MOLECULAR MECHANISMS IN WOUND HEALING IN THE HEART: REGULATION OF THE CARDIAC FIBROBLAST AT1 RECEPTOR

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SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY,

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A THESIS PRESENTED BY

ANUPAMA V

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM 695011

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

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DECLARATION BY THE STUDENT

I, Anupama V, hereby certify that I had personally carried out the work depicted in the thesis titled: **"Molecular mechanisms in wound healing in the heart: Regulation of the cardiac fibroblast AT1 receptor"**. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date 23.06.2016 Anupama V

CERTIFICATE OF THE GUIDE

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This is to certify that Anupama V of the Division of Cellular and Molecular Cardiology of this Institute has fulfilled the requirements prescribed for the PhD degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum. The study titled: **"Molecular mechanisms in wound healing in the heart: Regulation of the cardiac fibroblast AT1 receptor"** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Clearance was obtained from the Institutional Animal Ethics Committee for carrying out the study.

Date 23.06.2016 Dr K Shivakumar

The thesis titled

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Submitted by

ANUPAMA V

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has been evaluated and approved by

Dr K Shivakumar (Guide) (Examiner)

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List of abbreviations

ACE	Angiotensin-converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
AP-1	Activator protein-1
AT1 receptor	Angiotensin II type 1 receptor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
ChIP	Chromatin Immunoprecipitation
cIAP-2	Cellular inhibitor of apoptosis protein-2
DDR2	Discoidin domain receptor 2
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
ECM	Extracellular matrix
ECs	Endothelial cells
EMSA	Electrophoretic mobility shift assay
EMT	Epithelial-to-mesenchymal transformation
EPDCs	Epicardial-derived cells
ERK1/2	Extracellular signal-regulated kinase 1/2
ET-1	Endothelin-1
FITC	Fluorescein isothiocyanate
H_2O_2	Hydrogen peroxide
HRP	Horse Radish peroxidase
IL	Interleukin
IκB	Inhibitory-kappa B
JNK	Jun-N-terminal kinase

LDL	Low density lipoprotein
MAPK	Mitogen-activated protein kinase
MMPs	Matrix metalloproteinases
NF-κB	Nuclear Factor-kappa B
NOX	NADPH oxidase
PI3K	Phosphatidylinositide 3-kinases
PKC	Protein kinase C
RAS	Renin angiotensin system
ROS	Reactive oxygen species
RVLM	Rostral ventrolateral medulla
TGF-β	Transforming Growth Factor-β
TIMPs	Tissue inhibitors of matrix metalloproteinases
TNF-α	Tumour Necrosis Factor-α
VSMCs	Vascular smooth muscle cells
α-SMA	Alpha-smooth muscle actin

SYNOPSIS

Cardiac fibroblasts, the most abundant cell type in the heart, are the principal source of myocardial collagen and several biochemical mediators such as matrix metaloproteases, growth factors and cytokines that exert significant paracrine actions on co-resident cells. Advances in recent years have unequivocally shown that cardiac fibroblasts contribute substantially to multiple aspects of myocardial pathophysiology and are a major determinant of myocardial remodelling associated with conditions such as hypertension, myocardial infarction and cardiomyopathies. In response to myocardial injury, normally quiescent cardiac fibroblasts undergo phenotypic transformation into activated myofibroblasts that proliferate and produce matrix components to replace the damaged myocytes and facilitate healing.

There has been a lot of interest in various growth factors and cytokines that modulate cardiac fibroblast function in the normal myocardium and in a setting of myocardial injury. The most potent pro-fibrogenic factor among them is Angiotensin II (Ang II), which has marked stimulatory effects on collagen expression in cardiac fibroblasts. Ang II elicits its cellular effects by binding to and activating the Angiotensin II receptor of which Angiotensin II receptor type I (AT1) plays a predominant role in mediating various physiological and pathophysiological effects of Ang II. AT1 receptor expression is highly variable and many cardiovascular disease states are associated with dramatic increase in AT1 expression. Since abundance of AT1 receptors defines the biological efficacy of Ang II, AT1 receptor antagonists are a targeted therapeutic tool for reducing or delaying fibrotic remodelling. Surprisingly, there is very little information on the regulation of the AT1 receptors in myocardial cells, specifically in cardiac fibroblasts, although AT1 receptor density is very high in these cells.

Oxidative stress, a major component of ischemia-reperfusion injury and congestive heart failure, is reported to impact AT1 expression in some cell types. However, the factors and mechanisms that contribute to altered AT1 expression under oxidative stress conditions are poorly characterised in molecular terms in any cell type. Against this backdrop, this study sought to understand the molecular events involved in the regulation of AT1 gene expression in cardiac fibroblasts exposed to H_2O_2, which has been implicated in cardiac dysfunction attributable to ischemia-reperfusion. The findings show that oxidative stress enhances AT1 expression in cardiac fibroblasts by a mechanism involving the redox-sensitive transcription factors, AP-1 and NF-κB, which are activated by the co-ordinated action of ERK1/2 MAPK, p38 MAPK and JNK. Importantly, this study also provides evidence, for the first time, that oxidative stress induces Ang II production that can impact matrix production in cardiac fibroblasts.

Methods

Cardiac fibroblasts were isolated from the ventricular tissue of young adult male Sprague Dawley (2-3 months) rats by a series of enzymatic digestions using collagenase, trypsin, pancreatin and DNase. Cells were cultured in M199 with 10% FBS and characterized by morphological and immunocytochemical methods. Sub-confluent cultures of cardiac fibroblasts from passage 2 or 3, synchronized by serum-deprivation for 24 hours, were exposed to hydrogen peroxide at 25 µM. The experimental model was validated by viability analysis and ROS measurement by H_2DCFDA. Taqman quantitative real-time PCR analysis, Western blotting, Electrophoretic Mobility Shift Assay (EMSA), Dual luciferase assay, Chromatin Immunoprecipitation assay (ChIP), gene knockdown by RNA interference and ELISA were performed following standard protocols. Statistical significance was

assessed using one-way ANOVA and Student's *t*-test and $p \leq 0.05$ was considered significant.

Major findings

Oxidative stress enhances AT1 receptor gene expression in cardiac fibroblasts via NADPH oxidase-mediated ROS generation

Cardiac fibroblasts were exposed to different concentrations of H_2O_2 and the viability of cells was checked by Hoechst/ PI staining. There was no significant loss of viability in cardiac fibroblasts treated with 10 or 25 μM H_2O_2. To examine whether exposure to H_2O_2 results in generation of intracellular reactive oxygen species (ROS), cells were loaded with H_2DCFDA and exposed to different concentrations of H_2O_2 for 10 minutes. A significant increase in intracellular ROS levels was observed in cardiac fibroblasts treated with 25 μM, 50 μM and 100 μM H_2O_2. Based on the observations from viability assay and ROS measurement by H_2DCFDA, a concentration of 25 μM was chosen for subsequent experiments.

Effect of H_2O_2 on AT1 expression in cardiac fibroblasts was examined. Real time PCR analysis revealed a 6-fold increase in AT1 mRNA levels at 3 hours of treatment with H_2O_2. Consistent with the increase in mRNA levels, western blot analysis showed a 6-fold increase in AT1 protein expression at 6 and 12 hours of treatment with H_2O_2.

H_2O_2 is reported to trigger NADPH oxidase-mediated ROS release in cardiac fibroblasts. In the present study, the effect of H_2O_2 on AT1 mRNA and protein levels was attenuated when cardiac fibroblasts were pre-incubated with specific pharmacological inhibitors of NADPH oxidase, DPI or VAS2870, indicating

that NADPH oxidase-dependent ROS generation augments AT1 expression in H_2O_2-treated cells. Consistent with these observations, the increase in intracellular ROS levels in cardiac fibroblasts exposed to H_2O_2 was inhibited by DPI.

