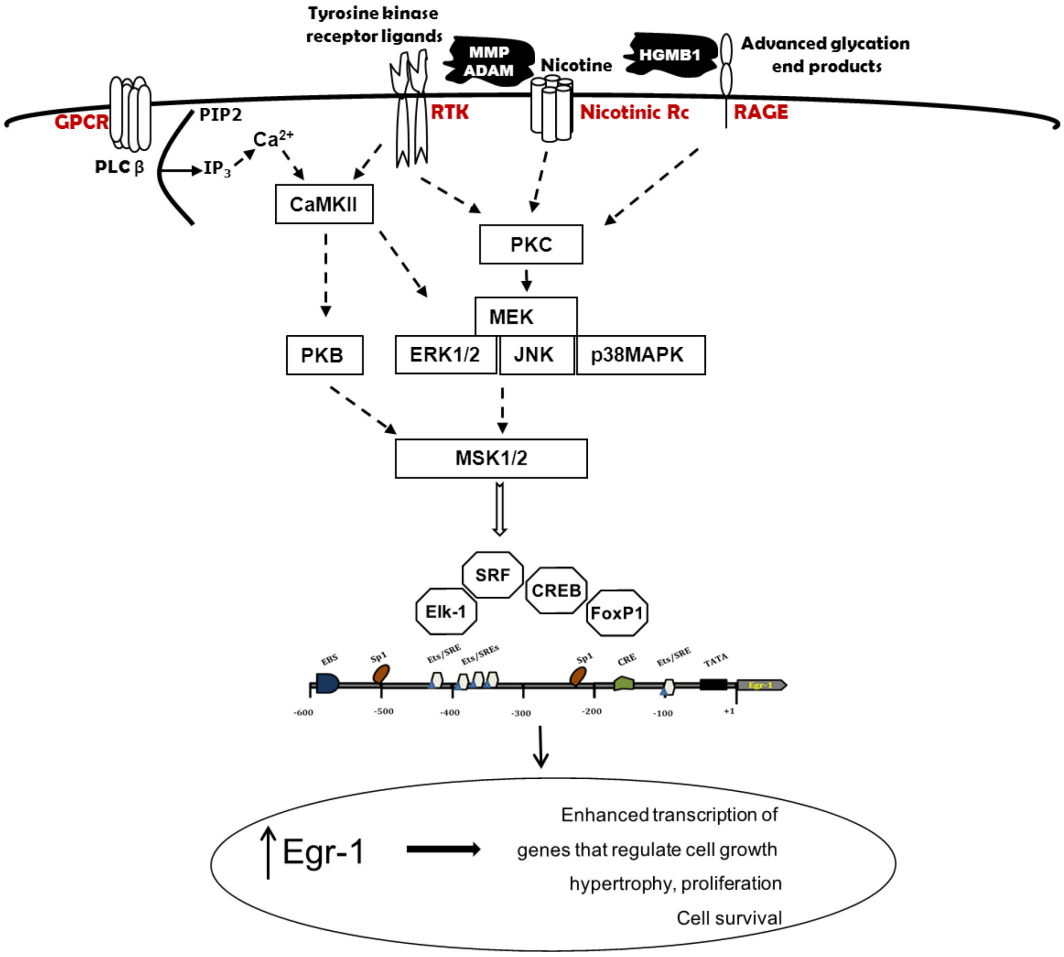

Figure 11: Signaling pathways upstream of Egr-1 expression

In response to elevated levels of hormones and external stimuli, activated receptors on vascular cell membranes (acetylcholine receptor (AchRc), G-protein coupled receptors (GPCR), receptor tyrosine kinases (RTK), advanced glycation end product receptors (RAGE)) trigger intracellular signaling either alone or in coordination with other proteins such as matrix metalloproteinases (ADAM, MMP) and high mobility box proteins (HGMB1). Receptor coupling or intrinsic kinase activation results in an increase in intracellular calcium that activates protein kinase B (PKB) , MEK and its targets ERK1/2, JNK and p38MAPK via the calcium calmodulin-dependent protein kinase II (CaMKII) or the protein kinase C isorforms (PKC). This results in the phosphorylation of the mitogen and stress-activated kinase (MSK1/2) that may coordinate the activity of the transcriptional cofactors such as Elk-1 and the serum response factor (SRF), the cAMP response element binding protein (CREB), and the forkhead box protein 1 (FoxP1) necessary for Egr-1 promoter activation leading to Egr-1 expression. Enhanced levels of Egr-1 lead to increased transcription of genes that promote vascular injury by modulating cellular growth, proliferation, hypertrophy, and survival. Figure and legend from (248).

1.3.4 Egr-1 and disease

The role of Egr-1 has been broadly studied in cancerogenesis where several groups have reported its contribution in the regulation of the expression of a large range of transcription factors involved in cell cycle and survival processes (203, 269-274). Recent investigations have focused on its role in the regulation of cardiovascular homeostasis as well as in neuronal plasticity.

1.3.4.1 Egr-1 in cardiovascular physiology

A role of Egr-1 in vascular disease was demonstrated by studies where LDL receptor null mice deficient in Egr-1 exhibited smaller atherosclerotic lesions and less macrophage infiltration following a high fat diet, compared to their littermates expressing Egr-1 (275). Egr-1 levels are increased in experimental models of cardiovascular diseases where it governs the expression of proteins known to control physiological responses in fibroblasts, endothelial cells and VSMC (276). In fact, many genes that are known to be upregulated in atherosclerotic lesions possess an EBS and their promoters are reported to be directly targeted by Egr-1 (228). These are mostly genes governing the expression of vascular growth factors, pro-inflammatory and pro-thrombotic markers, adhesion molecules, cytokines, matrix proteins, transcriptional regulators, and other signaling molecules involved in the remodeling of cardiovascular tissues (228, 277). Accordingly, upregulation of Egr-1 expression has been suggested to be among the mechanisms underlying the progression of vascular injury due to persistent stimuli like metabolic disturbances, increased oxidative stress, exaggerated activity of vasoactive peptides, mechanical stretch and fluid shear stress (Reviewed in (166).

Consequently, using strategies aiming at silencing Egr-1 mRNA (DNAzymes or RNA interference) or depleting Egr-1 protein (oligonucleotide decoys), Egr-1 loss of function studies have provided beneficial outcomes in experimental models of cardiovascular insults (278). In models of pulmonary hypertension (279), baloon injury (280, 281) or

permanent vessel ligation (234), loss of Egr-1 resulted in a decrease in intimal thickening, smooth muscle cell proliferation and migratory responses, in-stent restenosis, as well as a decrease in the expression of inflammatory and cell adhesion markers. Hypercholesterolemia is a well known inducer of vascular injury and therefore is accompanied with a robust upregulation of vascular Egr-1 levels (282). Depleting Egr-1 protein in models of vascular disease induced by hypercholesterolemia result in a decrease in macrophage population, vein graft hyperplasia, as well as in neointimal thickening reinforcing the role played by Egr-1 in the pathobiology of vascular remodeling (282, 283). In heart disease models like left coronary artery ligation-induced myocardial injury or myocardial ischemia reperfusion model, loss of Egr-1 is able to provide a better cardiac function, to reduce the size of the infarct, and to downregulate the inflammatory events in cardiomyocytes. In addition, oxidative disorders, apoptosis, and aberrant immune reactions are significantly attenuated by depletion of Egr-1 following heart injury induction (278).

Transcriptional targets of Egr-1	Functional involvement in vascular pathophysiology
CD44	Cell surface transmembrane receptor with demonstrated role in endothelial tubular network formation during angiogenesis
Cyclin D1	Protein that participates in cell cycle progression and is implicated in the regulation of cellular proliferative and survival responses
Interleukin-2	Pro-atherogenic inflammatory molecule
Macrophage colony stimulating factor-1	Growth factor that promotes monocyte/macrophage-dependent-inflammation and plays a role in vasoactive peptide-induced vessel remodeling
Tissue factor	Cellular receptor that initiates blood coagulation and which expression is inducible in vascular cells
Platelet-derived growth factor	VSMC growth and migration promoting factor involved in vessel remodeling
Vascular cell adhesion molecule 1	Pro-atherogenic adhesion molecule
Plasminogen activator inhibitor 1	Multifunctional protein that impairs fibrinolysis and promotes thrombosis Involved in intimal growth and VSMC migration
Tumor necrosis factor	Pro-atherogenic inflammatory molecule
Nuclear factor kappa B (NF-κB) (p65, p105)	Pro-atherogenic inflammatory molecule
Monocyte chemotractant protein-1	Chemokine that controls the migration and the infiltration of monocytes and macrophages during atherosclerotic plaque formation

Table 1: Egr-1 responsive gene products with relevant functions in the vasculature

1.3.4.2 Egr-1 role in brain plasticity

Egr-1 is expressed ubiquitously and has also been shown to participate in the pathogenesis neuronal disorders. A downregulation of Egr-1 expression in the hippocampus is among the mechanisms that relate neuro-inflammation or neurosepsis to aberrant cognitive function (284, 285). Studies showing that adult Egr-1 knockout animals present impairment in neuronal behavior demonstrate that hippocampal Egr-1 plays a crucial role in regulating neuroplasticity responses evidenced by long-term potentiation (286). Indeed, processes underlying synaptic plasticity associated, among other effects, with the formation of long term memory, have been shown to be closely linked with an upregulation of Egr-1 expression (287). In neurons, Egr-1 upregulation occurs following activity-dependent elevation in cytosolic calcium. There seems to be a clear relationship between Egr-1 and events linked with learning and memory responses that result from constant neuronal excitation and that require sustained changes in plasticity (288). Although it is now clear that these correspond to the long-term phenotypic changes (cell differentiation, structural changes, proliferation rate, etc.) transduced by immediate early genes in response to mitogens in other systems, the specific signaling pathways linking neuronal Egr-1 to the various markers of neuroplasticity remain elusive. However, in this regard, several signaling molecules have been suggested to interplay with Egr-1. The activity-regulated cytoskeletal associated (Arc) gene that plays a key role in memory formation has been shown to possess an EBS on his promoter region and to be directly targeted by Egr-1 in neurons suggesting one of the molecular mechanism by which Egr-1 contributes to long-term potentiation (289). In addition, Egr-1-dependent regulation of neuronal plasticity was shown to be related to the ability of Egr-1 to control the expression of proteasome and genes related to proteasome activity in nerve cells (290). Moreover, Egr-1 modulates the expression of synapsins (291, 292). Synapsins are essential for proper neurotransmitter release during synaptic communication and therefore are crucial regulators of neuronal plasticity. A decrease in synapsin expression and activity is associated with alterations in the processes underlying brain development. It is well known

that a deficiency in methyl donors such as folate and vitamin B12 during pregnancy is associated with impairments in fetal and postnatal brain development. Interestingly, this deficiency is accompanied by a decrease in Egr-1 expression and a consequent downregulation of synapsin levels in the brain of the offspring (291). The contribution of Egr-1 in the plasticity of the central nervous system indicates a potential effect on the sympathetic regulation of blood pressure thus providing research perspectives in vascular disease.

1.4 OBJECTIVES OF THE STUDY

A large body of evidence demonstrates that Ang-II contributes to vascular injury by inducing aberrant VSMC proliferation. Egr-1 is a calcium-dependent transcription factor upregulated in conditions of vascular injury where it controls the expression of several pro-atherogenic factors. The molecular aspects of Egr-1 regulation and its plausible implication in aberrant vascular function induced by LPA (145) and growth factors such as apelin-13 (109), thrombin (112), and PDGF (111) have been addressed in previous reports. However, the precise molecular features of this upregulation in response to Ang-II are yet to be fully characterized. We have previously reported a CaMKII-dependent upregulation of Egr-1 in VSMC in response to ET-1. However, the contribution of the SOCE molecules STIM-1 and Orai-1, as well as the potential involvement of the antimitogenic cAMP in the regulation of Egr-1 expression remain unexplored. Therefore, the main purpose of this thesis was to examine the Ca^{2+}-dependent signaling pathways that modulate Egr-1 expression in response to Ang-II and to examine the modulatory role of cAMP agonists.

We hypothesized that:

1. STIM-1/Orai-1-mediated intracellular Ca^{2+} signalling is critical for Ang-II-induced Egr-1 upregulation in VSMC.
2. cAMP exerts a negative regulation on Egr-1 expression in VSMC.

The first part of the study was dedicated to the characterization of Ang-II-induced response on Egr-1 expression and the role played by SOCE-related signaling. A putative role of the two SOCE-operating molecules STIM-1 and Orai-1 in hypertension and arterial remodeling has been suggested by studies showing an upregulation of their expression in animal models of hypertension. However their implication in Ang-II-induced regulation of Egr-1 has never been addressed. Therefore, studies conducted in this part assessed the role of intracellular Ca^{2+} signaling in Ang-II-induced response on Egr-1 expression and examined the modulatory effect of RNA interference targeting STIM-1 and Orai-1.

Since cAMP agonists have shown beneficial effect in the vasculature via their antiproliferative properties, in the second part of the study, we investigated the effect of cyclic AMP elevating agents on Ang-II-induced Egr-1 expression. Using the β-adrenergic agonist isoproterenol and the AC activator forskolin, as well as cAMP analogs and a broad spectrum PDE inhibitor, we compared the effects of receptor or non-receptor elevation of cAMP on Ang-II-induced Egr-1 expression and investigated the signalling changes associated with the response.

1.5 STUDY MODEL: A10 CELL LINE

A10 cells are isolated from thoracic aorta of 14-17 day old BD1X embryonic rat (293). Molecular and physiological similarities between VSMC *in vivo* and A10 cells support the extensive use of this cell line as a model to study the molecular mechanisms underlying the behavior of VSMC in vascular diseases. A major characteristic of adult medial VSMC is that they are not terminally differentiated. This property forms the basis of their propensity to change phenotype upon stimuli as occurs in vascular diseases (294) (described in section 3.1). Similarly, cultured A10 cells are prone to phenotypic modulation depending on the culture conditions (295). In addition to plasticity, mature VSMC are characterized by their contractility and the expression of SMC specific markers of differentiation including smooth muscle actin, smooth muscle myosin heavy chain, smooth muscle 22 α, and calponin (294). Based on that, de-differentiation has been widely considered as the underlying cause of the expansion of synthetic neointimal VSMC. In fact, neointimal VSMC are believed to be mature medial VSMC that have undergone changes leading to reduced levels of SMC markers, a lesser ability to contract as well as higher proliferative rates (294, 296). In addition to the theory of dedifferentiation of mature cells, studies have also reported that neointimal cells are SMC derived from resident multipotent stem cells that migrated toward the intima (44). A10 cells express SMC specific markers (295). Notably, when compared with adult VSMC, the expression levels of these markers tend to be smaller, reminiscent of progressive dedifferentiation of adult VSMC (295). Additionally, although A10 cells are contractile (297), this property is lost in

specific culture conditions (295) reminiscent of VSMC plasticity. In recent studies moreover, stem cell markers, including neural crest and glia markers, usually expressed in adventitial and medial progenitor cells, were reported to be expressed in A10 cells (298) as well as in primary cultures of VSMC isolated from adult animals (299). This suggests that A10 cells exhibit a molecular profile similar to both resident multipotent cells and mature de-differentiated SMC believed to populate the neointima in conditions of vascular diseases. This supports the use of A10 cells as a relevant model for *in vitro* studies addressing the molecular events underlying the role of VSMC in vascular pathophysiology.

Chapter 2 (Article 1): STIM-1 and ORAI-1 channel mediate Angiotensin-II-induced expression of Egr-1 in vascular smooth muscle cells.

2 STIM-1 and ORAI-1 channel mediate Angiotensin-II-induced expression of Egr-1 in vascular smooth muscle cells.

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2.1 ABSTRACT

An upregulation of Egr-1 expression has been reported in models of atherosclerosis and intimal hyperplasia and, various vasoactive peptides and growth promoting stimuli have been shown to induce the expression of Egr-1 in vascular smooth muscle cells (VSMC). Angiotensin-II (Ang-II) is a key vasoactive peptide that has been implicated in the pathogenesis of vascular diseases. Ang-II elevates intracellular Ca^{2+} through activation of the store-operated calcium entry (SOCE) involving an inositol-3-phosphate receptor (IP_3R)-coupled depletion of endoplasmic reticular Ca^{2+} and a subsequent activation of the stromal interaction molecule 1 (STIM-1) /Orai-1 complex. However, the involvement of IP_3R/STIM-1/Orai-1-Ca^{2+}-dependent signaling in Egr-1 expression in VSMC remains unexplored. Therefore, in the present studies, we have examined the role of Ca^{2+} signaling in Ang-II-induced Egr-1 expression in VSMC and investigated the contribution of STIM-1 or Orai-1 in mediating this response. 2-aminoethoxydiphenyl borate (2-APB), a dual non-competitive antagonist of IP_3R and inhibitor of SOCE, decreased Ang-II-induced Ca^{2+} release and attenuated Ang-II-induced enhanced expression of Egr-1 protein and mRNA levels. Egr-1 upregulation was also suppressed following blockade of calmodulin and CaMKII. Furthermore, RNA interference-mediated depletion of STIM-1 or Orai-1 attenuated Ang-II-induced Egr-1 expression as well as Ang-II-induced phosphorylation of ERK1/2 and CREB. In addition, siRNA-induced silencing of CREB resulted in a reduction in the expression of Egr-1 stimulated by Ang-II. In summary, our data demonstrate that Ang-II-induced Egr-1 expression is mediated by STIM-1/Orai-1/Ca^{2+}-dependent signaling pathways in A-10 VSMC.

2.2 INTRODUCTION

Exaggerated vascular smooth muscle cell (VSMC) proliferative responses have been widely described as underlying mechanisms of aberrant neointima formation in vascular diseases (Miao et al., (2000); Giachini et al., (2011)). A hallmark of vascular pathologies is the presence of elevated levels of vasoactive peptides, such as angiotensin-II (Ang-II), which is a major vasoconstrictor with a demonstrated causal role in the pathophysiology of vascular disorders (Montezano et al., 2014). Ang-II exerts its biological responses via the stimulation of its seven transmembrane heterotrimeric G-protein coupled receptors (GPCR) type 1 and type 2 (AT1 and AT2) (Murphy et al., 1991). Hyperactivation of Ang-II-induced signal transduction pathways has been demonstrated to contribute to vascular damage by promoting events, such as extracellular matrix accumulation, inflammation, oxidative stress, and, more importantly, VSMC proliferation, hypertrophy and migration (Nakashima et al., 2006; Touyz, 2005; Touyz and Schiffrin, 2000). An increase in the intracellular level of Ca^{2+} ($[Ca^{2+}]i$) is among the early events that occur following Ang-II stimulation of VSMC (Brock et al., 1985). This results in part from receptor-mediated activation of phospholipase C (PLC) and formation of inositol-3-phosphate (IP_3). IP_3 binds to and activates IP_3 receptors (IP_3R), which releases Ca^{2+} from the endoplasmic reticulum (ER) into the cytosol (Touyz and Schiffrin, 2000). Upon this release, subsequent ER Ca^{2+} depletion is sensed as a signal to trigger an influx of extracellular Ca^{2+} via a store-operated Ca^{2+} entry (SOCE) mechanism. The stromal interaction molecule 1 (STIM-1) located inside the ER membrane has been described as an essential member of the SOCE molecular machinery as it senses the Ca^{2+} depletion and, following conformational changes, associates with the transmembrane pore forming molecules, Orai, to mediate SOCE in the cytoplasm (Roos et al., 2005; Takahashi et al., 2007; Yang et al., 2012; S. L. Zhang et al., 2005). Among the different types of Orai channels, type 1 (Orai-1) have been shown to be involved in VSMC proliferation and migration (Potier et al., 2009).

Transcriptional and physiological responses triggered by a rise in $[Ca^{2+}]_i$ are in part mediated by specific Ca^{2+} handling proteins, like calmodulin (CaM), which forms a

complex with free Ca^{2+} and triggers the activation of downstream kinases, known as Ca^{2+}/calmodulin-dependent protein kinases (CaMK) (Cheyou et al., 2014). CaMK type II (CaMKII) has been implicated in Ang-II-induced VSMC proliferation (Li et al., 2010)

Early growth response protein-1 (Egr-1), a zinc finger transcription factor, has been shown to be upregulated in models of vascular injury (Khachigian, 2006), and heightened levels of Egr-1 are observed in atherosclerotic lesions of animal models of vascular diseases, as well as in response to growth stimuli in VSMC (Cheyou et al., 2014; M. Z. Cui et al., 2006; Goetze et al., 2001; Q. F. Liu et al., 2013; Midgley and Khachigian, 2004; Santiago et al., 1999; Vazquez-Padron et al., 2010) . Vasoactive peptides and growth factors have been shown to rapidly increase Egr-1 expression via mechanisms involving changes in $[Ca^{2+}]_i$ (Thiel et al., 2010), resulting either from a release from the intracellular stores (Jaimovich and Carrasco, 2002; Rossler and Thiel, 2009) or an influx of extracellular Ca^{2+} (Mayer et al., 2011; Mayer and Thiel, 2009; Stefano et al., 2006). However, the involvement of IP_3R/STIM-1/Orai-1-Ca^{2+}-dependent signalling in the upregulation of Egr-1 in VSMC has not been investigated.

In the present studies, by using pharmacological modulators of Ca^{2+} signaling, as well as RNA interference targeting STIM-1 and Orai-1, we investigated the involvement of SOCE-mediated signaling in Ang-II-induced Egr-1 expression in VSMC.

2.3 MATERIALS AND METHODS

Reagents: Ang-II (Cat# A9525) and 2-aminoethoxydiphenyl borate (2-APB) (Cat# D9754) were obtained from Sigma-Aldrich (St. Louis, MO, USA). W-7 (Cat# 681629), KN-93 (Cat# 422711), and KN-92 (Cat.# 422709) were obtained from EMD Millipore (Burlington, ON, Canada). U0126 (Cat# 109511-58-2) was from Calbiochem (San Diego, CA, USA).

Antibodies: Rabbit polyclonal primary antibodies were used to detect STIM-1 (Sigma-Aldrich Cat# SAB3500365, RRID:AB_10646327), Orai-1 (Santa Cruz Biotechnology Cat# sc-68895, RRID:AB_2283283), total ERK1/2 (Santa Cruz Biotechnology Cat# sc-154, RRID:AB_2141292), Thr202/Tyr204 phosphorylated ERK1/2 (Santa Cruz Biotechnology Cat# sc-16982, RRID:AB_2139990), Ser133 phosphorylated CREB (Cell Signaling Technology Cat# 9198, RRID:AB_2561044), Egr-1 (Cell Signaling Technology Cat# 4153, RRID:AB_2097038) and β-tubulin (Cell Signaling Technology Cat# 2146, RRID:AB_2210545). Rabbit monoclonal primary antibody directed against CREB (Cell Signaling Technology Cat# 9197, RRID:AB_331277) was also used to detect the total amount of CREB.