NF-κB and AP-1 mediate transcriptional regulation of AT1 expression in cardiac fibroblasts

NF-κB and AP-1 are redox-sensitive transcription factors that are found to act in concert to regulate many genes involved in myocardial pathophysiology. The involvement of NF-κB and AP-1 in the regulation of AT1 expression was probed next. H_2O_2 was found to induce NF-κB and AP-1 activation in cardiac fibroblasts, as shown by EMSA. Further, NF-κB and AP-1 inhibition by pharmacological inhibitors significantly attenuated AT1 mRNA and protein expression levels, suggesting that NF-κB and AP-1 mediate transcriptional regulation of AT1 expression in cardiac fibroblasts. The direct binding of NF-κB and AP-1 transcription factors to the AT1 promoter was analysed by Dual luciferase assay and ChIP. Cardiac fibroblasts were transfected with AT1 promoter-luciferase DNA construct carrying the NF-κB and AP-1 binding sites. Treatment with H_2O_2 caused a significant increase in luciferase activity over the control group, showing H_2O_2-dependent activation of the AT1 promoter. Further, ChIP results showed significantly higher NF-κB p65, c-Fos and c-Jun cross-linking with the AT1 chromatin in H_2O_2-treated cells, compared to the control group, confirming the presence of binding sites for these transcription factors on the AT1 gene promoter.

MAPKs regulate AT1 receptor gene expression in cardiac fibroblasts by modulating the activity of NF-κB and AP-1 transcription factors

Redox-sensitive protein kinase-driven pathways are known to regulate NF-κB and AP-1 activation. Western blot analysis revealed a significant increase in the levels of the phosphorylated forms of ERK1/2 MAPK, p38 MAPK and JNK following different durations of H_2O_2 treatment, pointing to their activation. EMSA showed that H_2O_2-induced NF-κB DNA binding activity was abolished in ERK1/2- and p38 MAPK-inhibited but not JNK-inhibited cardiac fibroblasts, suggesting that NF-κB activation is ERK1/2- and p38 MAPK-dependent. However, inhibition of any of the three MAPKs annenuated AP-1 activation, indicating a role for all three MAPKs in H_2O_2-induced AP-1 activation. Further, AT1 mRNA and protein levels were down-regulated in ERK1/2 MAPK, p38 MAPK or JNK-inhibited/silenced cells, suggesting that MAPKs have a positive regulatory role in AT1 expression under conditions of oxidative stress.

Oxidative stress triggers local angiotensin production in cardiac fibroblasts

Subsequent experiments led to the serendipitous finding that candesartan, the AT1 receptor antagonist, and enalapril, the ACE inhibitor, abolished H_2O_2-induced increase in AT1 mRNA and protein expression in cardiac fibroblasts, indicating that H_2O_2 triggers local Ang II production in these cells that in turn enhances AT1 expression. Consistent with these observations, H_2O_2 was found to cause a significant increase in Angiotensinogen mRNA expression and secreted levels of Ang II. To the best of our knowledge, existence of a local RAS in cardiac fibroblasts that is sensitive to altered redox potential has not hitherto been reported for any cell type.

Locally generated Ang II mediates oxidative stress-induced collagen production in cardiac fibroblasts via NADPH oxidase, MAPKs, NF-κB and AP-1

H_2O_2 significantly increased collagen mRNA and protein expression in cardiac fibroblasts, which was decreased by candesartan, confirming the involvement of Ang II in the H_2O_2 effect on collagen. Moreover, inhibition of NADPH oxidase, NF-κB, AP-1, ERK1/2 MAPK, p38 MAPK and JNK attenuated collagen protein expression, indicating that the same pathway mediates H_2O_2-induced increase in AT1 and collagen expression.

Significance of the study

Investigations on AT1 regulation in cardiac fibroblasts are of obvious importance as the AT1 receptor mediates the pro-fibrotic effects of Ang II on these cells that in turn profoundly impact myocardial growth and remodelling in pathological states. Apart from delineating the molecular basis of oxidative stress-induced increase in AT1 gene expression in cardiac fibroblasts, the study also provides evidence for the first time that H_2O_2 triggers Ang II production in cardiac fibroblasts. By causally linking oxidative stress to augmented expression of Ang II, AT1 and collagen in cardiac fibroblasts, this study expands our understanding of the implications of oxidative stress in cardiac pathobiology and provides a new paradigm to understand the pathogenesis of cardiovascular conditions associated with oxidative stress.

I. INTRODUCTION

The heart is a myogenic muscular organ in the circulatory system responsible for pumping blood by repeated, rhythmic contractions. The wall of the heart is made up of three layers, the epicardium, myocardium and endocardium. The myocardium (middle layer), which constitutes the bulk of the heart wall, is a highly organized structure consisting of two inter-dependent compartments, parenchyma and stroma (Braunwald, 1971; Weber et al., 1995). The cardiac parenchyma consists of fully differentiated post-mitotic cells, the myocytes or cardiocytes. Cardiac myocytes are the fundamental contractile units of the myocardium that confer pump function (Walker and Spinale, 1999). Although myocytes occupy 75% of the myocardial structural space, they constitute only one-third of the myocardial cell population. The remaining cells are found in the cardiac interstitium or the stromal compartment. Stroma is a dynamic metabolic entity consisting of endothelial cells, VSMCs, cardiac fibroblasts, macrophages and mast cells embedded in a protein scaffold (Weber, 1989). The acellular interstitial components of the heart, collectively referred to as the extracellular matrix (ECM), include interstitial collagens, proteoglycans, glycoproteins, matrix turnover-related enzymes, cytokines and growth factors (Corda et al., 2000). While the interstitial protein scaffold provides a structural and mechanical continuum for the normal functioning of the heart, the cellular components of the stroma interact in a dynamic fashion to maintain electrical, chemical and biochemical responses of the heart in diverse pathophysiological conditions (Weber, 1989).

Cardiac fibroblasts are undifferentiated cells that represent more than 90% of the cardiac interstitial cells and two-thirds of the myocardial cell population (Souders et al., 2009). They are the only intracardiac source of type I and III collagens, the major fibrillar collagens of the myocardium, that constitute its normal

structural protein network (Baudino et al., 2006; Fan et al., 2012). Further, they are an important “source and target” of a large array of bioactive molecules like growth factors, cytokines and immunomodulatory factors (Brown et al., 2005; Porter and Turner, 2009). In recent years, there has been increasing appreciation of the manifold contributions of fibroblasts to cardiac pathophysiology, particularly during tissue response to injury and myocardial remodelling *post injury.*

I.1. *Identification of the problem*

In a setting of myocyte loss, cardiac fibroblasts get phenotypically transformed into active myofibroblasts and converge on the site of myocyte injury where they proliferate, produce matrix proteins and facilitate wound healing (Baudino et al., 2006). Unlike fibroblasts in non-cardiac tissues that are removed by apoptosis upon completion of wound healing, cardiac fibroblasts resist apoptosis and remain active even after wound healing is completed (Porter and Turner, 2009); they continue to proliferate and lay down matrix components, which leads to stromal expansion and consequent impairment of pump function. Further, myocyte injury produces a dramatic surge in cytokine production in the myocardium that turns on cardiac fibroblasts, thereby setting the stage for myocardial repair. In this altered cytokine milieu, the factor that is reported to exert the most profound effects on cardiac fibroblasts is Angiotensin II (Ang II), which exerts pleiotropic effects on the heart.

Ang II, the main effector peptide of the renin-angiotensin system (RAS), is a multifunctional hormone implicated in cardiovascular pathology associated with hypertension, myocardial infarction, cardiomyopathies and heart failure (Griendling et al., 1993; Murphy et al., 2015). Although enhanced activity of circulating RAS and

excessive sympathetic outflow may contribute to many of these cardiovascular complications, there also exists an independent, functional, localised cardiac RAS whose effects on the myocardium can be more pronounced than it was previously acknowledged (Mitra et al., 2010; Xu et al., 2010). Studies on RAS expression in different cell types within the myocardium support a role for intra-cardiac RAS in the pathophysiology of cardiac remodelling. All the constituents of RAS, including Ang II, are expressed in the heart where they constitute an autocrine/paracrine tissue RAS (Dostal et al., 1992; Shivakumar et al., 2003).

Ang II exerts its physiological effects by activating a number of intracellular signal transduction pathways via the Ang II type 1 (AT1 receptor) (Matsoukas et al., 2013). AT1 receptor is a prototypical G protein-coupled receptor with seven trans-membrane spanning regions (Ichiki, 2013). Many cardiovascular events like myocardial infarction are associated with elevated expression of AT1 receptor, which can profoundly impact the recovery process (de Boer et al., 2003; Lefroy et al., 1996) by exacerbating Ang II action (Karnik et al., 2015). The widespread use of AT1 antagonists in patients with myocardial infarction and congestive heart failure provides a strong ground for studies on the molecular basis of AT1 gene regulation in the cardiovascular system. In this regard, AT1 regulation in the cardiovascular regulatory regions of the brain was addressed by Zucker and co-workers (Liu et al., 2008; Haack et al., 2013) who report a regulatory role for MAPKs, NF-kB and AP-1 in AT1 expression. Surprisingly, despite the cardinal role of cardiac tissue RAS in myocardial pathophysiology, the critical role of Ang II in the regulation of cardiac fibroblast function, AT1 receptor abundance in these cells and the clinical benefits of AT1 receptor block, molecular mechanisms involved in AT1 gene regulation in cardiac cells remain inadequately addressed.

I.2. *Heterologous receptor regulation of AT1 expression*

Since most Ang II actions are mediated by the AT1 receptor, AT1 receptor density defines the biological efficacy of Ang II (Nickenig and Harrison, 2002a). Previous studies have demonstrated that Ang II regulates AT1 expression via NADPH oxidase (NOX)-dependent reactive oxygen species (ROS) release (Liu et al., 2008). Interestingly, apart from Ang II, AT1 receptor expression is also subject to regulation by other agonists, a phenomenon referred to as 'heterologous receptor regulation' (Nickenig and Harrison, 2002b). In VSMCs, agonists like estrogen and cAMP-stimulating agents decrease AT1 expression (Nickenig et al., 2000; Wang et al., 1997) by stimulating the degradation of its mRNA whereas progesterone, LDL and insulin (Nickenig et al., 1997, 1998, 2000) up-regulate AT1 expression by decreasing mRNA decay. Tumour Necrosis Factor-α (TNF-α) and IL-1ß were also found to up-regulate AT1 expression in cardiac fibroblasts (Cowling et al., 2002). Although no uniform signal transduction pathway has been defined that modulates AT1 expression in all cell types, cAMP, p38 MAPK, ERK1/2 MAPK, PI3K, nitric oxide and superoxide are reported to modulate AT1 receptor expression positively or negatively in response to different stimuli (Nickenig and Harrison, 2002b). Sporadic reports suggest that oxidative stress, which has been implicated in many cardiovascular conditions (Tsutsui et al., 2011), modulates AT1 expression in macrophages, renal cells and VSMCs (Banday and Lokhandwala, 2008; Bhatt et al., 2014; Keidar et al., 2002). Less clear, however, is the molecular basis of AT1 gene regulation in any cell type under conditions of oxidative stress.

I.3. *Broad objective*

Against this backdrop, this study probed the mechanisms involved in the regulation of AT1 gene expression in cardiac fibroblasts in response to oxidative stress. Specifically, the role of redox-sensitive transcription factors and MAPKs in the regulation of AT1 expression was explored in cardiac fibroblasts exposed to H_2O_2, which has been recognized as a potent endogenous mediator and an important exogenous cardiovascular research tool to model oxidant-dependent changes in the cardiovascular system (Gough and Cotter, 2011).

I.4. *Specific objectives*

The following specific questions were addressed:

- Does oxidative stress enhance AT1 receptor mRNA and protein expression in cardiac fibroblasts?
- Is NOX-mediated ROS generation involved in AT1 receptor expression?
- Do NF-κB and AP-1 transcriptionally regulate AT1 gene expression in cardiac fibroblasts exposed to oxidative stress?
- Do ERK1/2 MAPK, p38 MAPK and JNK regulate NF-KB and AP-1 and impact AT1 expression in cardiac fibroblasts?

I.5. *Major findings*

H_2O_2 enhanced AT1 receptor gene expression in cardiac fibroblasts via NOX-dependent ROS induction. Activation of NF-κB and AP-1, abolition of AT1 expression upon their inhibition, and their binding to and activation of the AT1 promoter confirmed transcriptional control of AT1 by NF-κB and AP-1 in H_2O_2-

treated cells. Further, H_2O_2 activated ERK1/2 MAPK, p38 MAPK and JNK and their inhibition using pharmacological inhibitors and by siRNA attenuated AT1 expression. Inhibition of the MAPKs showed that while ERK1/2 MAPK and p38 MAPK suffice for NF-κB activation, all three kinases are required for AP-1 activation. Interestingly, the AT1 antagonist, candesartan, attenuated H_2O_2-stimulated AT1 and collagen mRNA and protein expression, showing, for the first time, that H_2O_2 up-regulates AT1 and collagen expression via local Ang II generation. This was corroborated by H_2O_2-induced transcriptional up-regulation of Angiotensinogen mRNA expression and elevated levels of secreted Ang II.

II. REVIEW OF LITERATURE

II.1. *Cardiac fibroblasts*

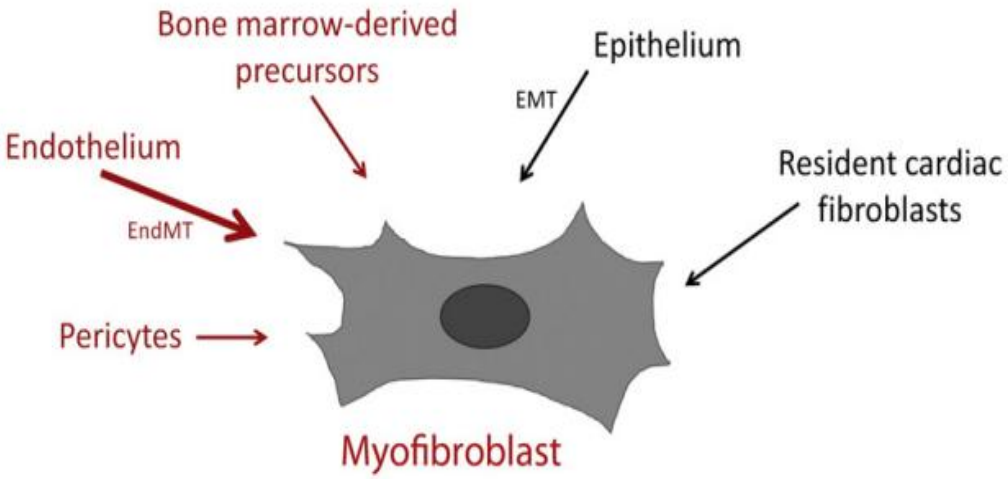
Maintaining the quantitative relationship between parenchyma and stroma is critically important to ensure optimal myocardial pump function (Brown et al., 2005). However, under conditions of cardiovascular stress, myocyte hypertrophy and apoptosis, fibroblast hyperplasia and interstitial fibrosis occur, which will disturb the ratio of functional parenchymal cells to structural stromal cells (Piek et al., 2016). Until the early 1990s, cardiac dysfunction was viewed solely in terms of damage to cardiac myocytes; cardiac fibroblasts, despite being the most abundant cell type in the heart, were historically overlooked in terms of their contributions to cardiac pathophysiology and disease pathogenesis (Lajiness and Conway, 2014). But today, it is recognized that fibroblasts act not only as a critical player in response to injury but also as an active participant in normal cardiac function.

Fibroblasts are cells of mesenchymal origin that produce interstitial collagen types I, III, IV and VI and fibronectin (Krenning et al., 2010). The specific marker for cardiac fibroblasts is the collagen receptor, DDR2, although vimentin, periostin and alpha-smooth muscle Actin (α-SMA) are also considered as cardiac fibroblast markers (Camelliti et al., 2005). Cardiac fibroblasts are spindle shaped, have multiple processes originating from their cell body and are the only cells in the heart that lack a basement membrane (Baudino et al., 2006).

II.1.1. Origin of cardiac fibroblasts

Fate-mapping studies, which throw light on the developmental history of each cell in the adult body, suggest that cardiac fibroblasts are derived from different progenitor cells depending on the stage of heart maturation and the cellular context (Zeisberg and Kalluri, 2010). Majority of embryonic cardiac fibroblasts are derived

from two principal sources: (1) the pro-epicardial organ and (2) the epithelial–mesenchymal transformation. Mesenchymal cells in the embryonic pro-epicardium give rise to a migratory cell population that eventually migrates over the surface of the embryonic heart and forms the epicardium (Lajiness and Conway, 2014). Some of these cells, under the influence of growth factors, then undergo epithelial-to-mesenchymal transition (EMT) to become epicardial-derived cells (EPDCs) that eventually invade the atrial and ventricular walls, progressively differentiate into a fibroblast phenotype and establish the compact myocardium (Snider et al., 2009). However, following injury to the adult heart, resident fibroblasts and cells from a variety of spatiotemporal sources such as endothelial cells, developing bone marrow and neural crest, pericytes or circulating progenitor cells are stimulated to differentiate into myofibroblasts and mediate tissue repair (Zeisberg and Kalluri, 2010).