Cell culture: Experiments were conducted in A-10 VSMC line derived from the medial layer of rat thoracic aorta (ATCC Cat# CRL-1476, RRID:CVCL_0130). The cells were maintained in culture with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC in a humidified atmosphere of 5% CO2, as described earlier (Bouallegue et al., 2007). Cells between passages 4 and 8 were grown to 80-90% confluence in 60-mm dishes and incubated in serum and antibiotic-free DMEM 5 hours prior to treatments.

Cell lysis and immunoblotting: Confluent serum-starved A-10 cells were incubated in the absence or presence of various reagents for 30 minutes followed by incubation with 100 nM Ang-II for indicated times. For dose response studies, cells were treated with increasing concentrations of Ang-II for one hour. The cells were washed three times with

ice-cold PBS and lysed in 100 µL radio-immunoprecipitation (RIPA) buffer. 35-45 µg of proteins measured by Bradford assay were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilion-P polyvinylidinedifluoride membranes (Millipore, USA) and incubated with respective primary antibodies, Egr-1 (1:1000), STIM-1 (1:1000), Orai-1 (1:1000), phospho-ERK1/2 (1:2000), total ERK1/2 (1:4000), phospho-CREB (1:1000), total CREB (1:4000) or β-tubulin (1:5000). The antigen-antibody complex was detected using a horseradish peroxidase-conjugated secondary anti-rabbit antibody (Cell Signaling Technology Cat# 7074, RRID:AB_2099233) and protein bands were visualized with the enhanced chemiluminescence detection kit (Perkin Elmer, Cat# NEL104, Montreal, QC, Canada).

Preparation of cDNA: After incubations, total RNA was isolated using Trizol reagent (Life Technologies, Burlington, ON, Canada). RNA concentration was quantified with the Eppendorf BioPhotometer D30 (Eppendorf, Mississauga, ON, Canada). Absorbances were measured at wavelengths of 260 nm and 280 nm. The purity of RNA preparation was confirmed when the ratio A260/A280 was comprised in the range of 1.8-2.0. cDNA was synthesized from 1 µg of total pure RNA using High Capacity RNA-to-cDNA Kit (Life Technologies, Cat# 4387406, Grand Island, NY, USA) as per manufacturer's instructions.

Real-time quantitative polymerase chain reaction (qRT-PCR): qRT-PCR was performed with SYBG (Life Technologies, Grand Island, NY, USA) using 1µL of cDNA in a 20 µL reaction. Amplification was performed using 7500 fast RT-PCR system (Applied Biosystems, Grand Island, NY). Sequences used to design Egr-1 primers were as follow: forward 5'-CTGCTTCATCGTCTTCCTCTG-3' and reverse 5'-GTCAGTGTTGGGAGTAGGAAAG-3'. Egr-1 mRNA expression was measured and normalized with β-actin (Primers: forward 5'-TCTTCCAGCCTTCCTTCCT-3' and reverse 5'-CAGCACTGTGTTGGCATAGA-3') mRNA levels.

Immunofluorescence: Serum-starved A-10 cells grown and treated on glass coverslips were washed with ice-cold PBS and fixed with paraformaldehyde 4% for 30 min at 4°C. Permeabilization was achieved by 10 minutes incubation with 0.1% Triton X-100, 0.1%

serum citrate pH 4.0 at room temperature (RT). Cells were then blocked with goat serum diluted in PBS (15 µL/mL PBS) for one hour and incubated overnight at 4°C with Egr-1 antibody diluted in the blocking solution (1:100). Coverslips were further incubated for two hours at RT with goat anti-rabbit IgG conjugated with Alexa Fluor 488 (1:150) (Thermo Fisher Scientific Cat# A-11034, RRID:AB_2576217). Nuclei were then labelled by staining the coverslips with DAPI (2 µL/1.5 mL H2O) before being mounted with a buffer made of 30% Glycerol in PBS. The images were taken using X-Cite Serie 120, TE2000-S fluorescence microscope (Youreva and Srivastava, 2016) .

Fura-2 [Ca^{2+}]i imaging: [Ca^{2+}]i was monitored in A-10 cells after Ang-II stimulation. Briefly, cells were loaded with 10 µM Fura-2-AM (1 hour, at 37 °C in dark), washed in DMEM containing 0.001% cremophor and 2.5 mM of probenecid, followed by washing in DMEM containing 2.5 mM probenecid to achieve de-esterification. Petri dishes containing fura-2-AM-treated A-10 cells were placed on the stage of an inverted microscope (Nikon TE300, Mississauga, ON, Canada). The cells were exposed to alternate (100 ms) excitatory wavelengths at 340 nm and 380 nm with a high-pressure mercury lamp (100 W) via interference filters (Chroma Technology, Brattleboro, VT, USA) mounted on a filter wheel (Sutter Lambda 10-C, Sutter Instrument, Novato, CA, USA) with a dichroic mirror. A cool-coupled device camera recorded fluorescent images from three to ten seconds intervals. Measurements are presented as the F340/F380 fluorescence ratio.

siRNA transfection protocol: Transfection was performed using lipofectamine RNAi max (Life Technologies, Cat# 13778-075, Burlington, ON, Canada). A-10 VSMC at 70% confluence were transfected with 10 nM rat siRNA constructs according to the manufacturer's protocol (Origene, Rockville, MD, USA). Briefly, distinct mixtures obtained by addition of lipofectamine to tubes containing siRNA against CREB (Origene, Cat# SR500635, Locus ID 298400), STIM-1 (Origene, Cat# SR512570, locus ID 361618), Orai-1 (Origene, Cat# SR508429, locus ID 84876) or non-targeting scrambled siRNA (Origene, Cat# SR30004) were used to transfect cells for 6 hours. The medium was

replaced afterward with normal supplemented culture medium and the cells were incubated for 48 additional hours at 37ºC before stimulation with Ang-II.

Data analysis: Images obtained from immunofluorescence assays were analyzed with the program ImageJ (http://rsb.info.nih.gov/ij/index.html RRID:SCR_003070). The intensity of the bands was quantified by densitometric analysis of immunoblots using Quantity One 1-D Analysis Software (http://www.bio-rad.com/en-us/product/quantity-one-1-d-analysis-software RRID:SCR_014280). Graphs and statistical analysis by one-way standard analysis of variance (ANOVA) were made with Graphpad Prism 5.0 software package (http://www.graphpad.com/ RRID:SCR_002798). Statistical significance of the differences between samples was assessed by a Tukey multiple comparison post hoc test. The differences between means were considered significant with $p<0.05$. All quantitative data are expressed as mean ± SEM from independent experiments.

2.4 RESULTS

Ang-II induces an increase in Egr-1 protein levels in a time- and dose-dependent fashion in A-10 VSMC.

Ang-II is a key vasoactive peptide with a well-established role in the pathogenesis of vascular diseases and upregulation of Egr-1 has been implicated in neointimal thickening and atherosclerosis. However, the effect of Ang-II on Egr-1 expression in VSMC is not fully characterized. Therefore, we sought to determine the effect of Ang-II on Egr-1 expression in VSMC. As shown in figure 1, Ang-II dose-dependently enhanced the expression of Egr-1 (Fig.1A). The increase in Egr-1 expression could be detected at 10 nM; however, at higher doses, the expression level was further enhanced. Time-course studies using 100 nM Ang-II demonstrated that Egr-1 protein expression was detectable after 30 min of treatment, reached a peak value at 60 min and rapidly declined to basal levels within a 2-hr period (Fig.1B), whereas Egr-1 mRNA was significantly upregulated after 30 min of treatment (Fig.1C).

Consistent with the immunoblotting data, immunofluorescence analysis revealed that the nuclear accumulation of Egr-1 protein also peaked at 60 min following stimulation of VSMC with Ang-II (Fig.1D). Further experiments using actinomycin D, an inhibitor of mRNA transcription, revealed that increased expression of Egr-1 in response to Ang-II required RNA transcription (Addendum, Figure 13). These data demonstrate that Ang-II induces Egr-1 expression, and this action involves an enhanced transcription of Egr-1 mRNA.

IP_3 receptor blockade attenuates Ang-II-induced Egr-1 expression and alters $[Ca^{2+}]i$ responses in A10 VSMC

IP_3 generation is among the earliest events subsequent to Ang-II receptor activation. Ang-II raises cytosolic Ca^{2+} concentration through release mechanisms involving PLC-dependent generation of IP_3 that activates IP_3R-coupled release of ER Ca^{2+} (Duff et al., 1995; Liu et al., 2009). IP_3R-dependent events have been widely shown to mediate

receptor-mediated signaling to Ca^{2+} entry in multiple cell types. Therefore, by using 2-APB, an allosteric non-competitive inhibitor of IP_3R that antagonizes SOCE (Ma et al., 2000; Peppiatt et al., 2003; van Rossum et al., 2000), we wished to determine whether IP_3R-dependent Ca^{2+} signaling plays a role in Egr-1 expression. As shown in Figure 2, 2-APB dose-dependently inhibited Ang-II-induced Egr-1 protein expression (Fig.2A) and significantly blunted Ang-II-induced upregulation of Egr-1 mRNA (Fig.2B). Consistent with the immunoblotting results, immunofluorescence localization of Egr-1 in cells also revealed that 2-APB-mediated blockade of IP_3R decreased the nuclear accumulation of Egr-1 in VSMC (Fig.2C) suggesting a role of IP_3R-mediated calcium signaling events in Ang-II-induced Egr-1 expression. It should be noted that 2-APB treatment alone resulted in the reduction of Egr-1 levels in unstimulated cells although in these cells, only trace amounts of Egr-1 were detectable.

We further investigated whether the attenuation of Egr-1 expression by IP_3R blockade could be attributed to the alterations in Ang-II-induced $[Ca^{2+}]i$ mobilization in VSMC. As shown in figures 3A and 3B, stimulation of Fura-2-AM-loaded A-10 VSMC with Ang-II resulted in a rapid rise in $[Ca^{2+}]_i$. Pre-incubation of the cells with 2-APB abolished the $[Ca^{2+}]_i$ response (Fig.3A and Fig.3B) confirming the ability of 2-APB to modify Ang-II-induced $[Ca^{2+}]_i$ dynamics.

siRNA-mediated silencing of STIM-1 or Orai-1 inhibits Ang-II-induced Egr-1 expression

IP_3R-mediated depletion of ER Ca^{2+} induces SOCE through a process where STIM-1/Orai-1 cooperation plays a pivotal role (Roos et al., 2005, Zhang et al., 2005, Zou et al., 2011). Our data using 2-APB suggested an involvement of IP_3R/SOCE in Ang-II-induced Ca^{2+} release and Egr-1 expression, but the contribution of STIM-1 and Orai-1 in Ang-II-induced expression of Egr-1 remains unexplored. Therefore, by using RNA interference technique, we assessed whether these key components of SOCE participate in this process. As shown in Figure 4, treatment of VSMC with 10 nM of siRNA targeting STIM-1 (siSTIM-1) (Fig.4A) or Orai-1 (siORAI-1) (Fig.4B) significantly reduced the expression of

these molecules, whereas scrambled siRNA (siSCR) exerted no effect on STIM-1 (Fig.4A) or Orai-1 (Fig.4B) expression. Furthermore, siRNA-induced silencing of either STIM-1 or Orai-1 significantly reduced Ang-II-induced expression of Egr-1 in VSMC, suggesting their involvement in this process.

Calmodulin and CaMKII inhibitors attenuate Ang-II-induced Egr-1 expression in VSMC

Since the downstream effects of Ca^{2+} are in part mediated by Ca^{2+} binding proteins, such as calmodulin (CaM) and CaMKinases (CaMKII), and because we have shown earlier that CaM/CaMKII plays a role in mediating endothelin-1 (ET-1)-induced signaling responses and Egr-1 expression in VSMC (Bouallegue et al., 2013), we investigated if these effectors of Ca^{2+} signaling also mediate Ang-II-induced expression of Egr-1. As shown in Figure 5, pre-treatment of the cells with 10 μM of W-7 (Fig. 5A) or KN-93 (Fig.5B), respective pharmacological inhibitors of calmodulin and CaMKII activity, significantly reduced Ang-II-induced expression of Egr-1. In contrast, KN-92, the inactive analog of KN-93, produced no effect on Ang-II-induced response. Collectively, these data support the involvement of Ca^{2+}/CaM/CaMKII-dependent signaling in Ang-II-induced Egr-1 expression.

siRNA-mediated silencing of STIM-1 or Orai-1 attenuates Ang-II-mediated activation of ERK1/2 and CREB

We have shown earlier that CaM/CaMKII pathway mediates MEK/ERK1/2 activation in response to ET-1 in VSMC (Bouallegue et al., 2013). Additionally, we and others have observed that the MEK/ERK1/2 pathway plays a key role in Egr-1 expression induced by several stimuli (Hasan and Schafer, 2008, Liu et al., 2013a, Youreva and Srivastava, 2016). Therefore, we investigated if the attenuation of Ang-II-induced Egr-1 expression due to STIM-1 and Orai-1 silencing in VSMC was associated with a change in ERK1/2 phosphorylation. As shown in Figure 6, siRNA-induced silencing of either STIM-1 (Fig.6A) or Orai-1 (Fig.6B) resulted in a significant reduction in Ang-II-induced

phosphorylation of ERK1/2 (Fig.6C and Fig.6D). This suggests that ERK1/2 is a downstream effector of STIM-1/Orai-1-mediated signaling in response to Ang-II in VSMC.

Two cyclicAMP response elements (CRE) are found in Egr-1 promoter (Cheyou et al., 2014, Cui et al., 2006) and the transcription factor CRE-binding protein (CREB) has previously been shown to regulate Egr-1 induction in response to GPCR agonist stimulation in VSMC (Cui et al., 2006). Additionally, a role of Ang-II-induced activation of CREB in mediating VSMC hypertrophy, proliferation and neointimal formation after vascular injury has been reported (Molnar et al., 2014, Funakoshi et al., 2002). Thus, we sought to investigate if attenuation of Ang-II-induced Egr-1 expression and ERK1/2 phosphorylation observed following STIM-1 and Orai-1 silencing were accompanied by a change in CREB activation as evidenced by an altered phosphorylation of CREB on Ser133 (Funakoshi et al., 2002, Liu et al., 2013b, Shaywitz and Greenberg, 1999). As shown in figure 6, siRNA-induced reduction in either STIM-1 (Fig.6A and Fig.6E) or Orai-1 (Fig. 6B and Fig.6F) expression also suppressed the phosphorylation of CREB in response to Ang-II, suggesting that STIM-1/Orai-1-mediated SOCE is required to trigger Ang-II-induced CREB phosphorylation in VSMC.

Activation of ERK1/2 and CREB is required to regulate Ang-II-induced Egr-1 expression

ERK1/2 has been shown to mediate the phosphorylation of CREB in response to Ang-II, (Cui et al., 2016, Funakoshi et al., 2002, Molnar et al., 2014) and CREB was demonstrated to mediate LPA-induced increase in Egr-1 expression (Cui et al., 2006). Therefore, to clarify the sequence of the molecular events regulating Ang-II-induced Egr-1 expression, it was of interest to investigate the effect of ERK1/2 blockade on Ang-II-mediated CREB phosphorylation and to assess the consequences of CREB depletion on Ang-II-induced Egr-1 expression. Therefore, cells were pretreated with the MEK/ERK1/2 inhibitor U0126 prior to stimulation with Ang-II. As shown in fig.7A, Ang-II induced a potent increase in CREB phosphorylation on Ser133 (Fig.7A) which was almost

completely attenuated by U0126. Similarly, Ang-II-induced Egr-1 expression was also suppressed by U0126 (Fig.7B). To further substantiate the involvement of CREB in Ang-II-induced Egr-1 expression we examined the effect of siRNA- induced depletion of CREB on Egr-1 expression. As shown in figure 7 C, CREB depletion resulted in almost total inhibition of Ang-II-induced Egr-1 expression in VSMC. Altogether, these data reveal for the first time that ERK1/2/CREB pathway is a downstream effector of SOCE-mediated signaling leading to Egr-1 induction in VSMC stimulated with Ang-II.

2.5 DISCUSSION

In this study, we have demonstrated a key role of Ca^{2+}-dependent signaling pathways in Ang-II-induced expression of Egr-1 in VSMC. Our data reveal that pharmacological blockade of IP_3R by 2-APB reduced Ang-II-induced increase in $[Ca^{2+}]i$ levels and Egr-1 expression in VSMC. In addition, our data showing that siRNA-induced silencing of either STIM-1 or Orai-1, key components of SOCE complex, resulted in a significant reduction in Ang-II-induced Egr-1 expression, indicate that a functional SOCE plays a critical role in triggering the signaling cascade leading to Egr-1 expression. Furthermore, by using pharmacological blockers of calmodulin and CaMKII, we have provided evidence that SOCE triggered by STIM-1/Orai-1 activation signals Egr-1 expression via CaM/CaMKII-dependent downstream pathways in VSMC. Moreover, we demonstrated that STIM-1/Orai-1 plays a key role in triggering Ang-II-induced activation of MEK/ERK1/2- and CREB-dependent signaling in VSMC.

Upregulated levels of IP_3 and IP_3R have been observed in VSMC and mesenteric arteries of hypertensive rats as compared to normotensive rats and correlated with heightened vascular reactivity in hypertension (Abou-Saleh et al., 2013). Studies have also reported an attenuation of VSMC proliferation following blockade of IP_3R (Wilkerson et al., 2006) suggesting the participation of IP_3R-mediated events in hypertension and vascular resistance. Our data obtained using 2-APB suggests that Egr-1 induction may be among the molecular events that underlie the importance of IP_3R in increased agonist-mediated vascular resistance in hypertension. 2-APB has been largely considered as a direct SOCE inhibitor (Peppiatt et al., 2003) and pharmacological inhibition of SOCE using SKF 96465 was recently shown to exert positive effects on blood pressure reduction, Ang-II-induced $[Ca^{2+}]i$ release and LPA-induced VSMC proliferation (Xu et al., 2015). Thus, our data demonstrating that 2-APB decreases Ang-II-induced Egr-1 expression suggest that Egr-1 downregulation may be one of the mechanism by which SOCE blockers exert their vasculoprotective effects.

Although earlier studies have reported that Ca^{2+} plays an important role in Egr-1 expression, the data presented here are the first to report an involvement of STIM-1/Orai-1 in enhancing the transcription of Egr-1 in response to Ang-II. A critical role of STIM-1/Orai-1-mediated SOCE in Ang-II-induced proliferation of VSMC has been reported (Guo et al., 2012). These authors also reported that siRNA-induced silencing of either STIM-1 or Orai-1 reduced Ang-II-induced neointimal growth and intimal thickening in balloon-injured carotid arteries (Guo et al., 2012). Interestingly, Egr-1 expression is enhanced in balloon-injured carotid arteries (Ohtani et al., 2004); thus, it is possible that the reduction of intimal thickening by silencing STIM-1/Orai-1 noted by Guo et al. may be occurring via a decrease in Egr-1 expression (Guo et al., 2012).

An exaggerated expression of STIM-1 and Orai-1 has been observed in the aorta isolated from stroke-prone spontaneously hypertensive rats (Giachini et al., 2009) and Ang-II has been reported to induce the expression of both STIM-1 and Orai-1 in carotid artery neointimal VSMC (Guo et al., 2012). Importantly, the knockdown of STIM-1 or Orai-1 reduced neointimal formation, and was also associated with a decreased VSMC proliferation and migration (Bisaillon et al., 2010). Considering that a similar reduction of Egr-1 either by antisense oligonucleotides or DNAzymes results in attenuation of neointimal growth (Bhindi et al., 2006, Chen et al., 2009, Ohtani et al., 2004), it may be suggested that modulation of Egr-1 expression by STIM-1/Orai-1-induced SOCE plays a key role in vascular damage. Our studies showing that pharmacological blockade of CaM/CaMKII pathways by using W-7 and KN-93 resulted in a significant reduction in Ang-II-induced Egr-1 implicated CaM/CaMKII as a downstream effector of SOCE-induced Ca^{2+} signals in VSMC. CaMKII has been demonstrated to participate in the proliferation of VSMC in response to GPCR ligands and contributes to neointimal growth in animal models of vascular injury (Li et al., 2010, Giachini et al., 2011). Notably, knock down of Orai-1, while reducing neointima formation, was also found to decrease the enhanced levels of CaMKII (Zhang et al., 2011) reinforcing the involvement of CaMKII as an effector of SOCE pathway.

Earlier work has demonstrated that MAP kinase signaling plays a key role in inducing Egr-1 expression in a wide variety of cell types in response to a large number of stimuli (Liu et al., 2013a, Stefano et al., 2007, Youreva and Srivastava, 2016). However, our data demonstrating that siRNA-induced silencing of either STIM-1 or Orai-1 resulted in the suppression of ERKas well as CREB phosphorylation indicated that Ang-II-induced SOCE is crucial to induce ERK and CREB phosphorylation in VSMC. A role of Ca^{2+} and CaM pathway in signaling ERK phosphorylation has been reported earlier, however, the data presented here provide the first evidence supporting a key role of SOCE in Ang-II-induced signaling pathways and reinforce the involvement of ERK signaling in Egr-1 expression in VSMC (Figure 8). Moreover, ERK has been demonstrated to control CREB activation via the mitogen and stress-activated kinase that directly phosphorylates the serine 133 motif on CREB. Our data showing that siRNA-induced silencing of CREB attenuated Ang-II –stimulated Egr-1 expression strengthens the notion that ERK-dependent CREB activation plays a key role in provoking Ang-II-induced signaling events leading to Egr-1 expression. In summary, we have demonstrated that STIM-1/Orai-1- induced SOCE, through ERK/CREB–dependent signaling pathways participates in the expression of Egr-1 in response to Ang-II in VSMC.

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2.7 DISCLOSURES

The authors of this manuscript do not have any conflict of interest to disclose.

2.8 FIGURE LEGENDS

Figure 1: Ang-II induces Egr-1 synthesis and accumulation in A-10 VSMC

Quiescent A-10 cells were stimulated with increasing concentrations of Ang-II for one hour (A) or with 100 nM Ang-II for the indicated time periods (B). Cell lysates were immunoblotted with Egr-1 antibody (top panels in A and B) or β-tubulin (middle panels). Bar diagrams in each section represent average data quantified by densitometric scanning of immunoblots. Bar diagrams represent the densitometric scanning of blots from six independent experiment where the control is defined as 1 and each value aside is expressed as fold increase compared to the control values. *p<0.05, **p<0.01, ***p<0.001 versus control values. C) Quiescent A-10 cells were incubated with 100 nM Ang-II for the indicated time periods. Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. ** p<0.01 versus control values from four independent experiments. D) Cells were treated with 100 nM Ang-II for the indicated time periods, fixed and stained with anti-Egr-1 antibody (green signal). Nuclei were stained with DAPI (blue signal). Merged pictures show the DAPI-stained image superimposed on the Egr-1-stained image.