(Lajiness and Conway, 2014)

Figure 1: Origin of cardiac fibroblasts

II.1.2. Structural organisation of cardiac fibroblasts

Cardiac fibroblasts are organized in a three dimensional fashion throughout the heart, surrounding group of myocytes and maintaining contact with each other, with cardiac myocytes and the ECM (Camelliti et al., 2005). Such an arrangement equips cardiac fibroblasts to respond to diverse stimuli arising during several pathophysiological states within the myocardium (Souders et al., 2009). Cardiac fibroblasts interact with the ECM through Integrins and DDR2 whereas intercellular connections appear to be through two families of cell surface proteins, connexins and cadherins. The connexin that connects fibroblasts to myocytes is Cx43 and that which connects fibroblasts to other fibroblasts is Cx45 (Goldsmith et al., 2004).

II.1.3. Pleotropic functions of cardiac fibroblasts

Cardiac fibroblasts are not just collagen-producing cells of stroma but an essential and dynamic cell population that are crucially involved in the development of the myocardium as well as its response to injury. Some relevant aspects of cardiac fibroblast function are as follows:

II.1.3.1. Maintenance of ECM homeostasis

The most critical role of cardiac fibroblasts is their contribution to the secretion, maintenance and remodelling of ECM (Porter and Turner, 2009). While improper matrix deposition can lead to inadequate mechanical strength, myocyte slippage and subsequent ventricular rupture, excessive fibroblast proliferation and increase in ECM protein content can induce myocardial stiffening. Thus, regulating the synthesis and secretion of ECM components (collagens type I and type III, elastin and laminin) as well as ECM regulatory and remodelling molecules such as

matricellular proteins, matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) is important in the maintenance of myocardial tissue structure (Fan et al., 2012).

II.1.3.2. Production of bioactive molecules

Cardiac fibroblasts produce and secrete proinflammatory cytokines such as IL-1β, IL-6, TNF-α and Transforming growth factor β (TGF-β). By creating a pro-fibrotic environment, these cytokines protect the injured myocardium and facilitate myocyte survival. In addition to pro-inflammatory cytokines, cardiac fibroblasts also secrete Ang II, Endothelin-1 (ET-1), natriuretic peptides, Vascular endothelial growth factor (VEGF) and hormones (noradrenalin) to facilitate the wound healing process (Baudino et al., 2006; Porter and Turner, 2009; Souders et al., 2009). The beneficial effects of Angiotensin-converting enzyme (ACE) inhibitors, ARBs (Angiotensin receptor blockers), statins and beta blockers on adverse ECM remodelling can be due to deterring the expression of these cardiac fibroblast-mediated cytokines and growth factors (Brown et al., 2005).

II.1.3.3. Role in cardiac electrophysiology

Cardiac fibroblasts were considered electrically inert and were historically overlooked in terms of their role in the cardiac electrical milieu. Recent reports, however, indicate that they contribute directly to cardiac electrophysiology (Lajiness and Conway, 2014). Cardiac fibroblasts affect electrophysiology passively by electrically coupling to myocytes through connexins; this coupling can alter the electrical properties of myocytes as well as help in synchronising contractions between individual myocytes (Porter and Turner, 2009; Vasquez et al., 2011). Cardiac fibroblasts can also act as mechano-electrical transducers by responding to

mechanical stimuli through changes in their membrane potential (Kamkin et al., 2003; Krenning et al., 2010).

II.1.3.4. Homeostasis of cardiac vessels

Cardiac fibroblasts produce and secrete fibroblast growth factor (FGF) and VEGF that can promote angiogenesis (Zhao and Eghbali-Webb, 2001). The tight association between fibroblasts and endothelial cells also seems to suggest that cardiac fibroblasts are important for blood vessel formation during development and disease. However, cardiac fibroblasts have also been reported to express and secrete connective tissue growth factor (CTGF) and platelet-derived growth factor (PDGF), which are considered to be anti-angiogenic (Inoki et al., 2002; Zhao and Eghbali-Webb, 2001). Thus, these cells are significant contributors to cardiac vessel homeostasis.

II.1.3.5. Wound healing in the heart

In a setting of myocardial injury, myocytes die and fail to regenerate whereas cardiac fibroblasts facilitate wound healing through a complex mechanism involving three stages, adaptive phase, inflammatory phase and granulomatous phase (Brown et al., 2005). In the early adaptive phase of wound healing response, the MMP/TIMP balance is altered leading to changes in net proteolytic activity (Spinale, 2007). This facilitates ECM degradation and allows inflammatory cells to infiltrate into the site of injury and clear the wound by phagocytizing dead myocytes and remnant cellular debris. Inflammatory phase is marked by significant neurohumoral changes and dramatic surge in cytokine production in the myocardium. As a consequence, cardiac fibroblasts undergo phenotypic transition into myofibroblasts characterized by hypersecretory capacity and contractile

properties due to the expression of α-SMA (Hinz et al., 2001). Activated cardiac fibroblasts chemotactically migrate from the wound margin into the zone of injury (Porter and Turner, 2009). Fibrogenic phase succeeds, which is marked by the accelerated synthesis of ECM proteins such as collagen types I and III, fibronectin and laminin causing the granulomatous tissue to progress to a mature scar (Gurtner et al., 2008). Unlike fibroblasts in non-cardiac tissues that are removed by apoptosis upon completion of wound healing, cardiac fibroblasts resist apoptosis (Philip and Shivakumar, 2013) and remain active even after wound healing; they continue to proliferate, release inflammatory signals and lay down matrix components, thereby altering the relative proportions of parenchyma and stroma (Krenning et al., 2010; Souders et al., 2009). Over a period of time, this reduces ventricular compliance and compromises cardiac function.

In the normal myocardium as well as in the infarct tissue, cardiac fibroblasts encompass pleotropic functions rather than just proliferating and contributing collagen to the ECM. In this regard, it is pertinent to note that among the various molecules that affect the phenotype and function of cardiac fibroblasts, the factor that exerts most profound effects on cardiac fibroblasts and sets the stage for myocardial repair and remodeling is Ang II, the effector of RAS cascade (Baudino et al., 2006; Regitz-Zagrosek et al., 1998; Sun, 2009).

II.2. *Renin-angiotensin system*

RAS is a co-ordinated hormonal cascade in the control of cardiovascular, renal and adrenal function that governs fluid and electrolyte balance and arterial pressure (Carey and Siragy, 2003). Classically defined as a peptidergic system with endocrine characteristics, proper functioning of RAS is essential to maintain

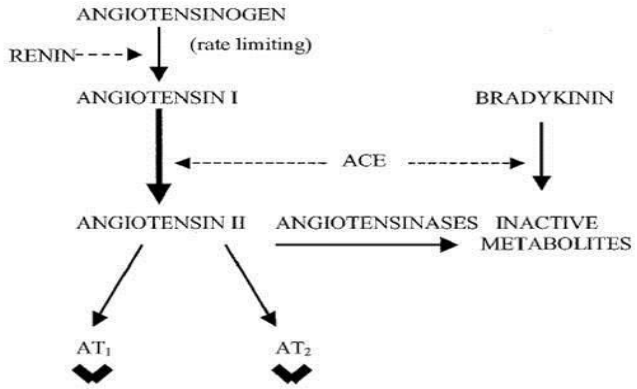
homodynamic stability, which is achieved through its complementary interactions with the hemodynamic effects produced by sympathetic nervous system and several vasoactive hormones (Alderman, 2004; Givertz, 2001). These complementary interactions allow RAS to respond to a variety of stimuli that can perturb blood pressure stability, ECF volume homeostasis and end organ perfusion.

Ang II, the biologically active hormone generated by RAS, binds to its specific receptors, triggering a broad range of biological actions impacting virtually every system in the body including the brain, heart, kidney, vasculature and immune system. In addition to the maintenance of blood pressure, RAS can also impact a range of processes including cardiovascular growth and remodelling (van Kesteren et al., 1997), ECM turnover (Ruiz-Ortega and Egido, 1997), inflammation and immune responses (Benigni et al., 2010).

II.2.1. Systemic RAS

The circulating/systemic RAS cascade begins with the synthesis and secretion of Renin, an enzyme belonging to the class of aspartyl proteinases, by juxtaglomerular apparatus of kidney (Griendling et al., 1993). Control of renin secretion is a key determinant of RAS activity. Renin cleaves substrate Angiotensinogen, synthesised and released into the circulation by liver, to form a biologically inert decapeptide, Angiotensin I (Ang I). This reaction is considered to be the rate-limiting step of the RAS cascade (Brown, 2007). Ang I is hydrolysed by ACE to form a biologically active octapeptide, Ang II. ACE is a membrane-bound metalloproteinase expressed predominantly on the surface of endothelial cells in the pulmonary circulation. Apart from generating vasoconstrictive Ang II, ACE also metabolizes vasodilator peptides like bradykinin and kallidin to inactive metabolites

and so it is also known as kininase II (Hooper, 1991). Ang II activates Ang II receptor, the main transducer of the cellular effects of Ang II, (Dinh et al., 2001) and induces either vasoconstriction or release of aldosterone from the adrenal cortex.



(Carey and Siragy, 2003)

Figure 2: Schematic representation of RAS cascade

RAS was historically viewed as a classical humoral system, wherein the various components secreted from dedicated glands are delivered to their site of action by the circulatory system. But now several organs or tissues are found to contain/synthesise the components of RAS, which expands the traditional circulating RAS paradigm to include “local RAS or tissue RAS” (Paul et al., 2006). The current concept is that RAS can function both as a circulating system and as a tissue paracrine/autocrine system.

II.2.2. Local tissue RAS

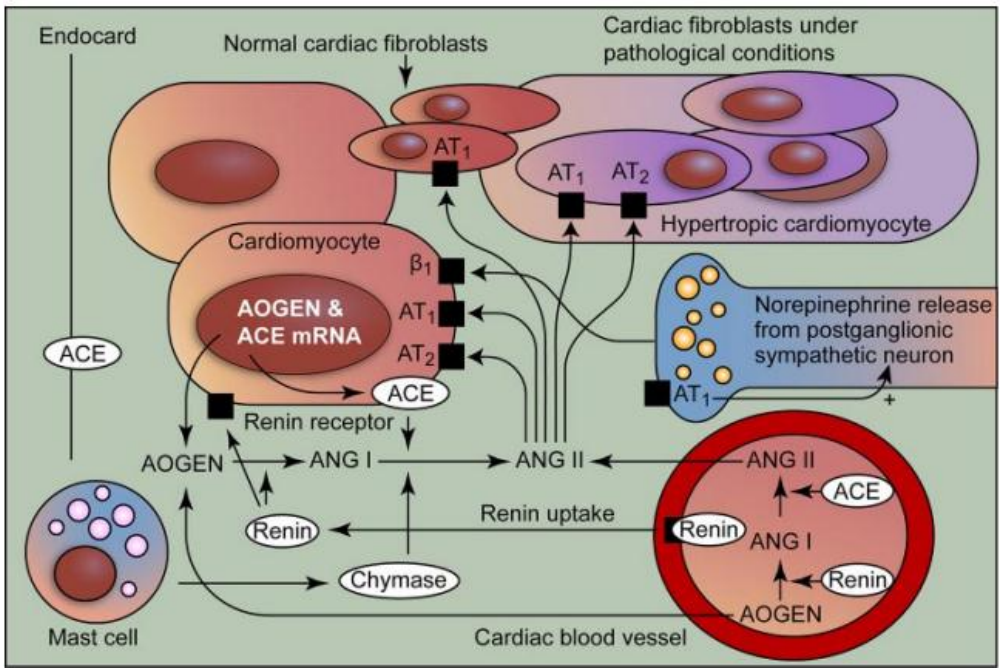
Several lines of evidence, including the identification of Ang II biosynthesis occurring locally in several tissues, characterisation of angiotensin receptors, identification of the unique actions of truncated sequences of angiotensin together

with the recent identification of putative cell surface receptors for renin and pro-renin, have emerged to support the presence of a self-sufficient tissue RAS (Paul et al., 2006). Pre-requisite for a tissue RAS is that all of the components necessary for the biosynthesis of Ang II must reside within the tissue, although some of the components can be taken up from the circulation (Bader et al., 2001). Local tissue RAS is particularly important in the development of disease processes since they can flank the action of the endocrine system by playing autocrine and paracrine functions (Campbell, 2014). The Ang II generated locally in the tissues will either interact with the Ang II receptors on the same (autocrine) cell or on adjacent (paracrine) cells. These putative local systems thus help in stringently regulating the actions of Ang II to a specific organ system or physiological event (Paul et al., 2006).

Independent Ang II-generating tissue RAS has been postulated to exist in the heart (Dostal and Baker, 1999), adipose tissue (Engeli et al., 2000), brain (Sakai and Sigmund, 2005), adrenal glands (Carey and Siragy, 2003), peripheral blood vessels, kidney, pituitary, testes, ovaries and skin (Paul et al., 2006).

II.2.2.1. Intra-cardiac RAS

Recognition of a localised RAS in the heart with all of the components necessary for synthesising Ang II and of responding to its effect has shed light on the role of this system in cardiac growth and development. The components required for Ang II generation in the myocardium are distributed among fibroblasts, cardiomyocytes, endothelium and vascular smooth muscle of the coronary arteries and veins (Dostal and Baker, 1999).



(Paul et al., 2006)

Figure 3: The Renin Angiotensin System in the heart

Renin has been identified in the whole heart as well as in primary cultures of neonatal and adult ventricular myocytes isolated from various species including humans (Barlucchi et al., 2001; Dostal et al., 1992; Serneri et al., 1996). However, myocardial concentration of Renin is relatively low and is only 1–4% of that in plasma indicating that Renin could be the least abundant RAS precursor in the myocardium. Some reports suggest that under physiological conditions, Renin is not synthesized in the heart and that Renin gene expression may be turned on only in pathophysiological situations (Mello and Danser, 2000). Another point of debate is whether local synthesis is the primary source of cardiac Renin since circulating Renin and pro-renin can be easily sequestered from blood by cardiac tissue. It is also pertinent to note that in adult rat myofibroblasts isolated from ventricular scar

tissue, “an aspartyl protease with cathepsin D–like properties” mediated the conversion of Angiotensinogen to Ang I, pointing to the involvement of a renin-independent pathway in Ang I synthesis in cardiac cells (Katwa et al., 1997).

Angiotensinogen and ACE are distributed heterogeneously in the heart. Angiotensinogen is expressed in all parts of the heart and in cultured cardiac myocytes and fibroblasts (Carey and Siragy, 2003). ACE is primarily expressed by coronary endothelial cells and cardiac fibroblasts (Hafizi et al., 1998). Though various studies suggest that ACE is the primary pathway leading to the production of Ang ll in the heart, intracardiac conversion of Ang I to Ang II may also occur via chymase, a chymostatin-sensitive serine protease secreted by mast cells (Urata et al., 1993). Chymase represents the dominant Ang II-generating pathway in the heart, coronary arteries and atherosclerotic aorta of certain species like human and dog.

Ang I and Ang II are localized in atria and/or ventricles of various species. Myocytes, fibroblasts and microvascular endothelial cells can synthesise Ang II under culture conditions suggesting that these cell types can independently contribute to Ang II production (Dostal et al., 1992; Fischer et al., 1997). However, the cardiac interstitial fluid concentrations of Ang I and Ang II are over 100-fold greater than in plasma, suggesting that the majority of the Ang II found in cardiac tissue is derived from myocardial synthesis rather than from the systemic circulation.

Both subtypes of Ang II receptors, AT1 and AT2, are expressed in the heart and cultured myocytes (Dinh et al., 2001). Under normal conditions, cardiac fibroblasts express only AT1 receptor but can recruit AT2 receptor under certain pathological conditions like heart failure (Asano et al., 1999; Ohkubo et al., 1997).