Figure 2: Attenuation of Ang-II-induced Egr-1 upregulation by 2-APB in A-10 VSMC

A) Quiescent A-10 cells were pre-treated with increasing concentrations of 2-APB for 30 minutes, followed by stimulation with 100 nM Ang-II for one hour. Cell lysates were probed with Egr-1 antibody (top panel) and β-tubulin (middle panel). Bar diagrams represent the densitometric quantifications of Egr-1 blots from five independent experiments. Values are expressed as fold increase compared to the control value (CTL) defined as 1. *p<0.05 versus control values; #p<0.05 versus VSMC treated with Ang-II alone. B) Quiescent A-10 cells were treated without (CTL) or with 2-APB (50 μM) for 30 minutes followed by stimulation with Ang-II for one hour. Analysis of relative Egr-1

mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. ***p<0.001 versus control values. #p<0.001 versus VSMC treated with Ang-II alone. C) Cells were treated without (CTL) or with 2-APB (50 μM) for 30 minutes followed by stimulation with Ang-II for one hour. Cells were fixed and stained with anti-Egr-1 antibody (green signal). Nuclei were stained with DAPI (blue signal). Merged pictures show the DAPI-stained image superimposed on the Egr-1-stained image.

Figure 3: Attenuation of Ang-II-induced [Ca^{2+}]i mobilization by 2-APB in A-10 VSMC

Quiescent VSMC were labeled with Fura 2-AM prior to treatments and imaging was conducted by alternating excitation wavelengths to excite Ca^{2+}-bound Fura 2 (340 nm) and Ca^{2+}-free Fura 2 (380 nm). The corresponding emissions were recorded and analyzed for the Fura 2 ratio (F340/F380). Graph in A represents the average measures of intracellular ratios F340/F380 obatined after stimulation with either 100 nM Ang-II alone (Black) or 50 μM 2-APB (Blue), prior to Ang-II stimulation. The bar diagrams in B correspond to measurements from 10 cells from selected regions. *** p<0.001.

Figure 4: Knockdown of STIM-1 or Orai-1 inhibited Ang-II-induced Egr-1 expression in A-10 VSMC.

Cells were transfected with 10 nM STIM-1 siRNA (siSTIM-1), 10 nM Orai-1 siRNA (siORAI-1) or 10 nM control siRNA (siSCR) prior to stimulation with 100 nM Ang-II for one hour. Cell lysates were immunoblotted with STIM-1 (top panel in A), Orai-1 (top panel in B), Egr-1 (middle panels in A and B) or β-tubulin. Bar diagrams in C, D, E and F represent average data quantified by densitometric scanning of immunoblots from six independent experiments. Values are expressed as fold increase compared to the control value (CTL) defined as 1. *p<0.05, **p<0.01, ***p<0.001 versus CTL. #p<0.05, ###p<0.001 versus siSCR+Ang-II.

Figure 5: Pharmacological blockade of calmodulin and CaMKII inhibited Ang-II-induced Egr-1 expression in A-10 VSMC

Quiescent A-10 cells were pre-treated with or without 10 μM of the calmodulin inhibitor W-7 (A) or the CaMKII inhibitor KN-93 (B), as well as its inactive analog KN-92 for 30 minutes, followed by stimulation with 100 nM Ang-II for one hour. Cell lysates were probed with Egr-1 antibody (top panels in A and B) or β-tubulin (middle panels). Bar diagrams in each section represent average data quantified by densitometric scanning of immunoblots from three independent experiements. The control is defined as 1 and each value aside is expressed as fold increase compared to the control value defined as 1. *p<0.05, ***p<0.001 versus CTL values. #*p*<0.05, ###*p*<0.001 versus VSMC treated with Ang-II alone.

Figure 6: STIM-1 and Orai-1 are required for Ang-II–induced activation of ERK1/2 and CREB in A-10 VSMC

Cells were transfected with 10 nM STIM-1 siRNA (siSTIM-1), 10 nM Orai-1 (siORAI-1) or 10 nM control siRNA (siSCR) prior to stimulation with 100 nM Ang-II for five minutes. A and B show immunoblotting of cell lysates with antibodies corresponding to ERK1/2 and CREB respectively phosphorylated on Thr202/Tyr204 and Ser133. Blots were also analyzed for total ERK and β-tubulin. Bar diagrams in C, D, E and F represent average data quantified by densitometric scanning of phospho-ERK1/2 and phospho-CREB immunoblots from six independent experiments. Values are expressed as fold increase compared to the control value (CTL) defined as 1. **p*<0.05, ***p*<0.01, ****p*<0.001 versus CTL. #*p*<0.05, ##*p*<0.01, ###*p*<0.001, versus siSCR+Ang-II.

Figure 7: Activation of ERK1/2 and CREB is required to regulate Ang-II-induced Egr-1 expression in A-10 VSMC

A) Quiescent A-10 cells were pre-treated with or without U0126 (10 μM) followed by stimulation with 100 nM Ang-II for five minutes. Cell lysates were immunoblotted with an antibody corresponding to CREB phosphorylated on Ser133 (top panel). The blots were

also analyzed for total CREB (middle panel). B) Quiescent A-10 cells were pre-treated with or without U0126 (10 μM) followed by stimulation with 100 nM Ang-II for one hour. Cell lysates were immunoblotted with Egr-1 (top panel) or β-tubulin (middle panel). Bar diagrams represent average data quantified by densitometric scanning of immunoblots from three independent experiments.Values are expressed as fold increase compared to the control value (CTL) defined as 1. ** $p<0.01$, *** $p<0.001$, versus CTL values. ## $p<0.01$, ###p<0.001, versus sample with Ang-II alone. C) Cells were transfected with 10 nM CREB siRNA (siCREB) prior to stimulation with 100 nM Ang-II for one hour. Cell lysates were immunoblotted with Egr-1, total CREB or β-tubulin. Bar diagrams represent average data quantified by densitometric scanning of Egr-1 immunoblots from six independent experiments. Values are expressed as fold increase compared to the control value (CTL) defined as 1. ** $p<0.01$ versus CTL. ## $p<0.01$ versus siSCR+Ang-II.

Figure 8: Schematic model of the involvement of STIM-1/Orai-1 and Ca^{2+} signaling in Ang-II-induced Egr-1 expression in A-10 VSMC

Ang-II binds to its G-protein coupled receptor, activates the Gq subunit and increases the intracellular levels of inositol-1,4,5-trisphosphate (IP_3) through a phospholipase C-β(PLC-β)-dependent hydrolysis of phosphatidylinositol-,4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and IP_3. IP_3 binds to its ligand-activated receptor (IP_3R) located within the endoplasmic reticulum (ER) membrane and triggers Ca^{2+} efflux inside the cytosol. Resulting ER Ca^{2+} depletion activates the stromal interaction molecule 1 (STIM1) known to mediate Ca^{2+} entry via a change in conformation that results in its accumulation near the plasma membrane, where it can activate Orai-1 calcium channels. Calcium diffusion from the extracellular compartment through Orai-1 channels results in heightened intracellular Ca^{2+} that binds to calmodulin (CaM) and interacts with CaMKII further leading to ERK1/2 and CREB activation and subsequent induction of Egr-1.

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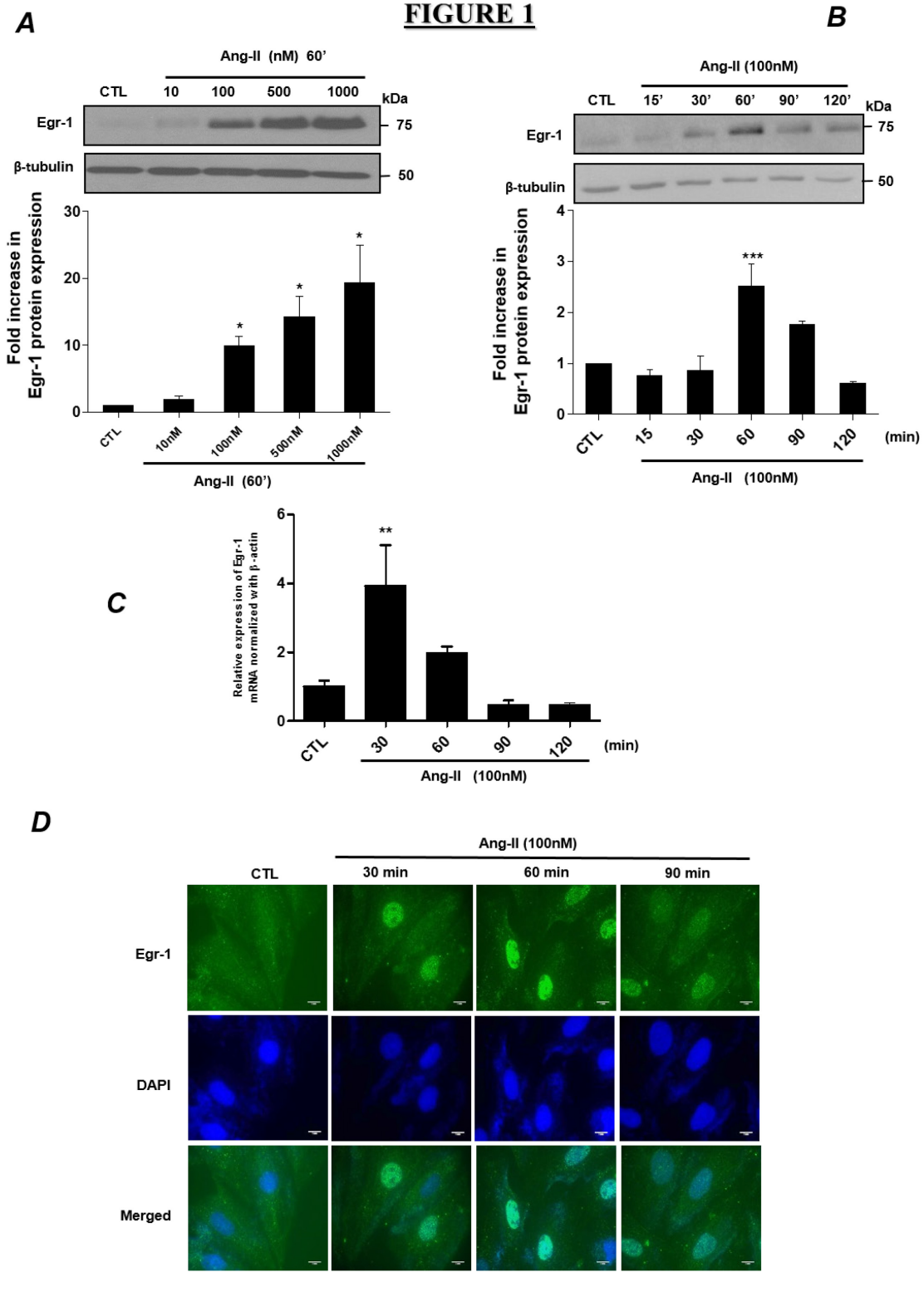
2.10 FIGURES

FIGURE 1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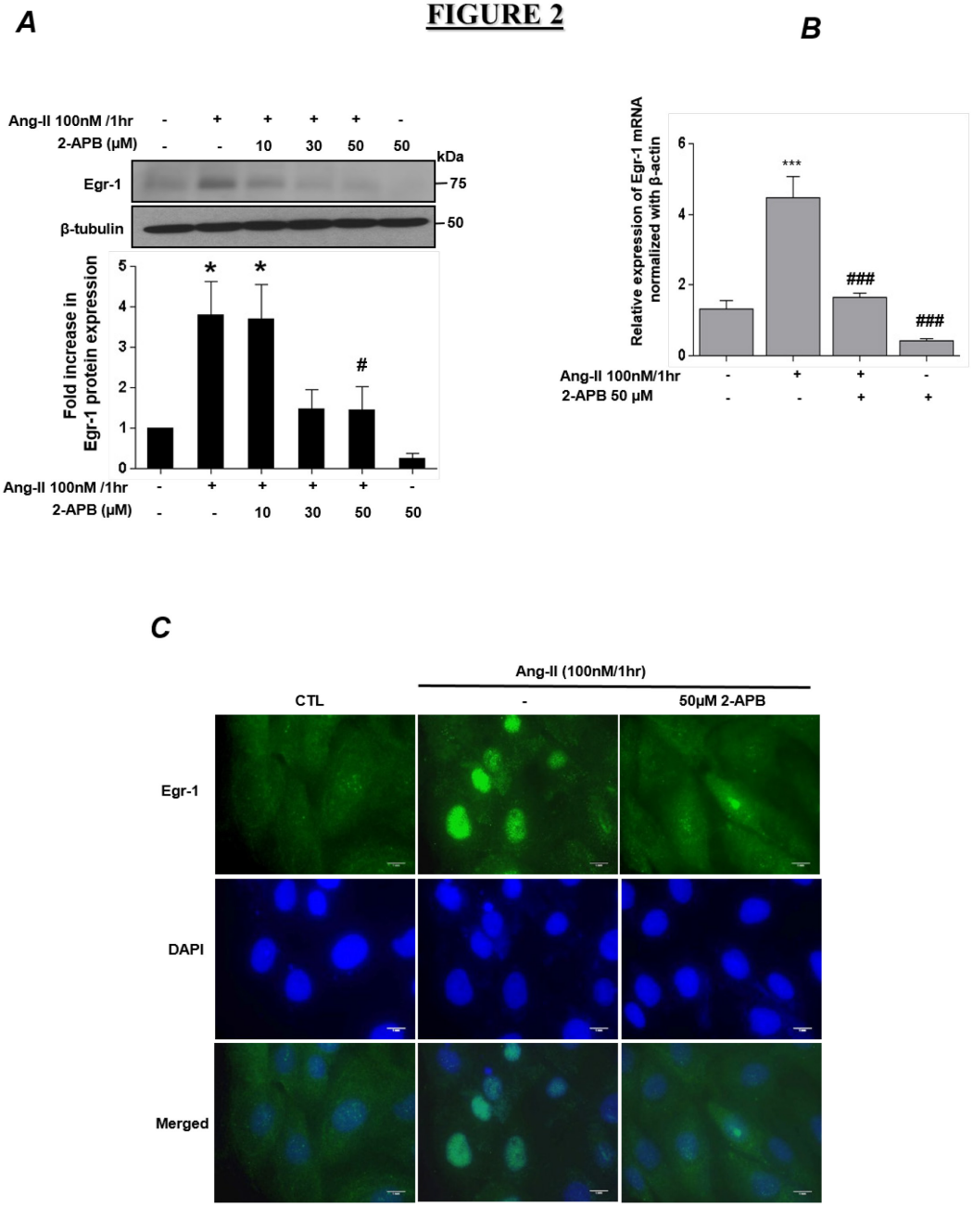
10
100
500
1000
kDa
Egr-1
75
β-tubulin
50
Fold increase in Egr-1 protein expression
30
20
10
0
*
*
*
CTL
10nM
100nM
500nM
1000nM
Ang-II (60')
B
Ang-II (100nM)
CTL
15'
30'
60'
90'
120'
kDa
Egr-1
75
β-tubulin
50
Fold increase in Egr-1 protein expression
4
3
2
1
0
***
CTL
15
30
60
90
120
(min)
Ang-II (100nM)
C
Relative expression of Egr-1 mRNA normalized with β-actin
6
4
2
0
**
CTL
30
60
90
120
(min)
Ang-II (100nM)
D
Ang-II (100nM)
CTL
30 min
60 min
90 min
Egr-1
DAPI
Merged

FIGURE 2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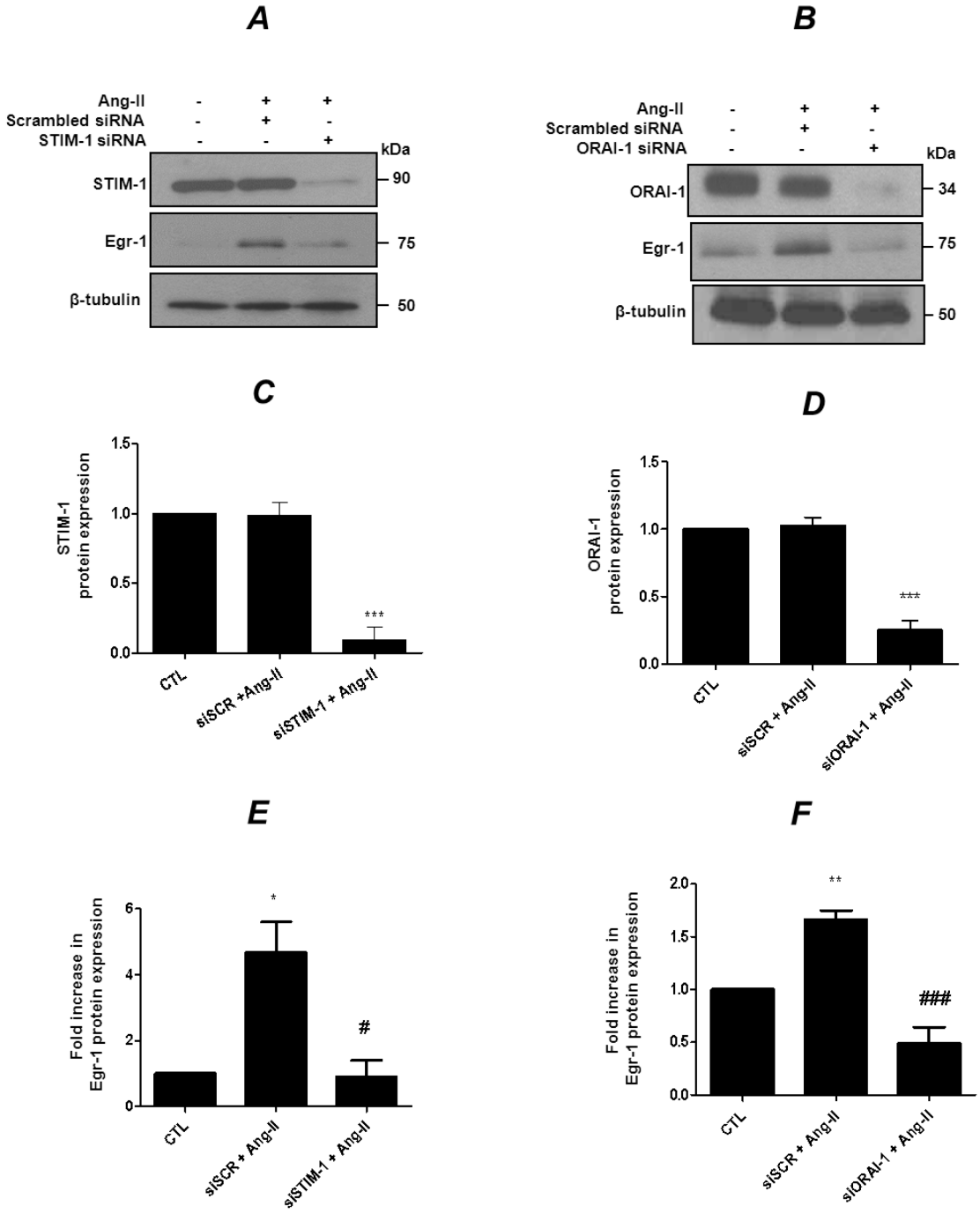
kDa
Egr-1
75
β-tubulin
50
Fold increase in Egr-1 protein expression
Ang-II 100nM /1hr - + + + + -
2-APB (μM) - - 10 30 50 50
B
Relative expression of Egr-1 mRNA normalized with β-actin
***
###
###
Ang-II 100nM/1hr - + + -
2-APB 50 μM - - + +
C
Ang-II (100nM/1hr)
CTL
-
50μM 2-APB
Egr-1
DAPI
Merged

FIGURE 4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STIM-1 siRNA - - +
kDa
STIM-1
90
Egr-1
75
β-tubulin
50
B
Ang-II - + +
Scrambled siRNA - + -
ORAI-1 siRNA - - +
kDa
ORAI-1
34
Egr-1
75
β-tubulin
50
C
STIM-1 protein expression
1.5
1.0
0.5
0.0
***
CTL
siSCR +Ang-II
siSTIM-1 + Ang-II
D
ORAI-1 protein expression
1.5
1.0
0.5
0.0
***
CTL
siSCR + Ang-II
siORAI-1 + Ang-II
E
Fold increase in Egr-1 protein expression
6
4
2
0
*
#
CTL
siSCR + Ang-II
siSTIM-1 + Ang-II
F
Fold increase in Egr-1 protein expression
2.0
1.5
1.0
0.5
0.0
**
###
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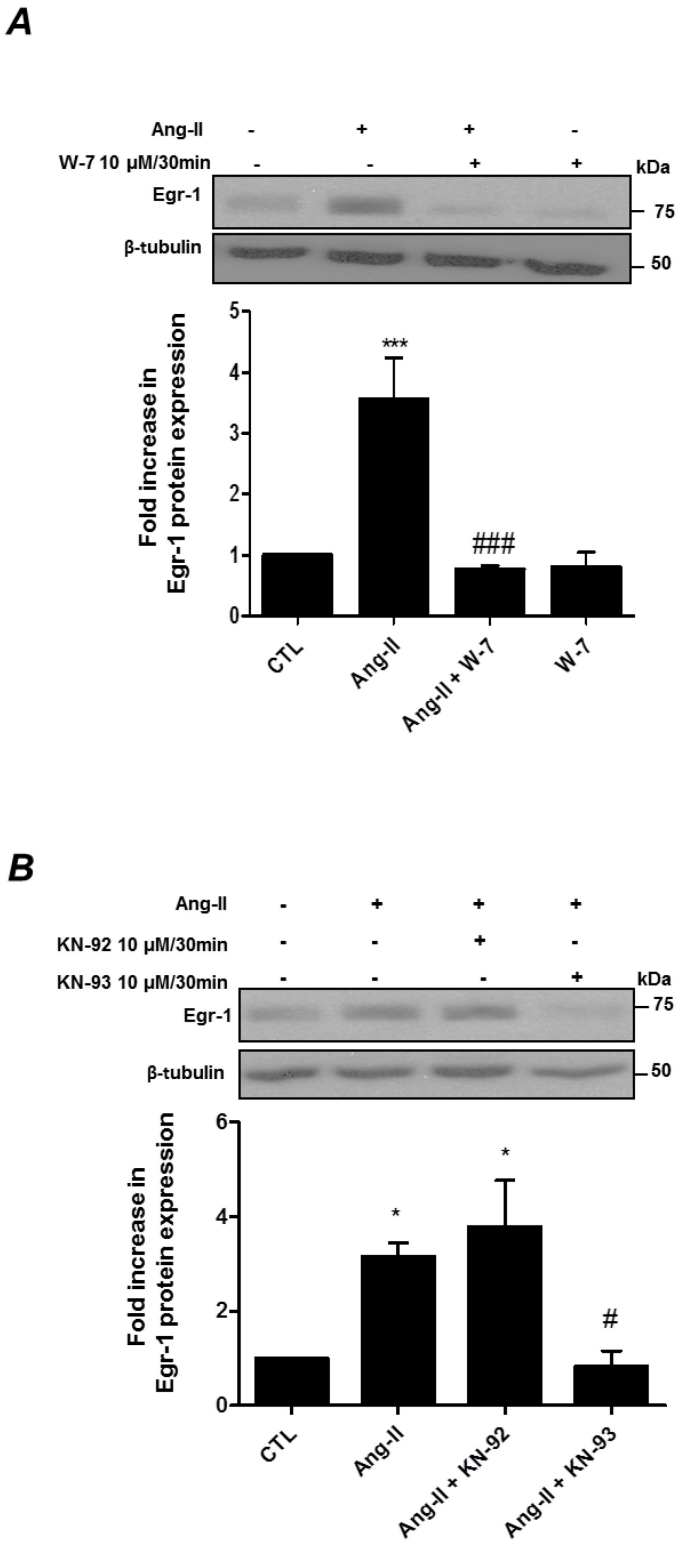
siSCR + Ang-II
siORAI-1 + Ang-II

FIGURE 5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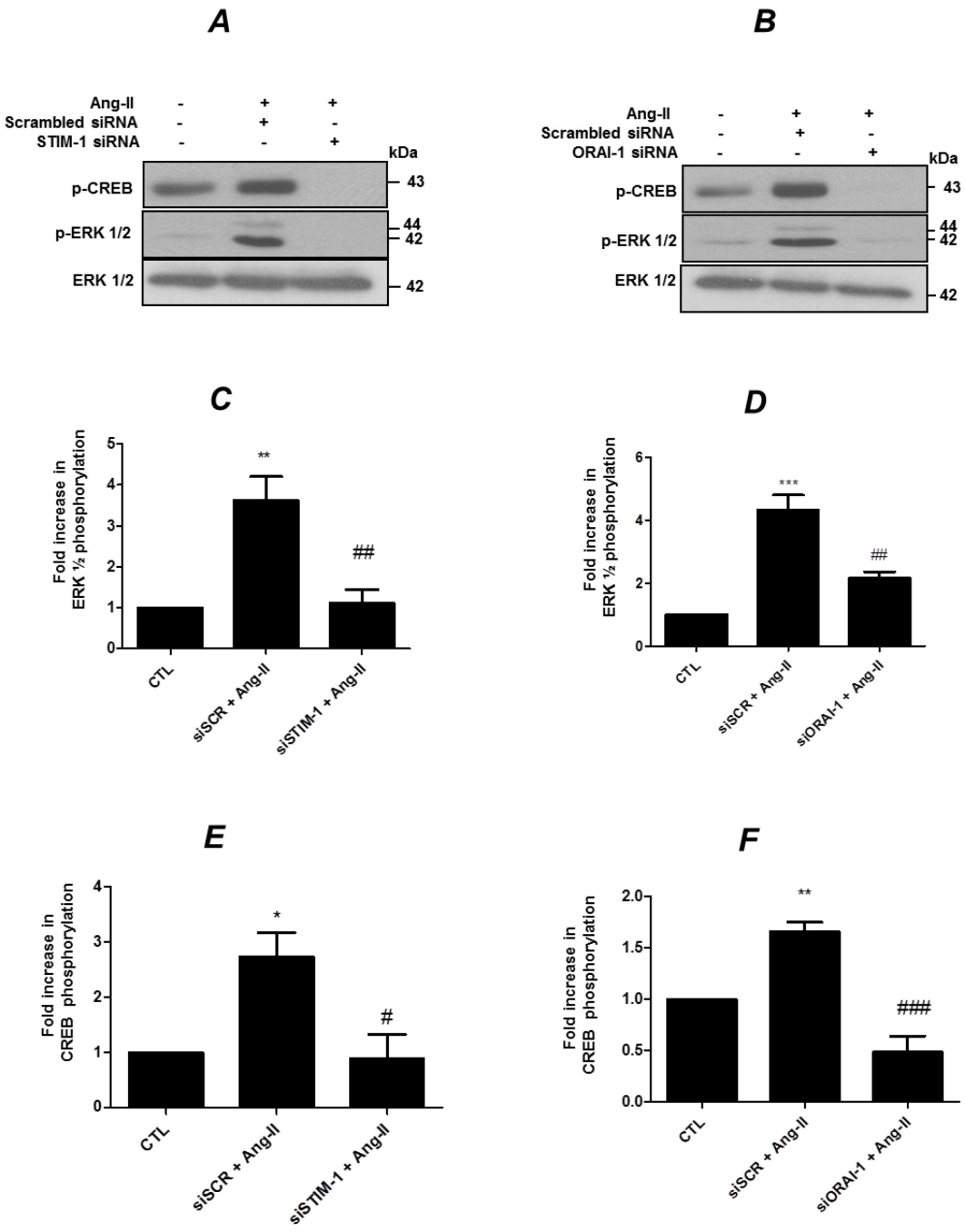
Ang-II - + + -
W-7 10 µM/30min - - + +
kDa
Egr-1
75
β-tubulin
50
Fold increase in Egr-1 protein expression
***
###
CTL
Ang-II
Ang-II + W-7
W-7
B
Ang-II - + + +
KN-92 10 µM/30min - - + -
KN-93 10 µM/30min - - - +
kDa
Egr-1
75
β-tubulin
50
Fold increase in Egr-1 protein expression
*
*
#
CTL
Ang-II
Ang-II + KN-92
Ang-II + KN-93

FIGURE 6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43
p-ERK 1/2
44
42
ERK 1/2
42
B
Ang-II - + +
Scrambled siRNA - + -
ORAI-1 siRNA - - +
kDa
p-CREB
43
p-ERK 1/2
44
42
ERK 1/2
42
C
Fold increase in ERK ½ phosphorylation
**
##
CTL
siSCR + Ang-II
siSTIM-1 + Ang-II
D
Fold increase in ERK ½ phosphorylation
***
##
CTL
siSCR + Ang-II
siORAI-1 + Ang-II
E
Fold increase in CREB phosphorylation
*
#
CTL
siSCR + Ang-II
siSTIM-1 + Ang-II
F
Fold increase in CREB phosphorylation
**
###
CTL
siSCR + Ang-II
siORAI-1 + Ang-II

FIGURE 7

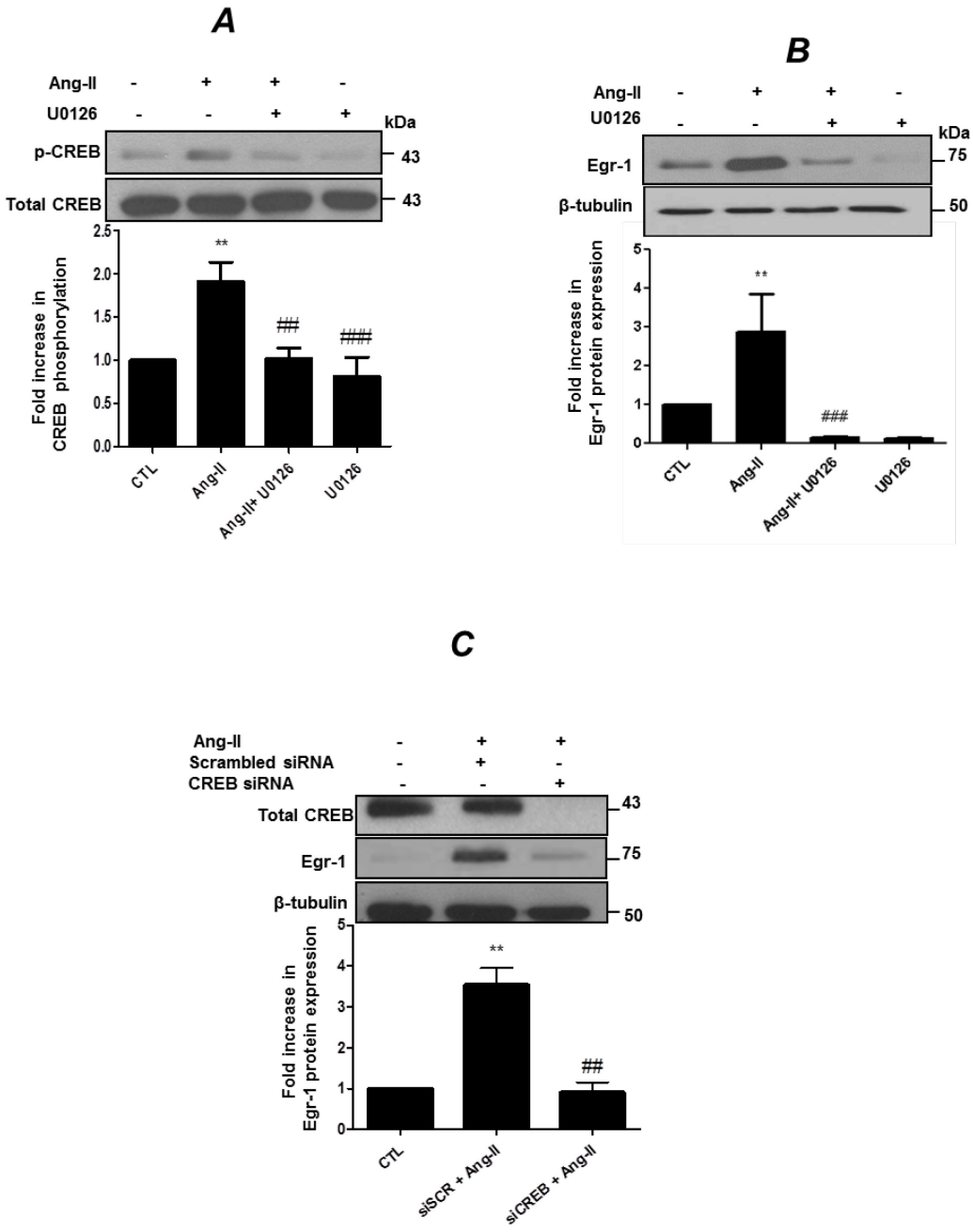
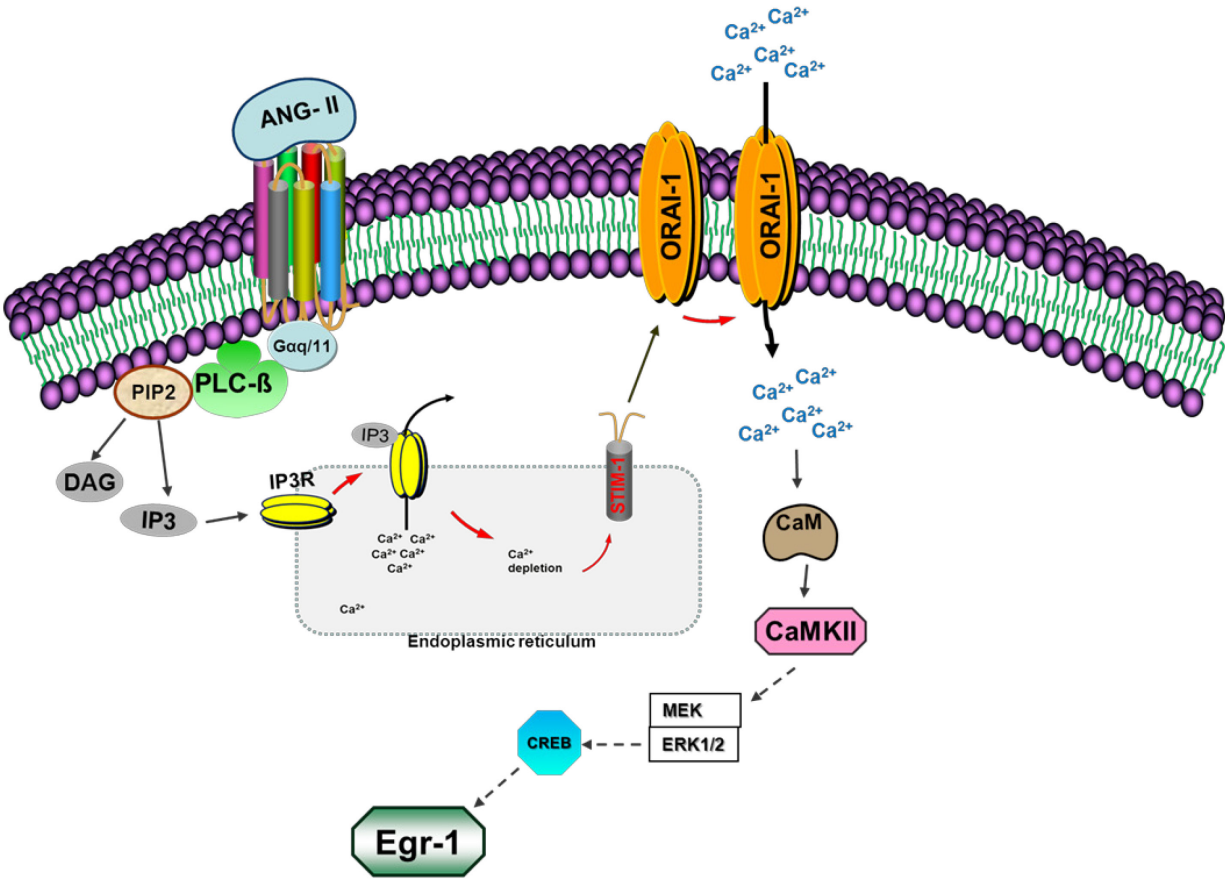

A
Ang-II - + + -
U0126 - - + +
kDa
p-CREB
43
Total CREB
43
Fold increase in CREB phosphorylation
2.5
2.0
1.5
1.0
0.5
0.0
**
##
###
CTL
Ang-II
Ang-II+ U0126
U0126
B
Ang-II - + + -
U0126 - - + +
kDa
Egr-1
75
β-tubulin
50
Fold increase in Egr-1 protein expression
5
4
3
2
1
0
**
###
CTL
Ang-II
Ang-II+ U0126
U0126
C
Ang-II - + +
Scrambled siRNA - + -
CREB siRNA - - +
Total CREB
43
Egr-1
75
β-tubulin
50
Fold increase in Egr-1 protein expression
5
4
3
2
1
0
**
##
CTL
siSCR + Ang-II
siCREB + Ang-II

FIGURE 8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PIP2
DAG
IP3
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STIM-1
Ca2+ depletion
Endoplasmic reticulum
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ORAI-1
Ca2+
CaM
CaMKII
MEK
ERK1/2
CREB
Egr-1

Chapter 3 (Article 2): cAMP attenuates angiotensin-II-induced Egr-1 expression via PKA-dependent signaling pathway in vascular smooth muscle cells.

3 cAMP attenuates angiotensin-II-induced Egr-1 expression via PKA-dependent signaling pathway in vascular smooth muscle cells

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ABSTRACT

Aberrant VSMC proliferative responses contribute to the development of intimal lesion. cAMP has been shown to inhibit vascular smooth muscle cell proliferation and exerts a vasculoprotective effect. An upregulation of the early growth response protein-1 (Egr-1) expression has been linked with the development of atherosclerosis and intimal hyperplasia. We have recently demonstrated that angiotensin-II (Ang-II) stimulates Egr-1 expression via $Ca^{2+/}$ERK-mediated cAMP-response element binding protein (CREB) activation. However, whether Ang-II-induced signaling leading to Egr-1 expression is modulated by cAMP remains unexplored. Therefore, in the present studies, we have examined the effect of cAMP on Ang-II-induced expression of Egr-1 and associated signalling pathways. Isoproterenol (ISO) and forskolin (FSK) attenuated Ang-II-induced Egr-1 expression in a dose-dependent fashion. In addition, dibutyryl-cAMP and benzoyl-cAMP, as well as isobutylmethylxanthine, attenuated Ang-II-induced Egr-1 expression. Moreover, inhibition of Ang-II-induced Egr-1 expression was accompanied by an increase in the phosphorylation of the vasodilator-activated phosphoprotein (VASP), and this was associated with a concomitant decrease in ERK phosphorylation. Blockade of PKA using H89 decreased VASP phosphorylation, restored Ang-II-induced ERK phosphorylation, and abolished ISO- and FSK-mediated inhibition of Ang-II-induced Egr-1 expression. In summary, these results suggest that PKA-mediated suppression of Ang-II-induced Egr-1 expression and phosphorylation of ERK may be among the mechanisms by which cAMP exerts its vasculoprotective effects.

3.1 INTRODUCTION

Aberrant proliferation and migration of vascular smooth muscle cells (VSMC) are believed to contribute to abnormal vascular function leading to the pathogenesis of vascular diseases (Rivard and Andres, 2000). These events are promoted by elevated concentrations of vasoactive peptides such as angiotensin-II (Ang-II) which plays an important role in vascular function (Montezano et al., 2014; Nakashima et al., 2006). At the cellular level, exaggerated stimulation by Ang-II results in a phenotypic switch of VSMC that progressively differentiate from a contractile state to a synthetic state (Campbell and Campbell, 1985). Synthetic VSMC are characterized by hyperproliferative properties and enhanced migratory capacities (Potier et al., 2009). These features induce pathological neointima formation and subsequent vessel narrowing. At the signaling level, elevated levels of Ang-II mediate the hyperactivation of growth promoting signaling pathways involving the mitogen-activated protein kinase (MAPK)-mediated cascade and related transcriptional events (Touyz and Berry, 2002) that contribute to the regulation of genes implicated in vascular remodeling (Cipolletta et al., 2010; Duff et al., 1995; Hartney et al., 2011).

Enhanced levels of 3'-5'-cyclic adenosine monophosphate (cAMP) produced via adenylate cyclase (AC) activity have been reported to antagonize vasoactive peptide- and mitogen-induced proliferative responses in VSMC (Begum et al., 2011; Graves et al., 1993; Hewer et al., 2011). One of the major effector involved in transducing cAMP-induced events is protein kinase A (PKA). The β-agonist isoproterenol (ISO), known to increase cAMP via G-protein-coupled receptor (GPCR)-mediated AC activation, has been demonstrated to inhibit Ang-II-induced VSMC proliferation (Kim et al., 2009). In accordance with this, functional abnormalities in the coupling of β-adrenergic receptor agonism are associated with alterations in the signaling events regulating VSMC proliferation (Gros et al., 2006). Impairments in cAMP-induced signaling have also been correlated with a rise in blood pressure and related dysfunctional features in VSMC (Shahid et al., 2010).

An involvement of the early growth response protein-1 (Egr-1), a zinc finger containing transcription factor, in the pathogenesis of vascular diseases has recently been demonstrated in experimental models of atherosclerosis and vascular injury (Khachigian, 2006; Ohtani et al., 2004; Santiago et al., 1999; Vazquez-Padron et al., 2010). The presence of Egr-1 binding motifs within the structure of several pro-atherogenic genes has suggested that Egr-1 modulates vascular physiology in response to several stimuli (McCaffrey et al., 2000). Growth-promoting stimuli and several vasoactive peptides have been demonstrated to enhance Egr-1 expression and activity in VSMC and other vascular cells (Bouallegue et al., 2013; Iyoda et al., 2012; Liu et al., 2013; Youreva et al., 2013; Youreva and Srivastava, 2016). With regard to the molecular mechanisms underlying Egr-1 expression, we recently demonstrated that Ang-II upregulates Egr-1 levels in VSMC via a pathway that involves calcium signaling components upstream of $ERK_{1/2}$-mediated molecular events (Simo-Cheyou et al., 2017).

Previous studies showing that deletion of Egr-1 suppresses the vasculoprotective effects of cAMP elevating agents in VSMC have suggested a link between cAMP-dependent signaling and Egr-1 expression and activity (Kimura et al., 2014). However, the molecular mechanism by which cAMP-mediated pathway can modulate Ang-II-induced signaling cascade leading to Egr-1 expression in VSMC remains unexplored. Therefore, in the present studies, we have examined the effects of an elevation in the intracellular levels of cAMP on Ang-II–induced signaling upstream of Egr-1 expression in VSMC.

3.2 MATERIALS AND METHODS

Antibodies and reagents

Ang-II (#A9525), forskolin (#F6886), dibutyryl-cAMP (#D0260), were purchased from Sigma-Aldrich (St. Louis, MO, USA). N6-Benzoyl-adenosine 3', 5'-cyclic monophosphate sodium salt (BNZ) (#116802) was purchased from EMD Millipore (Etobicoke, ON, Canada). Phosphorylated $ERK_{1/2}$ (#SC16982-R) antibody was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Egr-1 (#4153S), total (#3112S) and

Ser257 phosphorylated VASP (#3111S), β-tubulin (#2146S), total (#9272) and anti-rabbit (#7074S) antibodies were purchased from Cell Signaling (Beverly, Massachusetts, USA). The enhanced chemiluminescence (ECL) detection system kit was purchased from Perkin Elmer (Montreal, QC, Canada).

Cell culture

VSMC derived from the medial layer of rat thoracic aortae (A-10 cell line (CRL-1476) from ATCC, Manassas, USA) were maintained in culture with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37ºC in a humidified atmosphere of 5% CO_2, as described earlier (Bouallegue et al., 2007). Cells between passages 4 and 8 were grown to 80-90% confluence in 60-mm dishes and incubated in serum and antibiotic-free DMEM 5 hours prior to treatments.

Cell lysis and immunoblotting

Quiescent A-10 VSMC were incubated in the absence or presence of various reagents for 30 minutes followed by stimulation with 100 nM Ang-II for indicated time periods. The cells were washed three times with ice-cold PBS and lysed in 100 µL radio-immunoprecipitation (RIPA) buffer. 35-45µg of proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilion-P polyvinylidinedifluoride membranes (Millipore, USA) and incubated with respective primary antibodies. The antigen-antibody complex was detected by horseradish peroxidase-conjugated secondary anti-rabbit and protein bands were visualized with ECL kit. The intensity of the bands was quantified by densitometric analysis using Quantity One Bio-Rad Corp. imaging and Graphpad Prism 5 (San Diego, CA, USA) software programs.

Preparation of cDNA

Following incubations, total RNA was isolated with Trizol Reagent (Life Technologies, Burlington, ON). RNA concentration was quantified with the Biophotometer (Eppendorf, Mississauga, ON). Absorbances were measured at wavelengths of 260 nm and 280 nm. The purity of RNA preparation was confirmed when the ratio A260/A280 was comprised in the range 1.8-2.0. cDNA was synthesized from 1

μg of total pure RNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Grand Island, NY) as per manufacturer's instructions.

Real-time quantitative polymerase chain reaction (qRT-PCR)

qRT-PCR was performed with SYBG (Life Technologies, Grand Island, NY, USA) using 1μL of cDNA in a 20 μL reaction. Amplification was performed using 7500 fast RT-PCR system (Applied Biosystems, Grand Island, NY). Sequences used to design Egr-1 primers were as follows: forward 5'-CTGCTTCATCGTCTTCCTCTG-3' and reverse 5'-GTCAGTGTTGGGAGTAGGAAAG-3'. Egr-1 mRNA expression was measured and normalized with β-actin mRNA levels using primers: forward 5'-TCTTCCAGCCTTCCTTCCT-3' and reverse 5'-CAGCACTGTGTTGGCATAGA-3'.