Modulation of expression of RAS components in cardiac fibroblasts

Fibroblasts are the major RAS-positive cells in the heart that are involved in maintaining cardiac structure and function. In neonatal rat cardiac fibroblasts, while Atrial natriuretic factor (ANP) and isoproterenol positively regulate Renin and Angiotensinogen synthesis (Dostal et al., 2000; Sanghi et al., 2005), high glucose-induced intracellular Ang II synthesis from Angiotensinogen is catalysed by Renin and ACE (but not chymase) (Singh, Baker, et al., 2008). Cardiac fibroblasts from senescent rats also express the transcripts for Renin, Angiotensinogen and the AT1 receptor, thereby bringing about significant modifications in the response of old rats to Ang II (Shivakumar et al., 2003).

II.3. *Effects of RAS on the heart*

Ang II exerts both direct and indirect effects on the heart, which can affect its rate, contractility and cell growth (K M Baker et al., 1992). While indirect effects of Ang II are mediated through its effects on cardiovascular regulatory regions in the brain, alteration of sympathetic outflow and stimulation of aldosterone synthesis and release, direct actions of Ang II on the heart include chronotropic, inotropic and growth-promoting effects and effects on metabolism and remodelling (Goldsmith, 1999; Kats et al., 2000; Schorb et al., 1993).

II.3.1. Inotropic and chronotropic effects

Ang II is known to induce a positive inotropic effect, independent of β-adrenergic system, on cardiomyocytes of a variety of species including humans (Atlas, 2007). This dose-dependent response of Ang II is suppressed by ARBs,

suggesting that these effects are receptor-mediated and involve alterations in calcium homeostasis and trans-membrane calcium conductance, most likely via activation of protein kinase C (PKC) (Lindpaintner and Ganten, 1991). There are many reports for and against the chronotropic effects of Ang II on various cardiac cells and tissues isolated from different species (Atlas, 2007).

II.3.2. Growth-promoting effects

Apart from its classical actions on heart rate and blood pressure, Ang II drives cell growth and replication in a number of myocardial cell types, which may result in myocyte hypertrophy (Liu et al., 1998), cellular hyperplasia/ hypertrophy of VSMCs (Rosendorff, 1996) and fibroblast hyperplasia (Bouzegrhane and Thibault, 2002). The differential growth effects of Ang II (proliferation vs hypertrophy) are dependent on the cell type and cytokine milieu. Further, Ang II is also reported to be involved in remodelling processes through the modulation of matrix turnover and collagen production by acting on several components of ECM formation like MMPs and TIMPs (Fan et al., 2012).

Ang II has been shown to stimulate growth and induce protein synthesis and gene expression in isolated cardiac myocytes, which result in a hypertrophic phenotype, either by a direct action mediated by the AT1 receptor or by stimulating the release of paracrine factors like TGF-β and ET-1 from cardiac fibroblasts (Booz and Baker, 1998; Gray et al., 1998; Miyata and Haneda, 1994). Ang II has growth-stimulating effects on VSMCs and exerts profound effect on the composition of the ECM including effects on the synthesis and secretion of thrombospondin, fibronectin and tenascin (Griendling et al., 1997; Hahn and Bühler, 1992). In genetic hypertension, Ang II alters the structure of small arteries due to cellular hyperplasia

or remodelling whereas in renovascular hypertension it is due to hypertrophy of VSMCs (Rosendorff, 1996). Ang ll-mediated hypertrophy in VSMCs may initially be an adaptive mechanism to regulate its mass and maintain optimal vascular tone in response to work load. However, these hypertrophic signals when sustained for longer periods lead to the development of hypertension or cause structural vascular alterations (Heeneman et al., 1997).

By modulating various aspects of cardiac fibroblast function like growth, proliferation, migration and ECM protein synthesis, Ang II can exert profound effects on myocardial pathophysiology and act as a critical component of the cardiac remodeling process (Regitz-Zagrosek et al., 1998). Ang II is reported to act as a mitogenic stimulus and induce increase in protein synthesis, DNA synthesis and cell number in cardiac fibroblasts (Sadoshima and Izumo, 1993; Schorb et al., 1993). The most profound effect of Ang II on cardiac fibroblasts is its effect on the synthesis, deposition, and degradation of matrix proteins (Fan et al., 2012; Sullivan and Black, 2013). Ang II enhances synthesis and expression of collagen I and III, the major collagens of the heart. Concurrently, Ang II attenuates the expression of collagen-degrading enzyme MMP-1 (Chen et al., 2004; Lijnen et al., 2006; Stacy et al., 2007) and increases expression of TIMPs that control the activities of MMPs, causing fibrosis and increased collagen deposition in the cardiac interstitium (Pan et al., 2008; Stacy et al., 2007). Recent studies suggest that Ang II can also augment the expression of collagen receptor, DDR2, which can positively modulate collagen production in cardiac fibroblasts and therefore play a crucial role in Ang II-induced cardiac fibrosis (George et al., 2016). Further, Ang II is considered to be the most important factor that exerts profound effects on cardiac fibroblasts to facilitate wound healing. Treatment with Ang II is reported to influence cardiac fibroblast proliferation

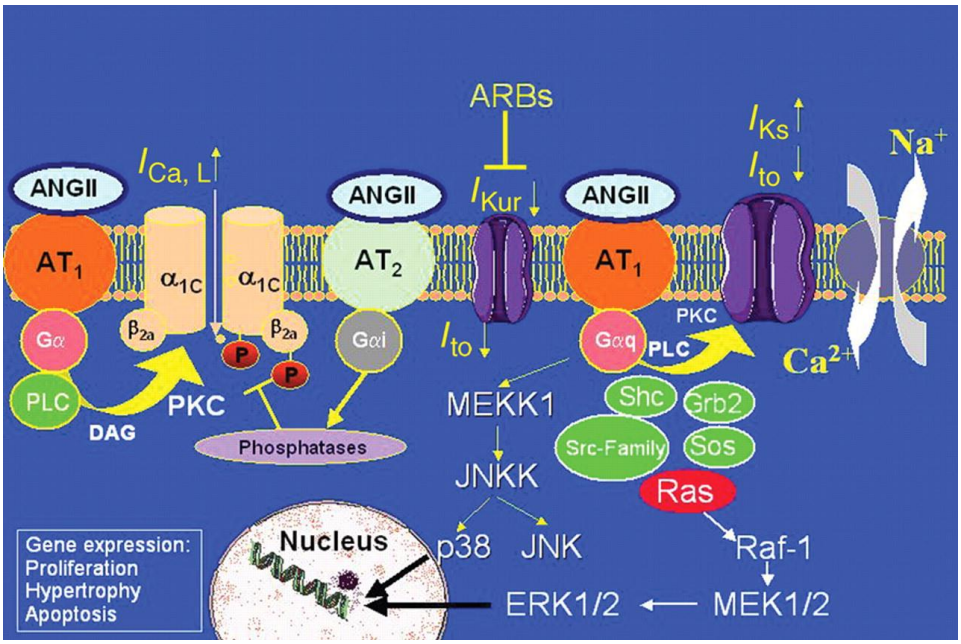
and migration in various models of myocardial injury (Deb and Ubil, 2014). Ang II stimulation induces differentiation of cardiac fibroblasts into myofibroblasts with increased expression of α-SMA (Bai et al., 2013). Further, Ang II induces osteopontin, a prominent cell adhesion molecule, and integrin receptor, which facilitates cardiac fibroblast proliferation and contraction of collagen gels in *in vitro* culture model of cardiac fibroblasts, thereby facilitating wound healing (Burgess et al., 1994; Regitz-Zagrosek et al., 1998).

Although it is not clear as to how Ang II is coupled to cellular growth and proliferation, it is speculated that Ang II could be acting as an amplifier of other cytokines and growth factors like TGF-β by activating signal transduction pathways similar to growth factor and cytokine signalling (Rosenkranz, 2004). Growth-regulating effects of Ang II are also regulated, in part, by the suppression of cyclin-dependent kinase inhibitor, p27, leading to G1-phase arrest and cellular hypertrophy (Wolf and Wenzel, 2004). In view of the high density of the Ang II receptors in the heart, excessive RAS activity can have pathological consequences for myocardial structure and function (Brilla, 2000). Thus, pharmacological interference with RAS activity using either β-blocker therapy, ACE inhibitors or ARBs, which will either prevent or delay Ang II formation or antagonise its binding to cell membrane receptors, is an effective treatment modality in preventing and ameliorating adverse myocardial growth and remodelling (Atlas, 2007; Hale, 2016).