Statistics

Statistical analysis was performed by one-way, standard analysis of variance (ANOVA) in conjunction with a Tukey post hoc test. All data are expressed as mean ± SEM of independent experiments. The differences between means were considered statistically significant at $p< 0.05$.

3.3 RESULTS

Isoproterenol attenuates Ang-II-induced expression of Egr-1 in A-10 VSMC

ISO inhibits Ang-II-induced VSMC proliferation and mitogenic signaling in a heme oxygenase-dependent pathway via β2 adrenoreceptor-mediated PKA activity (Kim et al., 2009). We have shown earlier that Ang-II is potent inducer of Egr-1 expression in VSMC (Simo-Cheyou et al., 2017) and, since serum-induced expression of Egr-1 was reported to be downregulated by cAMP-induced signaling in VSMC (Kimura et al., 2014), we examined the effect of ISO on Ang-II-induced Egr-1 expression. As shown in Figure 1, treatment of VSMC with different concentrations of ISO attenuated Ang-II-induced expression of Egr-1 in a dose-dependent fashion with the maximal inhibitory effect observed at 10μM (Fig.1A). Similar to the Egr-1 protein levels, treatment of VSMC with ISO reduced the Ang-II-induced increase in Egr-1 mRNA levels in VSMC (Fig.1B).

Adenylate cyclase (AC) activation by forskolin mimics the effect of isoproterenol on Ang-II-induced upregulation of Egr-1

Gαs-coupled receptor ligands activate AC and thereby elevate the intracellular levels of cAMP. A rise in intracellular cAMP has been reported following treatment of VSMC with ISO (Zhang et al., 1997) and anti-mitogenic properties are attributed to cAMP in VSMC (Kimura et al., 2014). Thus, to confirm the involvement of cAMP in ISO-mediated response, it was of interest to test whether a non-receptor-mediated activation of AC would exhibit a similar response on the regulation of Ang-II-induced Egr-1 expression. Therefore, we used the AC activator forskolin (FSK), and, as shown in Figure 2A, pre-treatment of VSMC with increasing concentrations of FSK resulted in a significant attenuation of Ang-II-induced upregulation of Egr-1 in a dose-dependent fashion. Treatment with FSK alone did not exert any significant effect as compared to untreated cells where Egr-1 protein was barely detectable (Fig.2A). Total β-tubulin protein levels were not affected by FSK treatment. Similar to isoproterenol, FSK induced an attenuation of Ang-II-induced upregulation of Egr-1 mRNA (Fig.2B). These data suggest the involvement of cAMP in ISO-induced downregulation of Ang-II-induced Egr-1 expression.

Isoproterenol-mediated decrease in Ang-II-induced Egr-1 expression is potentiated by phosphodiesterase inhibition and inversely correlates with VASP phosphorylation

By catalyzing the transformation of cAMP into AMP, phosphodiesterase (PDE) activity reduces the level of intracellular cAMP. PDE inhibitors delay this degradation and thus prolong the presence of cAMP at elevated concentrations in cells treated with cAMP elevating agents. Therefore, to further define the role of cAMP in ISO-mediated response, it was of interest to test the effect of PDE inhibition on Ang-II-induced Egr-1 expression in the presence or absence of ISO. Cells were pre-treated with 10μM of isobutylmethylxanthine (IBMX), a broad spectrum PDE inhibitor, prior to stimulation with Ang-II. As shown in Figure 3A, heightened levels of Egr-1 observed in response to Ang-II were attenuated by pre-treatment with IBMX suggesting that increase in cAMP levels by PDE inhibition can also attenuate Ang-II-induced signaling leading to Egr-1 expression (Fig.3A). Moreover, simultaneous addition of IBMX along with ISO resulted in a further decrease in Ang-II-induced Egr-1 expression (Fig.3B). Together, these data demonstrated that cAMP plays a key role in ISO-mediated inhibition of Ang-II-induced Egr-1 expression. In addition, since the phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) has been used as a marker of cAMP signaling and protein kinase A activation (Butt et al., 1994; Eckert and Jones, 2007; Harbeck et al., 2000; Joshi et al., 2011), it was of interest to examine the patterns of VASP phosphorylation associated with reduced Egr-1 levels. We therefore used an antibody able to detect both the 46 kDa total VASP protein and VASP phosphorylated at serine 157 exhibiting a shift at 50kDa due to phosphorylation-induced altered electrophoretic mobility (Butt et al., 1994; Harbeck et al., 2000). Figure 4A and B show that IBMX does not trigger a detectable increase in phosphorylation of VASP as compared to the unstimulated cells (Fig.4A and 4B). However, IBMX-mediated attenuation of Ang-II-induced Egr-1 expression is accompanied with an induction of VASP phosphorylation as measured by the ratio of serine 157 phosphorylated VASP over the total protein (Fig.4A). Furthermore, similar to the additive inhibitory effect on Egr-1 expression, simultaneous treatment with ISO and IBMX resulted in a more robust increase in Ser157 phosphorylation of VASP as compared with individual

treatment with either ISO or IBMX (Fig.4B). Taken together, these data demonstrate that sustained elevation of cAMP results in a more potent VASP phosphorylation that correlates with a potentiation of isoproterenol-mediated inhibitory effect on Ang-II-induced Egr-1 expression.

PKA signaling exerts an inhibitory effect on Ang-II-induced Egr-1 expression

Because increased VASP phosphorylation on serine 157 corresponds to an enhanced PKA activity in several types of cells and since PKA activity interferes with proliferative responses in VSMC (Hewer et al., 2011), we next sought to assess the role of PKA in the attenuation of Ang-II-induced Egr-1 expression. For this purpose, we examined the effects of the cell permeable analog of cAMP, dibutyryl cAMP (Db-cAMP) and a cAMP analog that specifically activates PKA in the cells, benzoyl-cAMP (BNZ-cAMP). As depicted in Figure 5, similar to ISO and FSK, both Db-cAMP and BNZ-cAMP attenuated Ang-II-induced Egr-1 expression in a dose-dependent fashion (Fig.5A) suggesting the contribution of PKA in downregulating Ang-II-induced Egr-1 expression (Fig.5B).

Blockade of PKA restores Ang-II-induced Egr-1 upregulation in the presence of ISO or FSK

Next, to further confirm the involvement of PKA in cAMP-induced suppression of Ang-II-induced Egr-1 expression, we investigated the consequence of pharmacological blockade of PKA on ISO- and FSK-mediated inhibitory responses. Cells were treated with 10μM of the PKA inhibitor H89, prior to ISO or FSK pre-treatments followed by Ang-II stimulation for one hour. As depicted in Figure 6 and Figure 7, whereas the increase in Egr-1 observed in the presence of Ang-II is totally blunted by ISO (Fig.6A) and FSK (Fig.7A), blockade of PKA with H89 restored it to an even higher level than that observed with Ang-II alone (Figs.6A and 7A). Interestingly, in the absence of ISO, PKA blockade also resulted in a potentiation of Egr-1 induction suggesting a high basal activity of PKA in A-10 VSMC. A similar effect of H89 treatment on the reversal of Egr-1 mRNA expression in the presence of ISO and FSK was also observed (Figs. 6B and 7B). These data indicated that PKA plays a critical role in transducing GPCR-activated or non-receptor-mediated

increase in cAMP leading to the suppression of Egr-1 expression in response to Ang-II in VSMC.

ISO or FSK-mediated phosphorylation of VASP is associated with suppression of Ang-II-induced ERK1/2 phosphorylation

Since our data demonstrated the participation of PKA in ISO- and FSK-mediated attenuation of Ang-II-induced Egr-1 expression, we sought to determine the signalling changes accompanying this response. ERK-mediated signalling pathways play a key role in mediating the migratory and proliferative responses in VSMC (Xi et al., 1999) and, has been implicated in Egr-1 induction in response to Ang-II as well as several growth-promoting stimuli in VSMC (Cui et al., 2006; Liu et al., 2013; Simo-Cheyou et al., 2017; Youreva and Srivastava, 2016). Therefore, based on our results showing that FSK and ISO- mediated suppression of Ang-II-induced Egr-1 expression was associated with an increase in the serine 157 phosphorylation of VASP (Fig.3), it was of interest to examine the effect of H89-mediated blockade of PKA on the degree of phosphorylation of VASP and ERK1/2 in response to the combination of Ang-II and ISO or FSK stimulation. Pre-treatments were made prior to Ang-II stimulation for 5 minutes. Results show that addition of ISO (Top panel Fig.8A) or FSK (Top panel Fig.9A) alters the mobility of total VASP (46 kDa to 50 kDa) depicting an increase in ser157 phosphorylation, and this increase was accompanied by a reduction in Ang-II-induced ERK1/2 phosphorylation (Middle panels Fig.8A and 9A). H89 treatment decreased VASP phosphorylation with a concomitant restoration of Ang-II-induced ERK1/2 phosphorylation (Fig.8A, C and 9A, C) suggesting that PKA-mediated downregulation of Egr-1 is preceded by a decrease in Ang-II-induced ERK1/2 phosphorylation. Interestingly, similar to Egr-1 expression, Ang-II-induced ERK1/2 phosphorylation was potentiated by blockade of PKA and this correlated with lower levels of serine 157 phosphorylation VASP (Figs.8B and 9B). These data suggest that PKA mediates the effects of ISO and FSK on Ang-II-induced Egr-1 expression by attenuating Ang-II-induced ERK1/2 phosphorylation in A-10 VSMC.

3.4 DISCUSSION

In this study, we have demonstrated that β-adrenergic receptor activation of VSMC by ISO suppressed both protein and mRNA expression of Egr-1 induced by Ang-II. We also show that FSK, that directly activates AC activity and increases cAMP levels in the cells, as well as Db-cAMP, a cell permeable analogue of cAMP, mimicked ISO in suppressing Ang-II-induced Egr-1 expression. We have also demonstrated that indirectly increasing cAMP by inhibiting PDE activity in VSMC exerts a similar effect. A large body of evidence has indicated that cAMP exerts a growth inhibitory effect in VSMC and reduces neointima formation in vascular injury (Takahashi et al., 1996; Indolfi et al., 1997; Palmer et al., 1998; Kim et al., 2009; Gusan and Anand-Srivastava, 2013; Lehrke et al., 2015). In view of the reported involvement of Egr-1 in neointima formation in vessel-injured models (Lowe et al., 2001; Wang et al., 2013), our studies showing that β-adrenergic stimulation or cAMP elevating agents potently suppressed Ang-II-induced Egr-1 expression in VSMC support the concept that reduction in the levels of transcription factors such as Egr-1 may be among the molecular mechanisms responsible for vasoprotective effects of cAMP (Gusan and Anand-Srivastava, 2013; Kimura et al., 2014; Dubey et al., 2015). Our results have also demonstrated that ISO or IBMX-induced reduction of Egr-1 expression was associated with an increase in serine 157 phosphorylation of VASP, which is a marker of PKA activity (Butt et al., 1994; Eckert and Jones, 2007; Harbeck et al., 2000; Joshi et al., 2011), suggesting the involvement of PKA activation in the this process. The findings that H89, a pharmacological inhibitor of PKA, restored the suppressive effect of ISO and FSK on Ang-II-induced Egr-1 expression have provided additional evidence for a role of PKA in transducing the downstream effects of cAMP in inhibiting Ang-II-stimulated expression of Egr-1. Although an attenuating effect of cAMP in serum-induced Egr-1 expression in VSMC has been reported earlier (Hewer et al., 2011), the present studies have demonstrated for the first time that cAMP signaling via PKA is able to block Ang- II- induced Egr-1 expression in VSMC.

We have shown earlier that ERK1/2 activation is critical to induce protein and mRNA expression of Egr-1 in response to endothelin-1 (ET-1), insulin-like growth factor-

1 and Ang-II (Simo-Cheyou et al., 2016; Simo-Cheyou et al., 2017; Youreva and Srivastava, 2016). Our data presented here suggest that cAMP/PKA-induced reduction in ERK1/2 activity may contribute to the suppressive effect cAMP elevating agents on Egr-1 expression because ISO- or FSK-induced suppression of Egr-1 expression caused a concomitant reduction in the level of the phosphorylation of ERK1/2. A similar reduction in ERK1/2 phosphorylation by Db-cAMP in VSMC from SHR aorta has also been reported (Gusan and Anand-Srivastava, 2013) and PDE-3A inhibition-induced increase in cAMP has been shown to attenuate ERK1/2 phosphorylation in VSMC (Begum et al., 2011). These authors also reported that PDE-3A–induced reduction in ERK1/2 phosphorylation was associated with an inhibition in the activity of Raf-1 and an increased activity of MAPK phosphatase-1 (MKP-1) (Begum et al., 2011). Raf-1 is an upstream kinase in the MEK/ERK1/2 signaling cascade and its inhibition results in impaired phosphorylation of ERK1/2. MKP-1, on the other hand, is an ERK1/2-specific-protein phosphatase that catalyzes the dephosphorylation and thereby inactivation of ERK1/2. Thus, it may be suggested that ISO- and FSK–induced reduction in ERK1/2 phosphorylation observed in our studies may be mediated through a similar mechanism. Furthermore, our results showing that H89 restored not only attenuated Egr-1 expression but also ERK1/2 phosphorylation indicated that PKA via ERK1/2 signaling pathway contributes to the suppressive effects of ISO and FSK. A similar role of ERK1/2 in adenosine-mediated suppression of VSMC proliferation and expression of cell cycle regulatory proteins in human coronary smooth muscle cells has been suggested (Dubey et al., 2015).

Ang-II signals its downstream responses through the generation of reactive oxygen species (ROS) by activating NAD(P)H oxidases (NOXes) (Nguyen Dinh Cat et al., 2013; Seshiah et al., 2002). Recent reports have shown that Ang-II-induced H_2O_2 production was attenuated by FSK or Db-cAMP in PKA-dependent fashion (Zhao et al., 2014). In addition, cAMP elevation by Db-cAMP has also been shown to suppress superoxide generation as well as NOX activity in VSMC isolated from SHR aorta (Gusan and Anand-Srivastava, 2013). These authors also noted that Db-cAMP treatment of VSMC resulted in

an attenuated tyrosine phosphorylation of the epidermal growth factor receptor (EGF-R) as well as c-Src. We have shown earlier that Ang-II signals protein and DNA synthesis, key markers of hypertrophic and proliferative responses in VSMC and have recently reported that c-Src is essential to trigger ET-1 induced Egr-1 expression (Bouallegue et al., 2009; Simo-Cheyou et al., 2016). Thus in view of the key role that ROS generation and tyrosine phosphorylation of EGF-R and c-Src plays in triggering Ang-II signaling pathways, it is possible that the ability of cAMP-PKA to inhibit these events may be responsible to inhibit ERK 1/2 phosphorylation observed in our studies.

In summary, we have demonstrated that elevating cellular cAMP levels either by GPCR-mediated activation or by other agents attenuated Ang-II-induced Egr-1 expression via a PKA-mediated inhibition of Ang-II-induced ERK1/2 phosphorylation in VSMC. Our findings have revealed a previously unidentified role of PKA system in regulating Ang-II-induced Egr-1 expression and suggest that suppression of Egr-1 expression may be one of the mechanisms by which cAMP exerts its vasculoprotective effects.

3.5 GRANTS

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3.6 DISCLOSURES

The authors of this manuscript do not have any conflict of interest to disclose.

3.8 FIGURE LEGENDS

Figure 1: Isoproterenol attenuates Ang-II-induced increase in Egr-1 protein and mRNA levels

Quiescent A-10 VSMC were pre-treated in the absence (CTL) or presence of indicated concentrations of ISO for 30 min, followed by stimulation with 100 nM Ang-II for 1 h. **A**) Cell lysates were immunoblotted with Egr-1 antibody (top panel) or β-tubulin (middle panel). Bar diagrams represent average data from five independent experiments quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. **B**) Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. $^{***}p<0.001$ versus control values. $^{###}p<0.001$ versus VSMC treated with Ang-II alone**.**

Figure 2: Forskolin attenuates Ang-II-induced increase in Egr-1 protein and mRNA levels in A-10 VSMC

Quiescent A-10 VSMC were pre-treated in the absence (CTL) or presence of indicated concentrations of FSK for 30 min, followed by stimulation with 100 nM Ang-II for 1 h. **A**) Cell lysates were immunoblotted with Egr-1 antibody (top panel) or β-tubulin (middle panel). Bar diagrams represent average data from three independent experiments quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. **B**) Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. $^{**}p<0.01$, $^{***}p<0.001$ versus control values. $^{##}p<0.01$, $^{###}p<0.001$ versus VSMC treated with Ang-II alone.

Figure 3: Blockade of phosphodiesterase activity potentiates the inhibitory effect of isoproterenol on Ang-II-induced Egr-1 expression

Quiescent A-10 cells were pre-treated with 10 μM of the PDE inhibitor, IBMX, alone (**A**) or in the presence of 10 μM of ISO (IBMX + ISO) (**B**) prior to stimulation with 100 nM Ang-II for 1 h. Cell lysates were probed with Egr-1 antibody (top panels in **A** and **B**) or β-tubulin (middle panels). Bar diagrams in each section represent average data from three independent experiments quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control value defined as 1. $^{**}p<0.01$, $^{***}p<0.001$ versus CTL values. $^{###}p<0.001$ versus VSMC treated with Ang-II alone. $^{+}p<0.05$ comparison between ISO and ISO+IBMX pre-treatments.

Figure 4: The phosphorylation levels of VASP inversely correlate with reduced levels of Egr-1 expression in the presence of isoproterenol and isobutylmethylxanthine

Quiescent A-10 cells were pre-treated with 10 μM of IBMX alone (A) or in the presence of 10 μM of ISO (IBMX + ISO) (B) prior to stimulation with 100 nM Ang-II for 1 h. Cell lysates were probed with total VASP antibody detecting the total VASP protein at 46 kDa and the serine 157 phosphorylated VASP at 50 kDa (top panels in A and B) or β-tubulin (middle panels). Bar diagrams represent average data from four independent experiments quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. $*p<0.05$, $**p<0.01$, $***p<0.001$ versus control values. $\#p<0.05$, $\#\#p<0.01$, $\#\#\#p<0.001$ versus VSMC treated with Ang-II alone.

Figure 5: Dibutyryl cAMP and Benzoyl-cAMP exert similar inhibitory effects on Ang-II-induced Egr-1 expression in A-10 VSMC

Quiescent A-10 VSMC were pre-treated in the absence (CTL) or presence of increasing concentrations of (**A**) Db-cAMP (50 μM, 100 μM and 500 μM) or (**B**) BNZ-cAMP (0.5μM, 5μM and 50 μM) for 30 min, followed by stimulation with 100 nM Ang-II for 1 h.

Cell lysates were immunoblotted with Egr-1 antibody (top panels) or β-tubulin (middle panels). Bar diagrams represent average data from five independent experiments quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control values. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ versus VSMC treated with Ang-II alone**.**

Figure 6: Role of PKA signaling in isoproterenol-mediated inhibitory effects on Ang-II-induced Egr-1 expression in A-10 VSMC

Quiescent A-10 VSMC were pre-treated in the absence (CTL) or presence of 10 μM of H89 for 30 min prior to pre-treatment with 10 μM of ISO for 30 min, followed by stimulation with 100 nM Ang-II for 1 h. **A**) Cell lysates were immunoblotted with Egr-1 antibody (top panel) or β-tubulin (middle panel). Bar diagrams represent average data from four independent experiments quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. **B**) Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR. Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control values. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ versus VSMC treated with Ang-II alone

Figure 7: Role of PKA signaling in forskolin-mediated inhibitory effects on Ang-II-induced Egr-1 expression in A-10 VSMC

Quiescent A-10 VSMC were pre-treated in the absence (CTL) or presence of 10 μM of H89 for 30 min prior to pre-treatment with 25 μM of FSK for 30 min, followed by stimulation with 100 nM Ang-II for 1 h. **A**) Cell lysates were immunoblotted with Egr-1 antibody (top panel) or β-tubulin (middle panel). Bar diagrams represent average data from four independent experiments quantified by densitometric scanning of immunoblots. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. **B**) Analysis of relative Egr-1 mRNA levels was performed by qRT-PCR.

Relative level of Egr-1 mRNA is measured as fold variation compared to the control and normalized with β-actin level taken as a standard. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control values. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ versus VSMC treated with Ang-II alone.

Figure 8: Blockade of PKA reduces isoproterenol-mediated VASP phosphorylation and restores Ang-II-induced ERK1/2 phosphorylation in A-10 VSMC

Quiescent A-10 VSMC were pre-treated in the absence (CTL) or presence of 10 μM of H89 for 30 min prior to pre-treatment with 10 μM of ISO for 30 min, followed by stimulation with 100 nM Ang-II for 5 min. **A**) Cell lysates were probed with total VASP antibody detecting the total VASP protein at 46 kDa and the serine 157 phosphorylated VASP at 50 kDa (top panel), with antibody detecting Thr202 and Tyr 204 phosphorylated ERK1/2 (middle panel) as well as with total ERK antibody (bottom panel). Bar diagrams obtained with average data from five independent experiments quantified by densitometric scanning of immunoblots represent the fold change in phosphoVASP/Total VASP ratio **B)** or in ERK phosphorylation **C)**. The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control values. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ versus VSMC treated with Ang-II+ ISO.

Figure 9: Blockade of PKA reduces forskolin-mediated VASP phosphorylation and restores Ang-II-induced ERK1/2 phosphorylation in A-10 VSMC

Quiescent A-10 VSMC were pre-treated in the absence (CTL) or presence of 10 μM of H89 for 30 min prior to pre-treatment with 25 μM of FSK for 30 min, followed by stimulation with 100 nM Ang-II for 5 min. **A**) Cell lysates were probed with total VASP antibody detecting the total VASP protein at 46 kDa and the serine 157 phosphorylated VASP at 50 kDa (top panel), with antibody detecting Thr202 and Tyr 204 phosphorylated ERK1/2 (middle panel) as well as with total ERK antibody (bottom panel). Bar diagrams obtained with average data five independent experiments quantified by densitometric scanning of immunoblots, represent the fold change in phosphoVASP/Total VASP ratio **B)**

or in ERK phosphorylation **C**). The control is defined as 1 and each value aside is expressed as fold increase compared to the control values. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ versus control values. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ versus VSMC treated with Ang-II + FSK.

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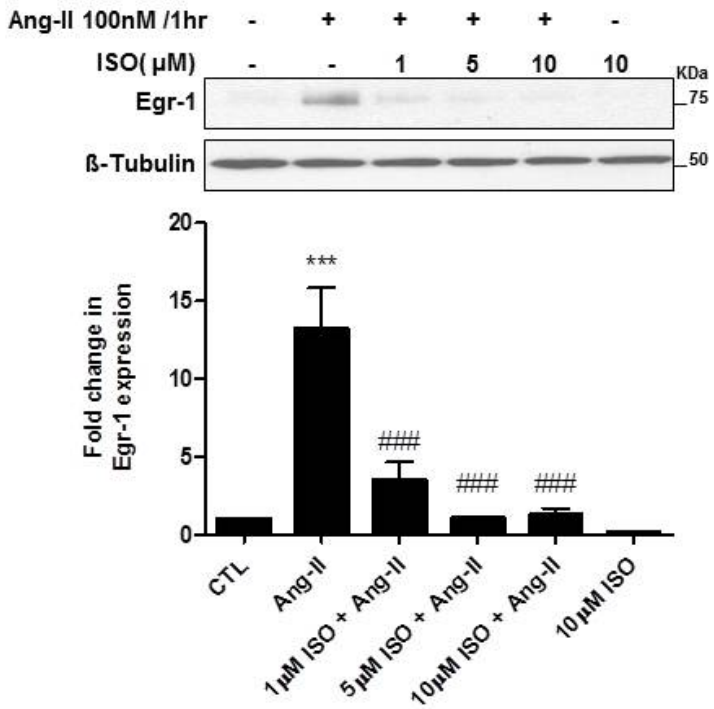
Zhao, L., Li, A. Q., Zhou, T. F., Zhang, M. Q., & Qin, X. M. (2014). Exendin-4 alleviates angiotensin II-induced senescence in vascular smooth muscle cells by inhibiting Rac1 activation via a cAMP/PKA-dependent pathway. *Am J Physiol Cell Physiol, 307*(12), C1130-1141.

3.10 FIGURES

FIGURE 1

A


Ang-II 100nM /1hr - + + + + -
ISO(μM) - - 1 5 10 10
KDa
Egr-1
75
ß-Tubulin
50
Fold change in Egr-1 expression
20
15
10
5
0
***
###
###
###
CTL
Ang-II
1μM ISO + Ang-II
5μM ISO + Ang-II
10μM ISO + Ang-II
10μM ISO

B

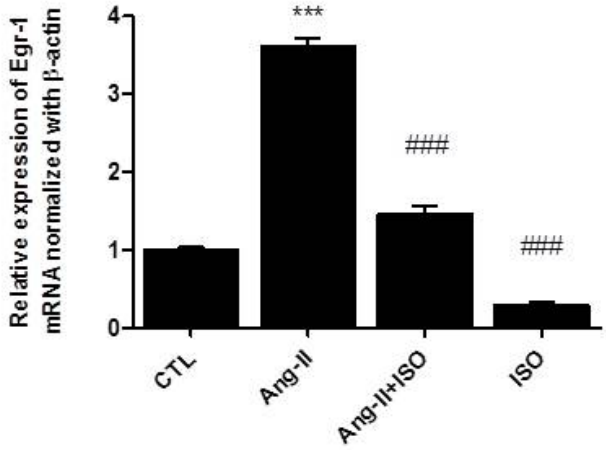

Relative expression of Egr-1 mRNA normalized with β-actin
4
3
2
1
0
***
###
###
CTL
Ang-II
Ang-II+ISO
ISO

FIGURE 2

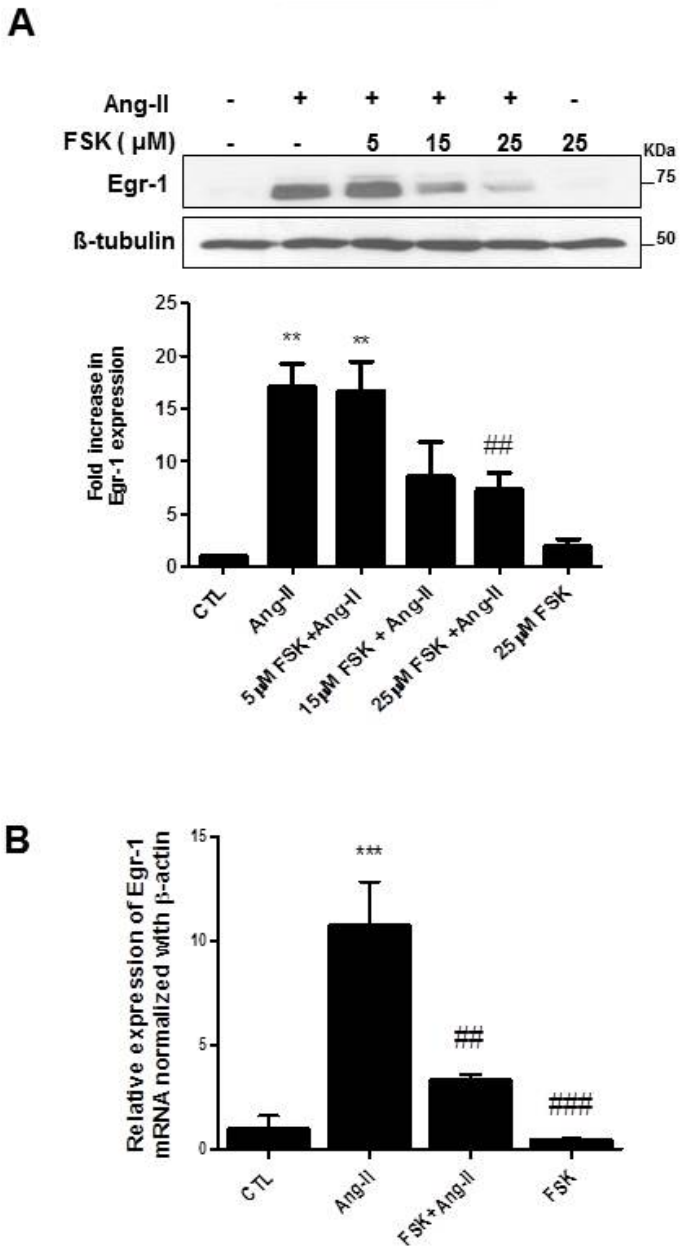
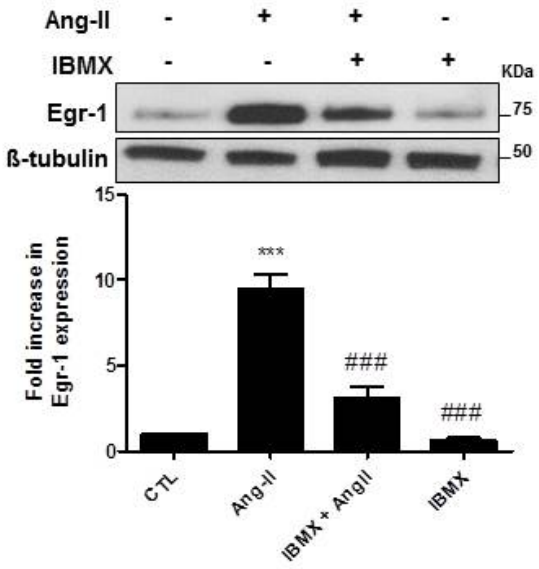

A
Ang-II - + + + + -
FSK (μM) - - 5 15 25 25
KDa
Egr-1
75
ß-tubulin
50
Fold increase in Egr-1 expression
25
20
15
10
5
0
**
**
##
CTL
Ang-II
5 μM FSK+Ang-II
15μM FSK + Ang-II
25μM FSK +Ang-II
25 μM FSK
B
Relative expression of Egr-1 mRNA normalized with β-actin
15
10
5
0
***
##
###
CTL
Ang-II
FSK+Ang-II
FSK

FIGURE 3

A


Ang-II - + + -
IBMX - - + +
KDa
Egr-1
75
ß-tubulin
50
15
10
5
0
Fold increase in Egr-1 expression
***
###
###
CTL
Ang-II
IBMX + AngII
IBMX

B

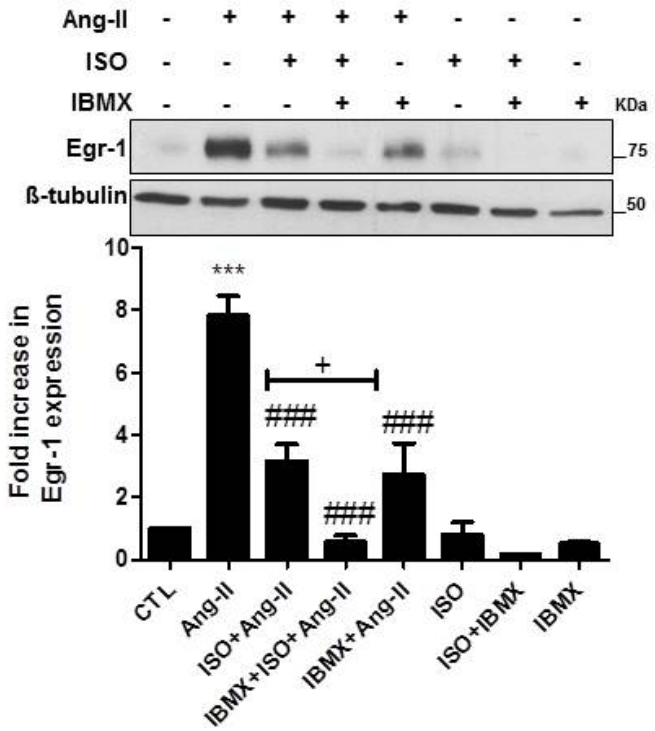

Ang-II - + + + + - - -
ISO - - + + - + + -
IBMX - - - + + - + +
KDa
Egr-1
75
ß-tubulin
50
10
8
6
4
2
0
Fold increase in Egr-1 expression
***
+
###
###
###
CTL
Ang-II
ISO+Ang-II
IBMX+ISO+Ang-II
IBMX+Ang-II
ISO
ISO+IBMX
IBMX

FIGURE 4

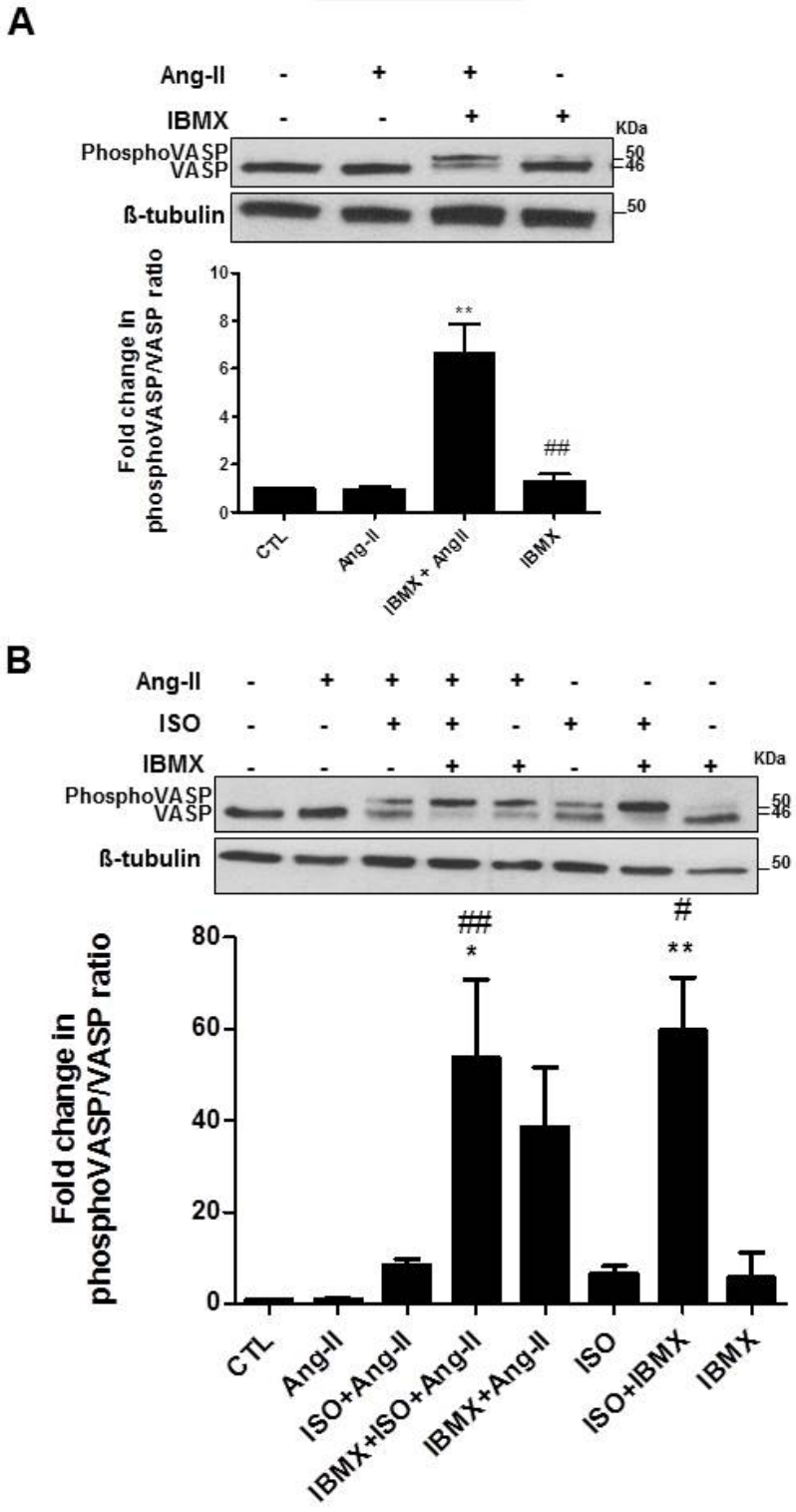

A
Ang-II - + + -
IBMX - - + +
KDa
PhosphoVASP
VASP
50
46
ß-tubulin
50
Fold change in phosphoVASP/VASP ratio
10
8
6
4
2
0
**
##
CTL
Ang-II
IBMX + AngII
IBMX
B
Ang-II - + + + + - - -
ISO - - + + - + + -
IBMX - - - + + - + +
KDa
PhosphoVASP
VASP
50
46
ß-tubulin
50
Fold change in phosphoVASP/VASP ratio
80
60
40
20
0
##
*
#
**
CTL
Ang-II
ISO+Ang-II
IBMX+ISO+Ang-II
IBMX+Ang-II
ISO
ISO+IBMX
IBMX

FIGURE 5

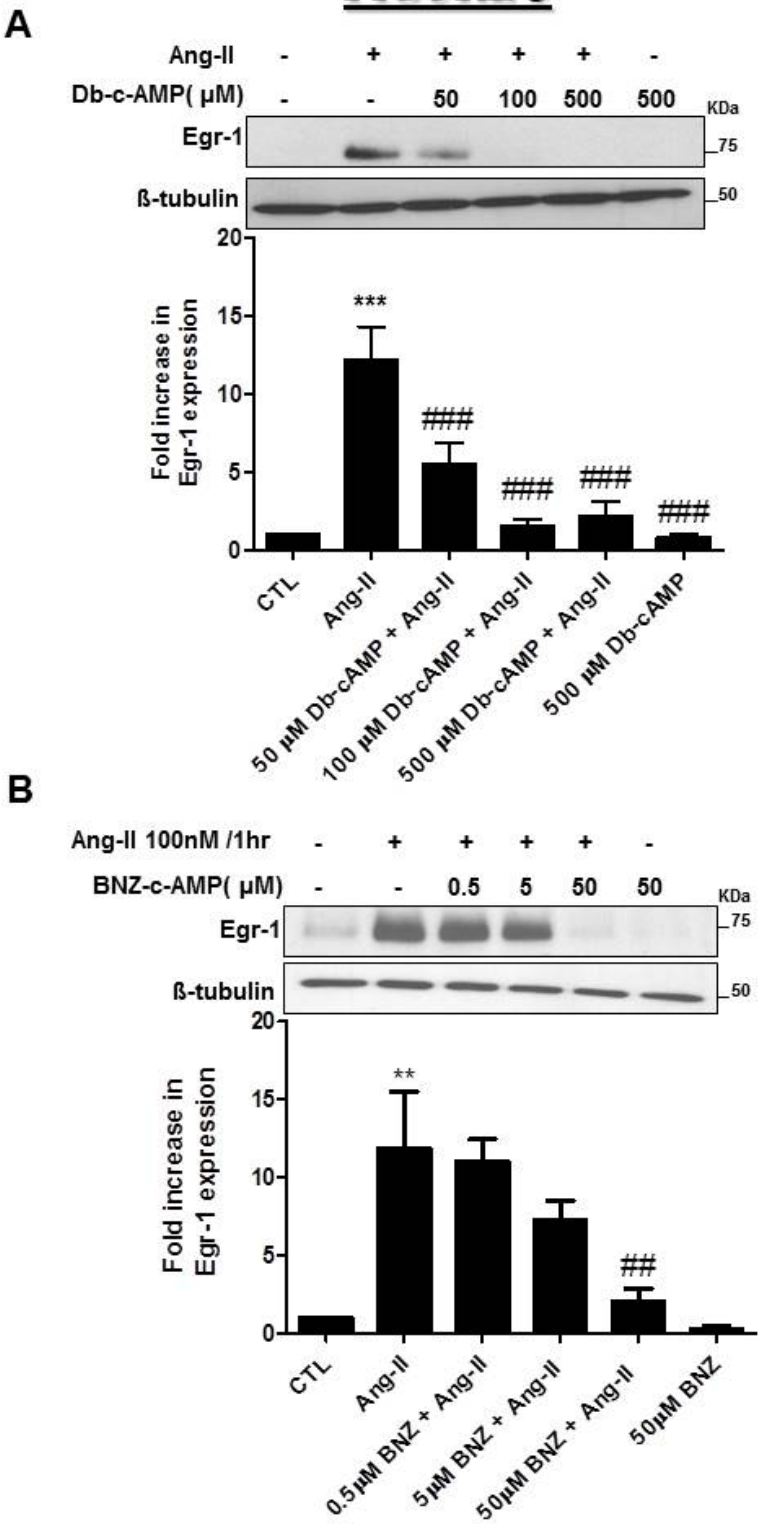

A
Ang-II - + + + + -
Db-c-AMP(µM) - - 50 100 500 500
KDa
Egr-1
75
ß-tubulin
50
Fold increase in Egr-1 expression
20
15
10
5
0
***
###
###
###
###
CTL
Ang-II
50 µM Db-cAMP + Ang-II
100 µM Db-cAMP + Ang-II
500 µM Db-cAMP + Ang-II
500 µM Db-cAMP
B
Ang-II 100nM /1hr - + + + + -
BNZ-c-AMP(µM) - - 0.5 5 50 50
KDa
Egr-1
75
ß-tubulin
50
Fold increase in Egr-1 expression
20
15
10
5
0
**
##
CTL
Ang-II
0.5µM BNZ + Ang-II
5µM BNZ + Ang-II
50µM BNZ + Ang-II
50µM BNZ

FIGURE 6

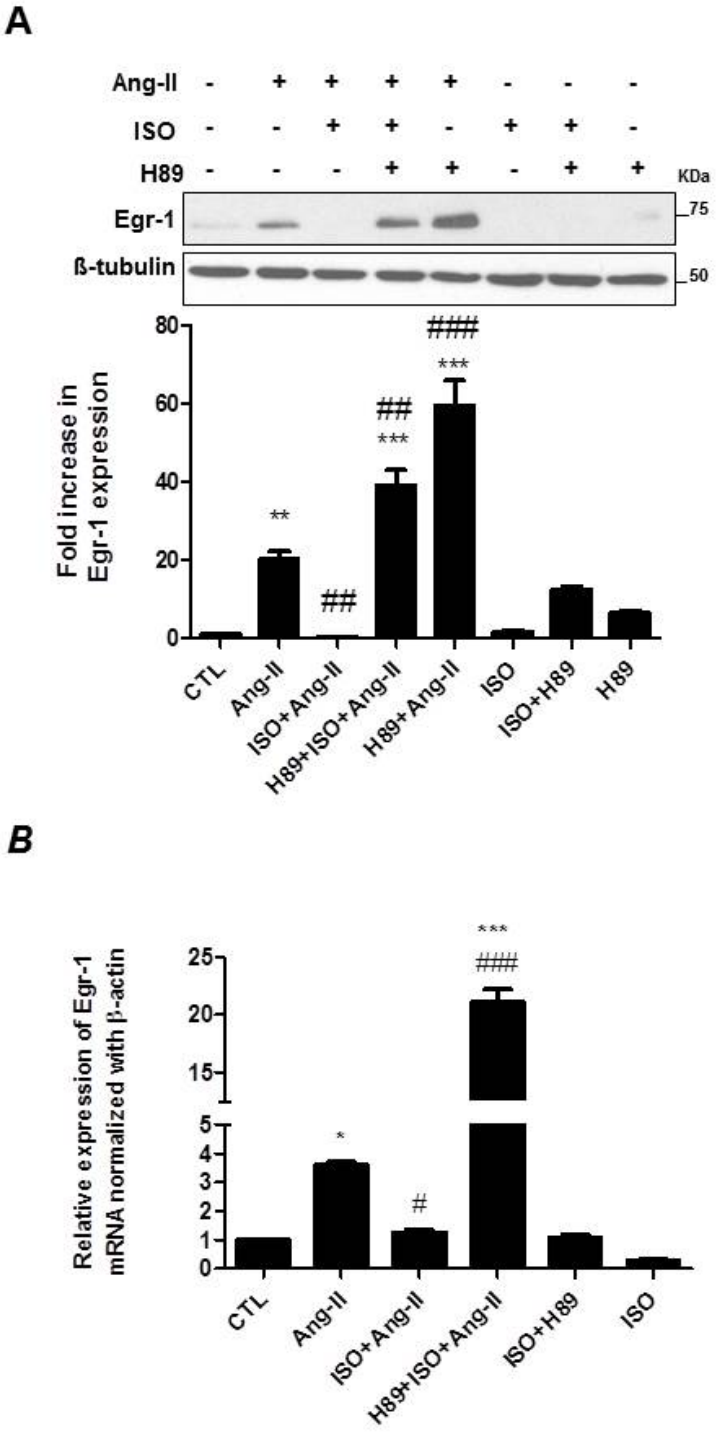

A
Ang-II - + + + + - - -
ISO - - + + - + + -
H89 - - - + + - + +
KDa
Egr-1
75
ß-tubulin
50
Fold increase in Egr-1 expression
80
60
40
20
0
**
##
***
##
###
***
CTL
Ang-II
ISO+Ang-II
H89+ISO+Ang-II
H89+Ang-II
ISO
ISO+H89
H89
B
Relative expression of Egr-1 mRNA normalized with β-actin
25
20
15
5
4
3
2
1
0
*
#
***
###
CTL
Ang-II
ISO+Ang-II
H89+ISO+Ang-II
ISO+H89
ISO