II.4. *Angiotensin signalling pathways*

The pathways activated by Ang II can be grouped under two categories - G-protein and non G-protein-mediated signalling (Dinh et al., 2001). When activated by an agonist, Ang II receptor, mainly AT1, couples to G-protein complex and

activates many downstream effectors including phospholipase C (PLC), PKC, Phospholipase A2 and D. Further, the duration and intensity of G protein-mediated signalling is also dependent on members of the regulators of G protein signalling family (RGS) (Mehta and Griendling, 2007). Apart from activating G-protein dependent pathways, Ang II also cross-talks with several tyrosine kinases including receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases.



(Goette and Lendeckel, 2008)

Figure 4: Ang II signalling pathway

RTKs, with intrinsic tyrosine kinase activity, influence growth-related signalling pathways involving transactivation of PDGF, Epidermal growth factor receptor **(**EGFR) and insulin receptor. They serve as docking sites for Grb2/Shc/Sos complexes and induce two major signal transduction pathways. The first pathway involving PI3K/PDK1/Akt mediates cellular metabolism, growth, survival and

remodelling whereas the second pathway involving Ras/Raf/ERK influences cell growth, hypertrophy and inflammation (Yin et al., 2003).

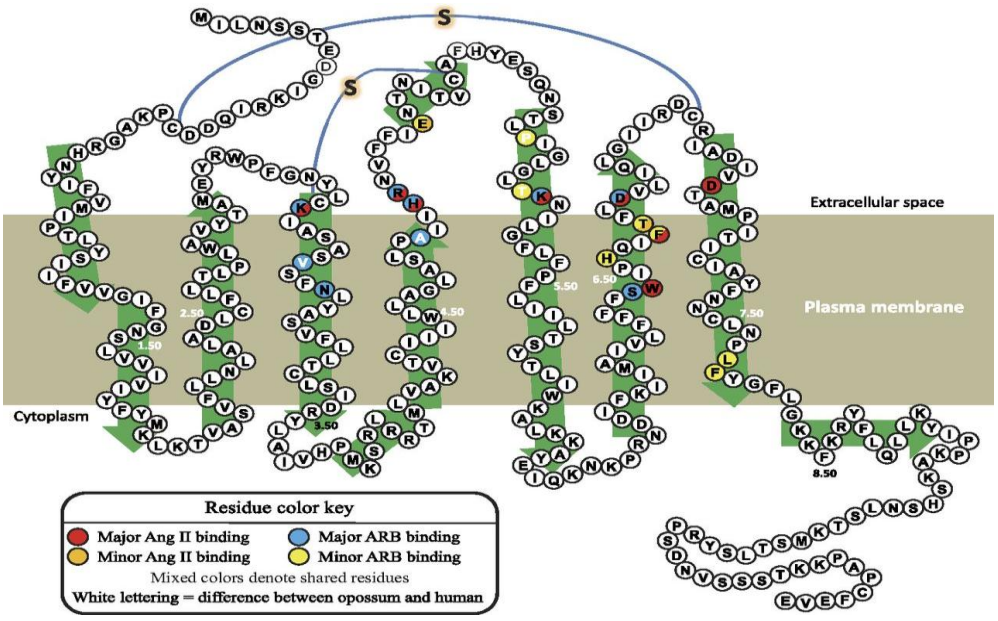
Non-receptor tyrosine kinases like c-src family kinases, Pyk2, FAK and JNK associate with AT1 receptor and initiate signalling cascades that lead to Ang II-mediated cell growth (Maric and Sandberg, 2004). Further, many of the pathological effects of Ang II, including the structural and functional changes in the vasculature, involve Ang II-mediated ROS that in turn can activate redox-sensitive transcription factors like NF-κB and AP-1 (Mehta and Griendling, 2007). Apart from the above mentioned pathways, Ang II has been shown to activate transcription factors like Krüppel-like factor 5 (KLF5) (Shindo et al., 2002) and protein kinases like ERK1/2 MAPK, JNK and p38 MAPK (Taniyama et al., 2004). These signalling cascades are tightly regulated and any event that causes over-stimulation of RAS can lead to initiation and propagation of pathological events.

II.5. *Angiotensin receptors*

Ang II activates a group of cell membrane receptors, which are grouped as AT1, AT2, AT3 and AT4 based on their pharmacological characteristics and binding affinity for non-peptide antagonists (Dostal and Baker, 1999). The two well-characterised Ang II receptor subtypes are AT1 and AT2. AT1 receptor is widely distributed in the heart, kidney, liver, brain and vasculature whereas AT2 receptor is found only in adrenal medulla, uterus, ovary, vascular endothelium and distinct brain areas (Dinh et al., 2001). While humans have only one AT1 subtype, rodents have two functionally and pharmacologically identical isoforms, AT1a and AT1b (Bouby et al., 1997). Both AT1 and AT2 are seven trans-membrane spanning serpentine receptors that elicit intracellular signals via heterodimeric G-proteins (Ichiki, 2013).

Which G-protein is coupled to Ang II receptor depends on the cell type on which the receptor is located. While Gq couples to AT1 in cardiac myocytes and VSMCs (Eguchi et al., 1996; Sadoshima and Izumo, 1996), Gi couples to AT1 in hepatocytes and cardiac fibroblasts (Klett et al., 1993; Zou et al., 1998).

Despite structural similarity, the genes for AT1 and AT2 are located on different chromosomes (AT1 on chromosome 3 and AT2 on X chromosome) and share only 30% sequence homology (Brewster et al., 2003). Importantly, these two receptors are functionally distinct. While AT1 receptor mediates most, if not all, of the physiological effects of Ang II, AT2 receptor is found to counter-regulate the AT1 receptor effects and elicit vasodilatory, antiproliferative and apoptotic effects (Dinh et al., 2001). Thus, the cellular expression and activation of the 40 kDa AT1 receptor is critical in regulating the effects of Ang II on many target tissues (Mehta and Griendling, 2007).



(Nistala et al., 2013)

Figure 5: Model of human AT1 receptor

II.5.1. AT1 receptor in the cardiovascular system

Expression of Ang II receptor sub-types in cardiac cells is determined by developmental stages of the organism. During embryonic stage, AT2 receptor expression is dominant over AT1 receptor expression in the heart, which is reversed during neonatal and adult stages (Regitz-Zagrosek et al., 1998). But, pathological stimuli can cause a shift in AT1 and AT2 receptor density. For instance, end stage failing human heart shows selective down-regulation of the AT1 receptor and unaltered or relative up-regulation of the AT2 receptor (Asano et al., 1999; Kurabayashi and Yazaki, 1997). In the heart, most of the effects of Ang II are reported to be mediated via the activation of AT1 receptor. AT1 receptors are widely distributed in fibroblasts, VSMCs, endothelial cells and myocytes. However, extravascular AT1 receptors in the heart reside predominantly on fibroblasts (Burchfield et al., 2013). Since expression levels of AT1 define the biological efficacy of Ang II (Sungkaworn et al., 2011), increasing AT1 expression is one mechanism by which Ang II can contribute to cardiovascular disorders. Elevated AT1 receptor expression is seen in many disease conditions like hypertension, atherosclerosis, myocardial infarction and heart failure (Billet et al., 2008). The beneficial effects of AT1 receptor antagonists in patients with cardiovascular diseases emphasize its emergence as an important drug target (Atlas, 2007; Ferrario, 2006).

II.5.2. Regulation of AT1 receptor

Since AT1 regulates Ang II effects on target tissues, it is important to understand the regulatory mechanisms that control AT1 receptor density. Four different routes of regulation of AT1 expression are reported: receptor internalization, desensitization, alternate splicing of receptor mRNA and modulation

of its gene expression (Nickenig and Harrison, 2002b). Within 10 minutes of its activation, AT1 receptors are internalised into catherin-coated pits or caveolin by endocytosis and about 25% of these internalised receptors are recycled back to the plasma membrane by β-arrestin-dependent mechanisms. Prolonged Ang II stimulation can desensitise the receptor and reduce Ang II signalling by phosphorylating the cytoplasmic surfaces via PKC (Mehta and Griendling, 2007). Alternate splicing of the AT1 receptor pre-mRNA can affect the translation of AT1 receptor protein.