FIGURE 7

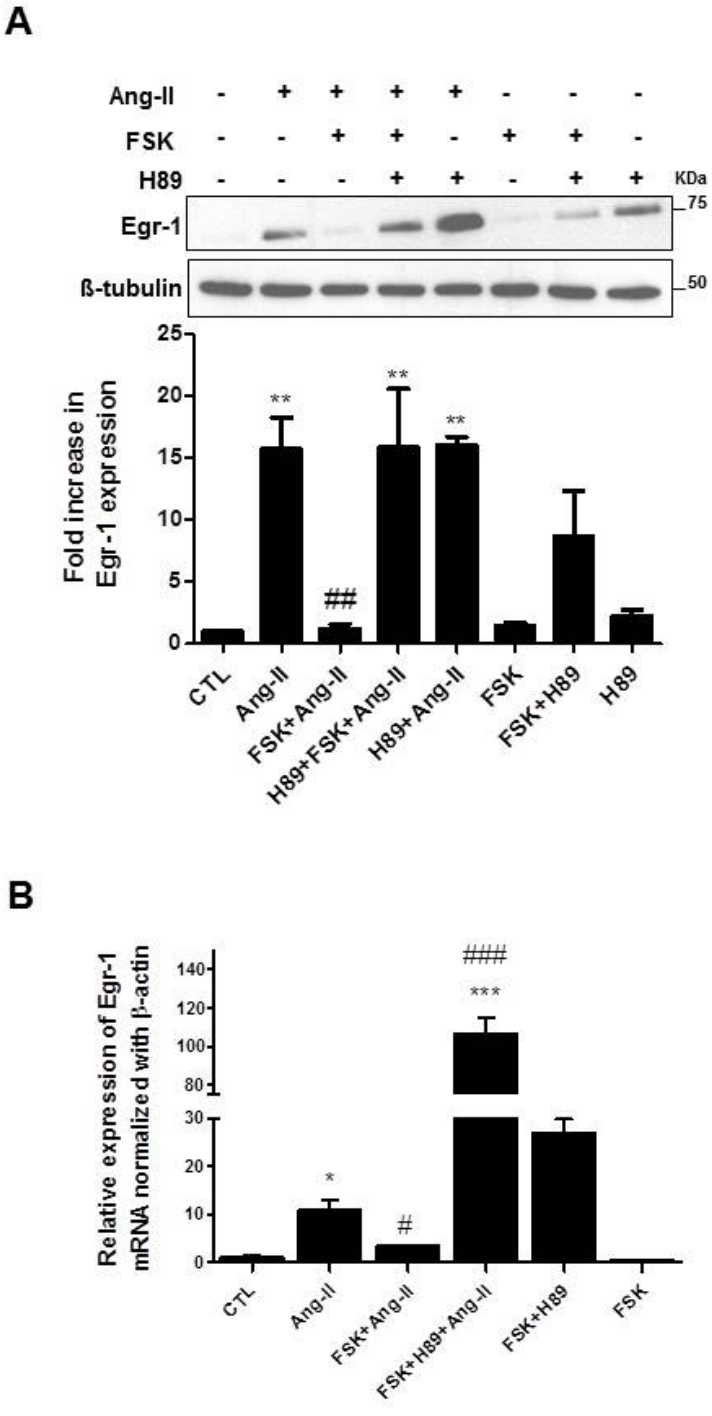

A
Ang-II
FSK
H89
KDa
Egr-1
75
ß-tubulin
50
Fold increase in Egr-1 expression
CTL
Ang-II
FSK+Ang-II
H89+FSK+Ang-II
H89+Ang-II
FSK
FSK+H89
H89
B
Relative expression of Egr-1 mRNA normalized with β-actin
CTL
Ang-II
FSK+Ang-II
FSK+H89+Ang-II
FSK+H89
FSK

FIGURE 8

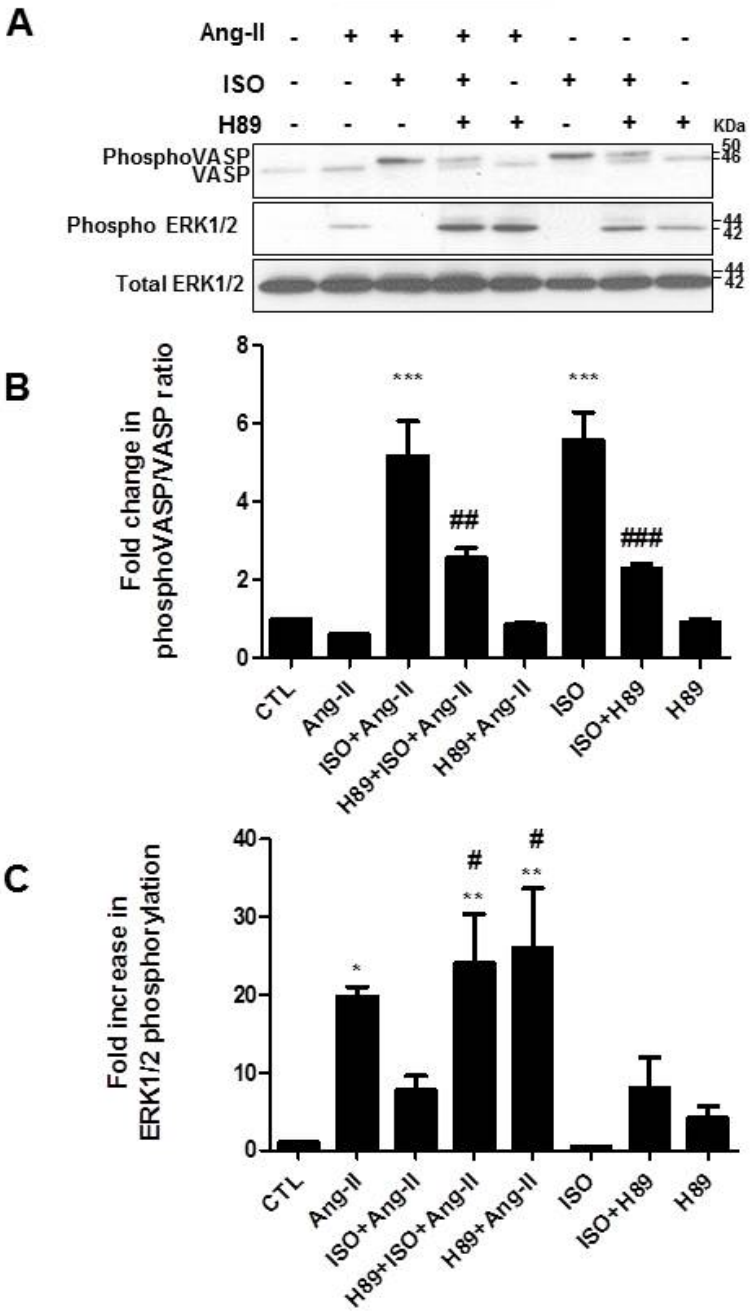
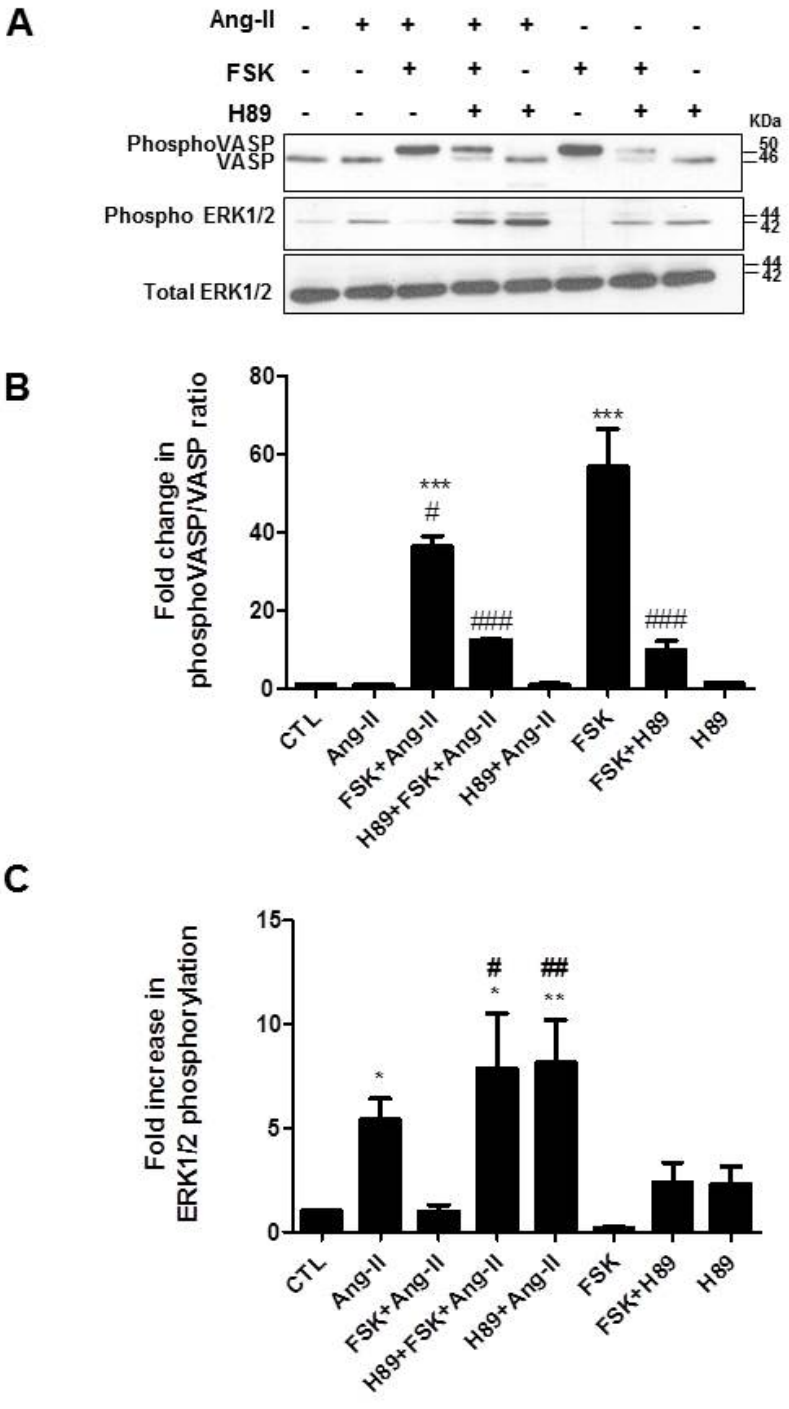

A
Ang-II - + + + + - - -
ISO - - + + - + + -
H89 - - - + + - + +
KDa
PhosphoVASP
VASP
50
46
Phospho ERK1/2
44
42
Total ERK1/2
44
42
B
Fold change in phosphoVASP/VASP ratio
8
6
4
2
0
***
***
##
###
CTL
Ang-II
ISO+Ang-II
H89+ISO+Ang-II
H89+Ang-II
ISO
ISO+H89
H89
C
Fold increase in ERK1/2 phosphorylation
40
30
20
10
0
*
**
**
#
#
CTL
Ang-II
ISO+Ang-II
H89+ISO+Ang-II
H89+Ang-II
ISO
ISO+H89
H89

FIGURE 9


A
Ang-II - + + + + - - -
FSK - - + + - + + -
H89 - - - + + - + +
KDa
PhosphoVASP
VASP
50
46
Phospho ERK1/2
44
42
Total ERK1/2
44
42
B
Fold change in phosphoVASP/VASP ratio
80
60
40
20
0
***
#
###
***
###
CTL
Ang-II
FSK+Ang-II
H89+FSK+Ang-II
H89+Ang-II
FSK
FSK+H89
H89
C
Fold increase in ERK1/2 phosphorylation
15
10
5
0
*
#
*
##
**
CTL
Ang-II
FSK+Ang-II
H89+FSK+Ang-II
H89+Ang-II
FSK
FSK+H89
H89

Chapter 4: General discussion

4.1 OVERVIEW OF THE RATIONALE OF THE THESIS

Studies by our group have suggested that hyperactivation of signaling cascades induced by vasoactive peptides like ET-1 and Ang-II underlies aberrant physiological responses in VSMC (256, 263, 264, 300). Most of the effects produced by Ang-II in VSMC are mediated via its AT1R which triggers growth and migratory responses (301). The signalling cascades that participate in these processes involve a Gαq11-dependent activation of PLC-β that catalyzes the formation of DAG and IP_3 from the membrane inositides. Further transduction of Ang-II signal involves the participation of Ca^{2+} originating from both an influx from the extracellular space and an efflux from the intracellular stores into the cytosol. The mechanism of SOCE connects these events linked with Ca^{2+} mobilization and thus represents an essential process for the outcome of calcium-dependent physiological responses in the cells (170). Interestingly, pharmacological inhibition of SOCE results in an attenuation of ET-1- and Ang-II-induced elevation of $[Ca^{2+}]i$ as well as it reduces LPA-mediated increase in blood pressure (302) and VSMC proliferation (302, 303). The molecular basis of SOCE has been uncovered by studies conducted in the past five years. STIM-1 and Orai-1 are well described to date as major functional components of SOCE (304). Notably, Ang-II has been shown to enhance the expression of STIM-1 and Orai-1 in neointimal VSMC (305) and changes in their expression were associated with aberrant VSMC physiological responses and hypertensive vascular diseases (305-307). Furthermore, handling of Ca^{2+} in response to Ang-II implicates the activity of a group of proteins including calmodulin and CaMKII that transduce Ca^{2+} signaling into VSMC pathophysiological responses via downstream activation of MAPK and transcription factors (167, 168).

Egr-1 was first described by Sukhatme and his collaborators as a protein rapidly induced by mitogens in atherosclerosis-relevant cells (macrophages, lymphocytes, fibroblasts) (239). Early evidences of Egr-1 induction in VSMC came from studies aiming at differentiating the effects of two Ang-II antagonists on VSMC proliferative responses (308, 309). In these studies, Ang-II was found to increase Egr-1 expression in a time-

dependent fashion and inhibiting Egr-1 in further experiments resulted in an abrogation of VSMC proliferation (310). Given the roles played by Ang-II and VSMC proliferation in the pathogenesis of vascular diseases, these early data generated a lot of interest in understanding the mechanisms and significance of Egr-1 expression in vascular biology and disease. Reports from several investigations have converged to these main conclusions: vascular remodeling is associated with elevated levels of Egr-1 expression (228, 311, 312) and, via its ability to regulate the expression of many genes involved in vascular injury and atherosclerotic events (228, 313, 314), Egr-1 contributes to the pathogenesis of vascular diseases (312, 315). Studies that address the signaling pathways underlying Egr-1 induction thus provide perspectives for molecular interventions. Yet, although several studies have demonstrated the role of Ca^{2+} in mediating Egr-1 induction (219, 225, 316, 317), there is no evidence of an involvement of STIM-1 and Orai-1 in Ang-II-induced Egr-1 expression in VSMC. In the first part of this thesis, our attempt to define the role played by Ca^{2+} mobilization and Ca^{2+} handling molecules in Egr-1 induction has provided data demonstrating that STIM-1 and Orai-1 are essential in mediating Ang-II-induced activation of ERK, CREB and Egr-1 expression. Pharmacological blockade of SOCE using 2-APB resulted in a decrease in $[Ca^{2+}]i$ and was accompanied by a downregulation of Ang-II-induced Egr-1 expression. Our studies reveal for the first time that STIM-1 and Orai-1 are required for the signaling responses upstream of Ang-II-induced Egr-1 expression in VSMC.

4.2 CALCIUM-DEPENDENT INDUCTION OF EGR-1 BY ANG-II

Heightened levels of $[Ca^{2+}]i$ are found in synthetic VSMC (180, 318). Vessels from hypertensive animals exhibit an abundance of synthetic VSMC (69) and upregulated levels of IP_3 and IP_3R (319). In accordance with reports suggesting that Egr-1 is a Ca^{2+}-dependent transcription factor (reviewed in (219)), our data reveal that the reduction in $[Ca^{2+}]i$ following IP_3R blockade is accompanied by an attenuation of Ang-II-induced increase in Egr-1 expression in VSMC. We suggest that this downregulation of Egr-1 may underlie the suppression of VSMC proliferation observed following similar treatment in

previous studies (320). Our data also reinforce the concept that elevation of Egr-1 may be among the mechanisms that underlie the mitogenic properties of Ca^{2+} (317). Furthermore, pharmacological blockers of CaM and CaMKII attenuate Ang-II-induced Egr-1 expression suggesting that Ca^{2+} signals Egr-1 expression via CaM/CaMKII-dependent signaling pathways in VSMC. CaM/CaMKII has emerged from several studies as a transducer of transcriptional events in VSMC (321-323) in animal models of vascular disease (168). Previous studies from our laboratory have demonstrated that CaMKII is required for ET-1-induced signaling and physiological responses in VSMC (256). CaMKII mediates the activity of transcription coactivators such as MEF-2 and HDAC during the process of neointima formation and in response to stimulation by Ang-II (167, 168, 323). Moreover, VSMC-specific blockade of CaMKII decreased aortic stiffness and blood pressure in a model of Ang-II-induced hypertension (324). Our data suggest that a decrease in Ang-II-induced Egr-1 expression in VSMC may account for the beneficial outcomes observed following blockade of CaMKII.

4.3 STIM-1 AND ORAI-1 IN ANG-II-INDUCED RESPONSE

Data provided in this thesis are the first to report an involvement of STIM-1/Orai-1 in enhancing the transcription of Egr-1 in response to Ang-II. We suggest that the participation of STIM-1 and Orai-1 in Ang-II-mediated signaling in VSMC underlies their contribution to the pathogenesis of vascular diseases. Enhanced levels of STIM-1 and Orai-1 are found in synthetic VSMC (175) and STIM-1/Orai-1 complex has evolved as a regulator of important vascular functions such as vessel reactivity (307, 325), VSMC proliferation (175, 305), ROS generation (325), thrombus formation (326) and vascular inflammation (327). While elevated levels of STIM-1 and Orai-1 correlate with impaired basal tonus in hypertensive models (307), SMC- or endothelial cell-specific knockout of STIM-1 impairs vessel responsiveness to epinephrine (325) and low levels of STIM-1 in injured rat carotid arteries protect against restenosis (318). In airway SMC, silencing of STIM-1/Orai-1 decreased PDGF-induced migratory and proliferative responses (328). Our data suggest that in vascular diseases, a prolonged activity of STIM-1/Orai-1 promotes the

events linked with vessel remodeling by enhancing Egr-1 expression indeed found at heightened concentrations under these conditions (282, 311, 329). The notion of prolonged activity of STIM-1/Orai-1 in vascular diseases is supported by studies that showed that in models of vascular injury, there is an alteration in the expression and activity of SERCA (105, 330) resulting in a defect in the refilling of ER Ca^{2+} (331). Since ER depletion is sensed by STIM-1, a sustained activation of STIM-1/Orai-1 interaction may follow and lead to aberrant induction of Egr-1 expression. Hence, experimental approaches aiming at the restoration of SERCA inhibit neointima formation in models of vascular injury (332, 333) and Egr-1 depletion produces similar effects (282, 317, 334). It may therefore be suggested that vascular damage observed in conditions of altered expression of SERCA is due sustained STIM-1/Orai-1 activity leading to enhanced Egr-1 expression. Moreover, we provide the first evidence that siRNA-induced silencing of either STIM-1 or Orai-1 results in the suppression of Ang-II-induced ERK1/2 and CREB phosphorylation. MAPK signaling plays a key role in inducing Egr-1 expression in a wide variety of cell types (222, 260, 335, 336) and a role of CREB in mediating Egr-1 expression (247) as well as Ang-II-induced transcriptional events and VSMC proliferation has been suggested (220, 337). Therefore, our data reinforce the involvement of ERK- and CREB-signaling in Egr-1 induction and indicate that SOCE is crucial for Ang-II-induced transcriptional events in VSMC. A recent report have shown a similar involvement of STIM-1/Orai-1 in mediating Ang-II-induced activity of the nuclear factor of activated T cells and protein expression in cardiac fibroblasts (338). Taken together, it can be suggested that via their participation in Ang-II-induced aberrant responses, STIM-1/Orai-1 contribute to the pathogenesis of CVD. Moreover, ERK1/2 -mediated CREB activation has recently been involved in Ang-II-induced modulation of Nur77 expression in VSMC (220). Our data showing that siRNA-induced silencing of CREB attenuated Ang-II –stimulated Egr-1 expression strengthen the notion that ERK-dependent CREB activation plays a key role in transducing Ang-II-induced signaling events in VSMC. In summary the first part of this thesis (Chapter 2) has demonstrated that STIM-1/Orai-1- induced SOCE, through ERK1/2/CREB–dependent signaling pathways, participates in the expression of Egr-1 in response to Ang-II in VSMC.

4.4 RATIONALE OF EGR-1 DOWNREGULATION AND POTENTIAL OF TARGETING STIM-1 AND ORAI-1

The major hallmark of atherosclerotic disease or restenosis following angioplasty is the increase in progressive vessel wall thickness due to aberrant VSMC proliferation (339). Previous studies have shown that attenuation in the progression of atherosclerosis following administration of telmisartan, an AT1R antagonist, to hyperlipidemic mice was accompanied with a reduction in Egr-1 expression and in the levels of vascular inflammation markers (340). Further investigations using transgenic models demonstrated that Egr-1 deficiency in atherosclerotic conditions protect against vascular lesions (275). Moreover, neointima formation was almost completely abrogated in vascular injured animals that received an Egr-1-specific DNAzyme blocking Egr-1 expression prior to injury, as compared to animals treated with the vehicle (341). These studies suggested that Egr-1 deficiency reduces VSMC proliferation in models of vascular injury. In flow-mediated pulmonary hypertension, oligonucleotide-mediated depletion of Egr-1 attenuated the levels of prothrombotic markers such as PDGF, interleukin-6, p-53 and transforming growth factors (279). These molecular effects were accompanied by a reduction in the remodeling of pulmonary arteries due to lower rates of VSMC proliferation. Notably, pharmacological inhibition of Egr-1 did not produce any effect on VSMC proliferation in control animals suggesting that Egr-1 deficiency or loss of function protects vascular function only in pathological conditions (279). Our data demonstrate that in proliferating A-10 cells, STIM-1/Orai-1 deficiency attenuates Egr-1 expression in response to Ang-II. This suggests for the first time that targeting these two molecules may attenuate vascular damage by decreasing Egr-1-mediated VSMC proliferation. However, given the ubiquitous expression of STIM-1 and Orai-1 (342) and, more importantly, given the crucial role played by SOCE in $[Ca^{2+}]_i$ homeostasis (343), one could expect either an invasive physiological defect or an incompatibility with life upon whole body deficiency in STIM-1 or Orai-1. In mice, ubiquitous loss function of STIM-1 and Orai-1 is lethal in early life (344). In humans, STIM-1 and Orai-1 deficiency does not lead to death but as per few

emerging studies (195, 345), resulting clinical phenotypes are restrained to immunodeficiency, muscular dystrophy and ectodermal disease. One of the explanation of the limited deleterious effects of a whole body deficiency in STIM-1 or Orai-1 in humans is the redundancy of their function as suggested by McCarl and her collaborators (346). This means for example that cellular arrangements involving other Orai isoforms may compensate a functional defect arising from ubiquitous lack of Orai-1 (191) (347). This limited phenotype may thus support the relative safety of future work in humans. Nevertheless, further studies using loss of function targeted to the cardiovascular system are more relevant and needed to provide sufficient data on the physiological outcomes of STIM-1 and Orai-1 deficiency in conditions of vascular injury.

Moreover, in view of molecular intervention, it is important to note that additional sources of Ca^{2+} are involved in Ca^{2+} -dependent responses mediated by Ang-II. Ang-II activates the voltage –operated calcium entry (VOCE) through PLC and PKC-mediated activation of the L-type calcium channels (LTCC) (348, 349) as well as transient Ca^{2+} sparks through intracellular ryanodine receptors activation (350). LTCC is among the major sources of cytosolic Ca^{2+} in VSMC stimulated by vasoactive peptides (351). Interestingly however, as discussed by Harraz and Altier (352), while STIM-1 impairment results in an increase in LTCC activity in VSMC, STIM-1 overexpression attenuates LTCC gating (189, 352, 353). This implies that, although the activities of both types of channels results in an increase in $[Ca^{2+}]_i$, LTCC channeling may either be an inappropriate source of Ca^{2+} with regard to STIM-1-dependent responses similar to Ang-II-induced Egr-1 expression, or be required in a proportion that is just enough to be balanced with STIM-1 activity. Therefore, in addition to loss of function studies, investigating the differential contribution of SOCE and VOCE in mediating Egr-1 expression in response to Ang-II may be helpful in supporting the pertinence of targeting STIM-1 or Orai-1.

Similar to the alterations found in the expression and activities of STIM-1/Orai-1 during vascular diseases (section 4.3), reports have suggested that hypertension and vessel remodeling are also associated with impairments in cAMP-related signaling responses

(150, 354-357). In contrast, elevation of cAMP levels attenuated Ang-II-induced proliferation and signaling responses in VSMC (358, 359). This suggests that activation of cAMP-mediated pathway can reduce Ang-II-induced Egr-1 expression and impairment in this inhibitory effect may underlie the vessel remodeling associated with altered cAMP signaling. In view of the potential in understanding the signaling cascades leading to Egr-1 downregulation in vessel remodeling, studies in the second part of this thesis aimed at investigating the effect of cAMP elevating agents on Ang-II-induced Egr-1 expression.

4.5 DOWNREGULATION OF ANG-II-INDUCED EGR-1 EXPRESSION: ROLE OF ERK

cAMP was first discovered in 1957 by Sutherland and Rall who described it as a heat stable second messenger produced by epinephrine in heart, brain, and muscle homogenates (360, 361). Because of their ability to induce β-adrenergic receptor mediated activation of Gαs and subsequent induction of AC activity, epinephrine and other catecholamines such as ISO have been widely used to study the functional role of cAMP in VSMC (359, 362-364). AC activity can also be triggered by a direct binding with FSK. Additionally, elevation of cAMP by exogenous addition of cell permeable cAMP analogs or by inhibition of PDE activity has also been useful in the study of the role cAMP in cellular responses. Our attempt to investigate the effect of cAMP elevation on Ang-II-induced Egr-1 expression reveals for the first time that β-adrenergic receptor activation of VSMC by ISO suppressed both protein and mRNA expression of Egr-1 induced by Ang-II. We also show that FSK, as well as the cAMP analog Db-cAMP, mimicked ISO in suppressing Ang-II-induced Egr-1 expression. Furthermore, inhibiting PDE activity in VSMC exerts a similar effect. Since antimitogenic properties have been attributed to the effect of cAMP in VSMC in conditions of vascular injury (147, 358, 365-368), our data support the concept that reduction in the levels of transcription factors such as Egr-1 may be among the molecular mechanisms responsible for vasoprotective effects of cAMP (147, 369, 370). Our results showing that ISO or IBMX-induced reduction of Egr-1 expression is

associated with an increase in serine 157 phosphorylation of VASP, which is a marker of PKA activity (371-374), suggest the involvement of PKA activation in this process. The findings that BNZ-cAMP, a PKA-specific analog of cAMP, exerts an effect similar to Db-cAMP and that H-89, a pharmacological inhibitor of PKA, restored the suppressive effect of ISO and FSK on Ang-II-induced Egr-1 expression has provided additional evidence for a role of PKA in transducing the downstream effects of cAMP in inhibiting Ang-II-stimulated expression of Egr-1. Although an attenuating effect of cAMP in serum-induced Egr-1 expression in VSMC has been reported earlier (155), the present studies have demonstrated for the first time that cAMP signaling via PKA is able to block Ang-II-induced Egr-1 expression in VSMC. Our group has earlier reported a critical involvement of ERK1/2 activation in inducing Egr-1 protein and mRNA expression in response to vasoactive peptides (260, 335, 375). The data presented in the first part of this thesis have also reinforced this notion. A direct implication of this is that downregulation of Egr-1 may be associated with changes in ERK1/2 signaling. Hence, we provide data showing that ISO- or FSK-induced suppression of Egr-1 expression caused a concomitant reduction in the level of ERK1/2 phosphorylation. Thus, in line with the role of ERK1/2 in the upstream regulation of Egr-1 expression, cAMP/PKA-induced reduction in ERK1/2 activity may contribute to the suppressive effect of cAMP elevating agents on Egr-1 expression. Similar reduction in ERK1/2 phosphorylation was reported from previous investigations where cAMP elevation was achieved in VSMC either by an exogenous addition of Db-cAMP (147) or by blockade of PDE-3A activity (376). PKA-dependent activity has been associated with an increase in the activity of cellular phosphatases. It is thus possible that the reduced amount of phosphorylated ERK associated with PKA activity is due to enhanced PKA-dependent phosphatase activation. Interestingly, studies by Begum and his group showed that MAPK phosphatase-1 (MKP-1) activity is increased concomitantly with PDE-3A–induced reduction in ERK1/2 phosphorylation (376). MKP-1 is an ERK1/2-specific-protein phosphatase that catalyzes the dephosphorylation of ERK1/2. A hyperactivation of MKP has been associated with antiproliferative events in VSMC. Furthermore, Begum et al also showed that PDE-3A treatment inhibited the

activity of Raf-1 (310). Raf-1 is an upstream kinase in the MEK/ERK1/2 signaling cascade and its inhibition results in impaired phosphorylation of ERK1/2. Thus, it may be suggested that a similar mechanism underlies ISO- and FSK–induced reduction in ERK1/2 phosphorylation observed in our studies. Furthermore, our results showing that H-89 restored not only attenuated Egr-1 expression but also ERK1/2 phosphorylation indicated that PKA via ERK1/2 signaling pathway contributes to the suppressive effects of ISO and FSK. Similar observations were made in studies that addressed the role of ERK1/2 in adenosine-mediated suppression of VSMC proliferation and expression of cell cycle regulatory proteins in human coronary smooth muscle cells (370). In these studies, the decrease in ERK1/2 phosphorylation following adenosine receptor-mediated Gi stimulation was accompanied by an increase in PKA activity as illustrated by high levels of VASP phosphorylation. Taken together (Figure 12), our data suggest for the first time that cAMP elevating agents downregulate Ang-II-induced Egr-1 expression and this effect is accompanied by PKA-induced changes in signaling pathways leading to reduced ERK1/2 phosphorylation.

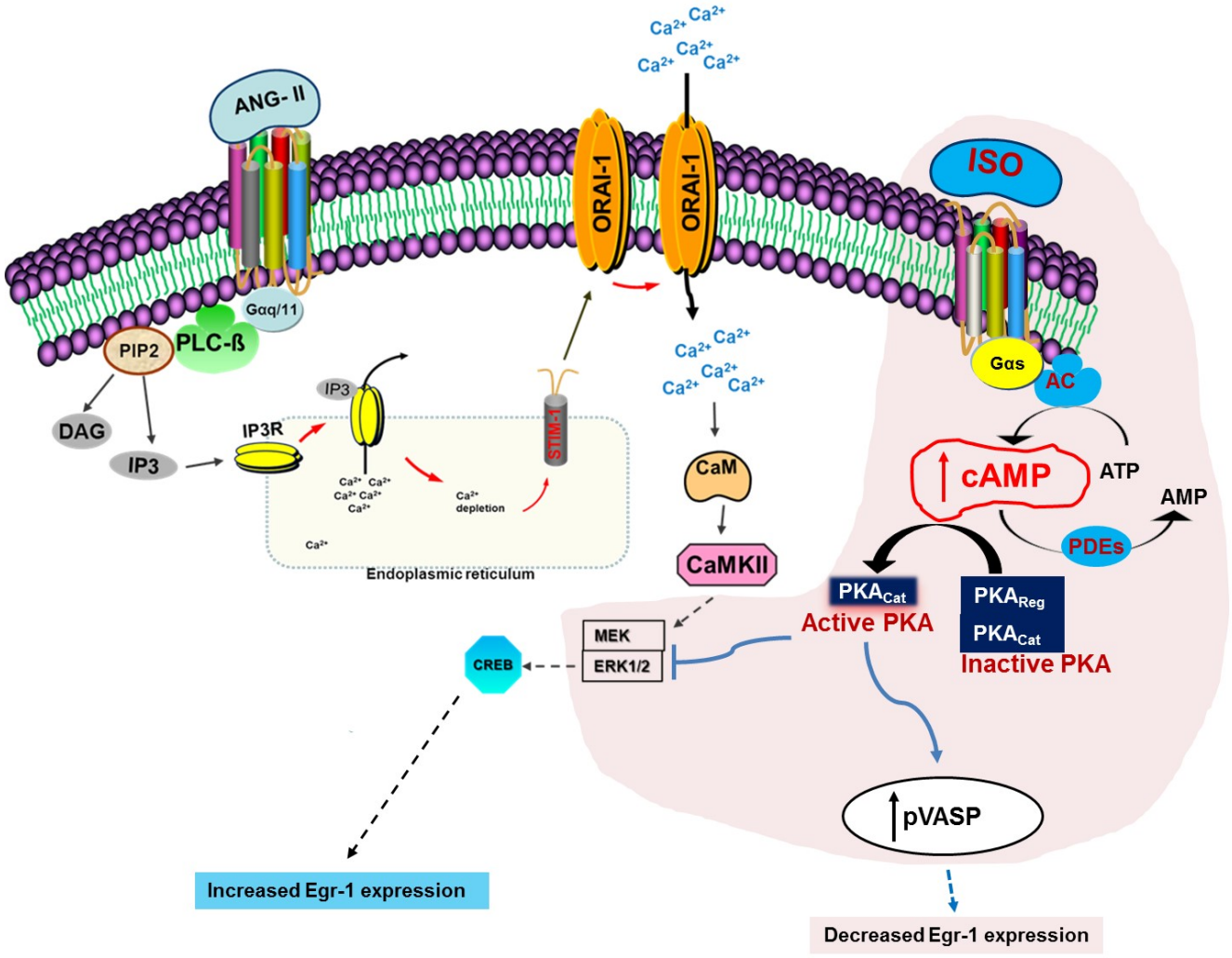


Figure 12: Modulation of Ang-II-induced Egr-1 expression in A10 VSMC

STIM-1 and Orai-1 mediate Ang-II-induced Egr-1 expression via ERK1/2 and CREB activation in A10 VSMC. Enhanced cAMP/PKA-induced signaling exhibited by heightened levels of VASP phosphorylation is associated with a decrease in Ang-II-induced ERK1/2 phosphorylation. This effect of cAMP is reflected by an attenuation of Ang-II-induced Egr-1 expression.

Conclusion and perspectives

As discussed, Egr-1 is implicated in the pathogenesis of vascular diseases via its ability to mediate proliferative events in VSMC. Understanding the regulation of Egr-1 expression has a potential therapeutic value in the treatment of vascular disease. Overall, the studies presented in this thesis demonstrate that the SOCE molecules, STIM-1 and Orai-1, participate in Ang-II-induced Egr-1 expression in VSMC and, cAMP elevation, via PKA-induced suppression of ERK1/2 activity, can attenuate Ang-II-induced response on Egr-1 expression. Although Ca^{2+} signalling has extensively been shown to participate in VSMC phenotypic switch that underlies the process of vessel remodeling, the role played by STIM-1 and Orai-1 in vascular disease is still progressively being uncovered. STIM-1/Orai-1 complex emerges as a novel target for the regulation of Egr-1 expression in VSMC. We provide proof of the inhibitory effect of cAMP-induced PKA signalling on Ang-II-induced Egr-1 and suggest that regulation of Egr-1 is the mechanism by which catecholamines or cAMP elevating agents may exert their protective effect on vascular function. However, there is a growing interest in differentiating PKA-mediated and Epac-mediated signaling in response to cAMP. Thus, further studies combining the use of specific selective Epac analogs such as 8-pCPT-2′-O-Me-cAMP, or Epac antagonist such as ESI-09, may provide additional data useful for molecular intervention in this context. Our data support the requirement of ERK1/2 and CREB in transducing STIM-1/Orai-1-mediated Ang-II-induced Egr-1 expression as well as we provide evidence that increase in PKA-induced VASP signalling is associated with attenuation in ERK1/2 activation leading to the decrease in Ang-II-induced Egr-1 expression. By showing that AC-mediated cAMP production and PLC-mediated STIM-1/Orai-1 activity exerts opposite effects on ERK1/2 activation and Egr-1 expression in the presence of Ang-II, this work highlights for the first time Egr-1 regulation as a target for a cross talk between GPCR-mediated signalling pathways in hypertension and atherosclerotic vascular diseases. However, molecular mechanisms involved in these responses still need to be elucidated. Interestingly, ghrelin, an intestinal peptide that has recently emerged as a protective peptide in cardiovascular homeostasis (377-381) exhibits antiproliferative properties in VSMC (382). Ghrelin also mediates a decrease in Ang-II-induced Ca^{2+} release in a PKA-dependent fashion in VSMC

(383). Given the crucial role played by Ang-II-induced Ca^{2+} in Egr-1 induction and since our data reveal that PKA mediates an attenuation in Ang-II-induced Egr-1 expression, it is possible that ghrelin-mediated antiproliferative responses result from a downregulation of Egr-1 expression. In this line, ghrelin-induced PKA-dependent attenuation of Ang-II-induced Ca^{2+} release observed in these studies (383) raises the question of the effect of cAMP/PKA on STIM-1/Orai-1-mediated SOCE in response to Ang-II in VSMC. Figure 12 shows the modulation of Ang-II-induced Egr-1 expression by cAMP-mediated PKA signaling. We provide data on the implication of SOCE in the upregulation of Egr-1 and regardless of Ca^{2+} signaling, we demonstrate that PKA-induced signaling is accompanied by a decrease in Ang-II-induced expression (Figure 12). In the cardiovascular system, PKA can exert opposite effects on $[Ca^{2+}]_i$ homeostasis and thus lead to different implications for Ca^{2+}-dependent gene transcription. In cardiomyocytes, PKA-induced signaling is accompanied by a hyperactivity LTCC (384-386) whereas in VSMC, PKA is involved in LTCC attenuation (387, 388). Given the Ca^{2+} dependence of Egr-1, this difference is to be taken into consideration in studies addressing whether cardiovascular protection exerted by cAMP is mediated by a decrease in the expression of Egr-1. The use of genetic silencing or targeted loss of function may help in providing insights in a potential crosstalk between SOCE/VOCE and PKA-dependent signal transduction in VSMC under conditions of vascular disorders.

Addendum

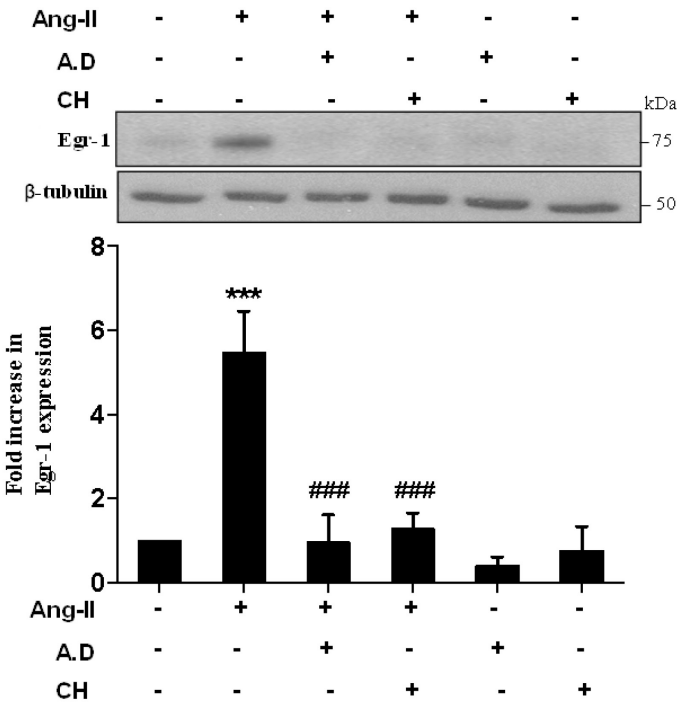


Figure 13: Attenuation of Ang-II-induced Egr-1 expression by actinomycin D and cycloheximide

Quiescent A-10 cells were pre-treated with 10 μM of actinomycin D (A.D) or cycloheximide (CH) for 30 minutes, followed by stimulation with 100 nM Ang-II for one hour. Cell lysates were probed with Egr-1 antibody (top panel) and β-tubulin (middle panel). Bar diagrams represent the densitometric quantifications of Egr-1. Values are the mean ± SEM of five independent experiments and expressed as fold increase compared to the control value (CTL) defined as 1. ***$p<0.001$, compared to control values. #$p<0.001$ compared to VSMC treated with Ang-II alone.

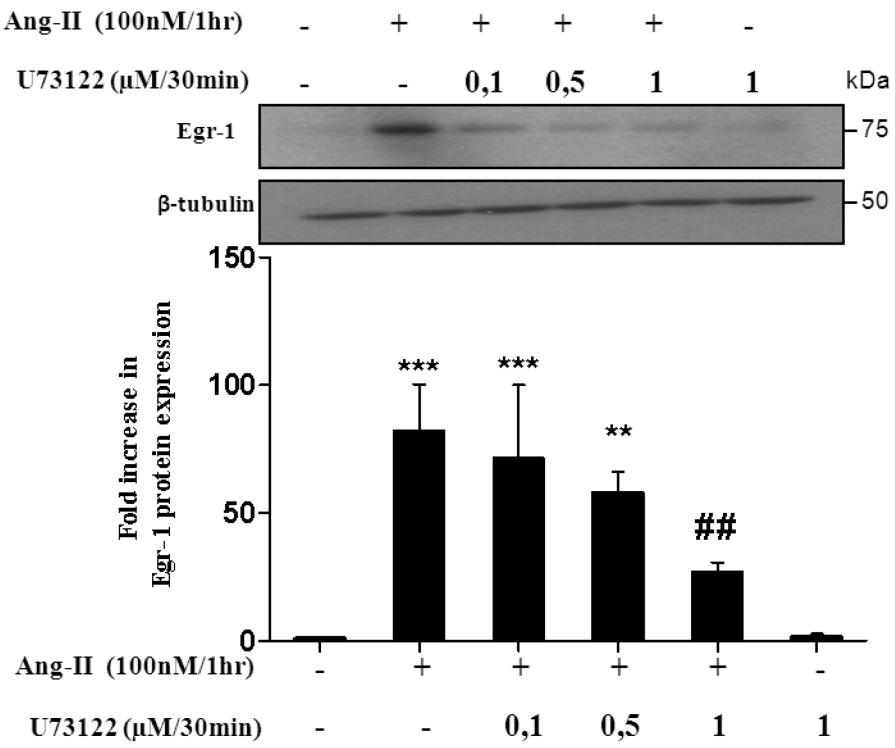


Figure 14: Inhibition of phospholipase C activity with U73122 attenuates Ang-II-induced Egr-1 expression

Quiescent A-10 cells were pre-treated with U73122 (0.1µM, 0.5µM and 1µM) for 30 min, followed by stimulation with 100nM Ang-II for one hour. Cell lysates were probed with Egr-1 antibody (top panel) and β-tubulin (middle panel). Bar diagrams (bottom panel) represent the densitometric quantifications of Egr-1. Values are the mean ± SEM of three independent experiments and expressed as fold increase compared to the control value (CTL). **$p<0.01$ compared with the control; ***$p<0.001$ compared to control values. $^{##}$ $p<0.01$ compared to VSMC treated with Ang-II alone.

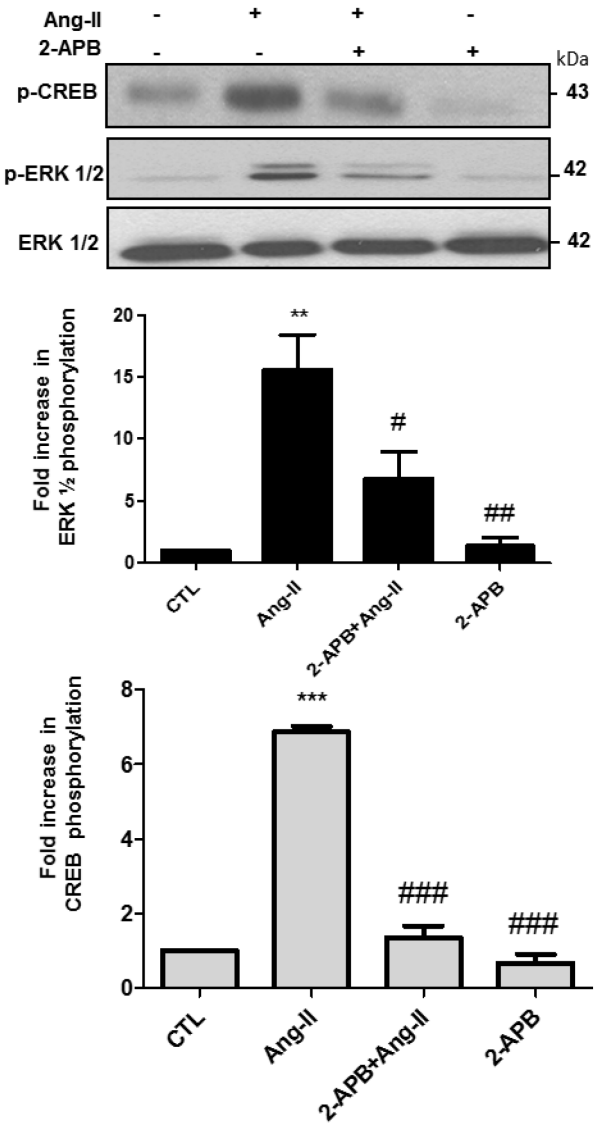


Figure 15: Attenuation of Ang-II-induced ERK and CREB phosphorylation by 2-APB

Quiescent A-10 cells were treated without (CTL) or with 2-APB (50 μM) for 30 minutes followed by stimulation with 100 nM Ang-II for five minutes. Panels show immunoblotting of cell lysates with antibodies corresponding to ERK1/2 and CREB respectively phosphorylated on Thr202/Tyr204 and Ser133. Blots were also analyzed for total ERK1/2. Bar diagrams represent average data quantified by densitometric scanning of immunoblots.Values are the mean ± SEM of three independent experiments and are expressed as fold increase compared to the CTL

defined as 1. **p<0.01, compared to control values. #p<0.05, ##p<0.01, ###p<0.001, compared to samples with Ang-II alone.

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