The most favoured mechanism of regulation of AT1 receptor is the modulation of AT1 gene expression. A potent regulator of AT1 expression is its own agonist, Ang II, which influences AT1 expression based on the duration of treatment and the cell type on which it acts. While exposure to acute levels of Ang II can up-regulate AT1, chronic exposure to Ang II leads to decreased levels of AT1 activation (Lassègue et al., 1995; Wei et al., 2009). In neuronal cell lines, cardiovascular regulatory regions of the brain like rostral ventrolateral medulla (RVLM) and brain areas crucial for controlling stress reaction, Ang II up-regulates AT1 receptor expression via MAPKs, cAMP response element binding protein (CREB), Elk-1, NF-KB and AP-1 (Haack et al., 2013; Liu et al., 2008; Mitra et al., 2010; Wei et al., 2009).

The current concept of heterologous AT1 receptor regulation, wherein multiple agonists apart from Ang II can modulate AT1 receptor expression, provides a mechanistic link between hypertension and various clinical conditions (Nickenig and Harrison, 2002b). Numerous agonists like TNF-α, insulin, estrogen, growth hormone, thyroid hormone (T3), can cause modulation of AT1 receptor expression (Fukuyama et al., 2003; Mok et al., 2003; Nickenig et al., 1998, 2000; Peng et al.,

2002). In vascular cells, while estrogen-induced, nitric oxide-dependent, PI3K-mediated suppression of AT1 expression contributes to atheroprotective effect in pre-menopausal women, progesterone-induced AT1 up-regulation may contribute to the steep increase in cardiovascular events in women after menopause (Nickenig et al., 2000). Insulin-mediated, ERK1/2 MAPK-dependent up-regulation of AT1 in VSMCs may underlie hypertension associated with hyperinsulinemia and non-insulin dependent diabetes mellitus (Nickenig et al., 1998). Further, exposure of VSMCs to LDL augments AT1 expression via PI3K and post-transcriptional mRNA stabilization, providing molecular evidence of an association between hypercholesterolemia and hypertension (Nickenig et al., 1997). Down-regulation of vascular AT1 expression in hyperthyroid state plays an important role in T3-induced vascular relaxation (Fukuyama et al., 2003). In cardiac fibroblasts, TNF-α and IL-β mediate up-regulation of AT1 via NF-κB (Cowling et al., 2002; Gurantz et al., 1999). Apart from these agonists, sporadic reports suggest that oxidative stress modulates AT1 expression in macrophages, renal proximal tubules and human aortic smooth muscle cells (Banday and Lokhandwala, 2008; Bhatt et al., 2014; Keidar et al., 2002).

II.6. *Oxidative stress*

Mammalian heart is an obligate aerobic organ and a constant supply of oxygen is indispensable to sustain cardiac function and viability. However, oxygen is also the substrate for generation of ROS, which is a normal component of oxidative phosphorylation in the heart. ROS can have dual roles; both as mediator of injury and as homeostatic signalling molecules based on the type, concentration and location of ROS generation (Taverne et al., 2013). A pathological imbalance, where an excess of ROS is generated that cannot be adequately countered by the intrinsic

antioxidant system, is termed oxidative stress. Excess ROS generation triggers cell dysfunction, lipid peroxidation and DNA mutagenesis, which can cause irreversible cell damage leading to apoptosis or generate other more reactive radicals (Morrell, 2008). Oxidative stress can also play a central role in the genesis of many components of myocardial abnormalities like contractile dysfunction, cardiomyocyte hypertrophy, cell death, myocardial fibrosis and ischemia-reperfusion injury, predisposing to heart failure (Grieve and Shah, 2003; Tsutsui et al., 2011).

ROS can mediate apoptosis of cardiomyocytes by redox regulation of the extrinsic apoptotic pathway, involving TNF-α, and the intrinsic apoptotic pathway, involving Bcl2, ASK-2 and p38 MAPK, all of which are found to be over-expressed via NOX2-dependent mechanisms in cells destined to undergo apoptosis (Circu and Aw, 2010). ROS is believed to contribute to the development of cardiac hypertrophy by inducing many hypertrophic signalling pathways (Giordano, 2005). ROS generation is triggered in the vessel wall as a result of uptake of oxy-LDL during the development of atherosclerosis causing MMP activation and formation of nitric oxide that prevents vascular relaxation leading to increased risk of thrombosis (Victor, 2014).

Though oxidative stress is generally considered to be deleterious to the heart, tightly regulated transient ROS production plays an important role in modulating several important biological processes in the myocardium and exerts cardio protective effects (Burgoyne et al., 2012; Kimura et al., 2014). Ischaemic preconditioning involving mitochondrial ROS or NOX-ROS prior to reperfusion of the ischaemic heart during coronary occlusion is believed to be cardio protective and prevents further cardiac damage by minimising ischaemia/reperfusion (I/R) injury (Kharbanda et al., 2001; Pasupathy and Homer-Vanniasinkam, 2005). ROS plays a

crucial role in the maturation and differentiation of cardiomyocytes from embryonic stem cells (Buggisch et al., 2007). Redox signalling is important in excitation-contraction coupling via NOX2 activation that facilitates the mechanotransduction of calcium release and contractile force in cardiomyocytes (Santos et al., 2011). Further, ROS helps in the regulation of vascular tone by regulating vasodilation and blood pressure (Burgoyne et al., 2012).

II.6.1. Effects of ROS on cardiac fibroblasts

ROS can influence several aspects of cardiac fibroblast biology, including activation, differentiation, proliferation and collagen metabolism. Cucoranu et al., 2005 observed that activation of NOX4 induces proliferation of cardiac fibroblasts and mediates cardiac fibrosis by stimulating myofibroblast differentiation. Further, as a regulator of the quantity and quality of ECM, ROS is considered to be important in fibrogenesis and matrix remodelling in the heart (Chen and Frangogiannis, 2013). However, there are contrasting reports on the effects of ROS on fibroblast-mediated matrix protein synthesis. While some studies on neonatal and adult rat cardiac fibroblasts suggest that ROS decreases collagen synthesis and increases MMP activity (Siwik et al., 2001), some other reports on adult rat cardiac fibroblasts demonstrate that ROS enhances collagen synthesis and decreases MMP expression by a mechanism mediated by NOX4 (Lijnen et al., 2006). Although ROS can mediate all these effects directly, it can also modulate these functions by triggering matrix-preserving pathways involving TGF-β or Ang II (Chen and Frangogiannis, 2013; Lijnen et al., 2006). It is pertinent to note that cardiac fibroblasts are relatively resistant to oxidative injury and hypoxia in contrast to

myocytes (Li et al., 1999; Philip and Shivakumar, 2013; Sangeetha et al., 2011; Zhang et al., 2001).

II.6.2. Cellular signalling pathways regulated by ROS

While there are several potential sources of ROS in the heart, NOX family of enzymes are considered to be specifically important in redox signalling (Murdoch et al., 2006). NOX2 (known previously as gp91phox) and NOX4 are the main isoforms of NOX expressed in the myocardium (Cave et al., 2006; Griendling et al., 2000). ROS signalling processes involve the activation of a variety of redox-sensitive signalling kinases (Berk, 2000) and transcription factors like Hypoxia-Inducible Factor (HIF)-1, Nuclear factor erythroid 2-related factor 2 (Nrf2), NF-κB, and AP-1 in the heart (Haddad, 2002, 2003; Seddon et al., 2007; Surh et al., 2005). NF-κB and AP-1 are best characterised transcription factors to be influenced by cellular oxidation-reduction (redox) state (Sano et al., 2001). Pro-inflammatory cytokines and oxidative stress cause concomitant activation of NF-κB together with elevated AP-1, suggesting that these transcription factors can function individually or act in tandem to orchestrate the expression of many genes involved in inflammation, cell differentiation, proliferation and apoptosis in response to a plethora of physiological and pathological stimuli (Fujioka et al., 2004). Under unstimulated conditions, these redox-sensitive heterodimeric proteins remain quiescent. AP-1 consists of jun/jun homodimers or heterodimers of jun and fos family and it requires c-Fos synthesis and c-Jun induction for AP-1 activation (Shaulian and Karin, 2001). NF-κB is made as a dimer of two proteins, p50 (which binds the κB motif) and p65 or RelA (which is required for transactivation of gene expression) (Guo et al., 2005; Jefferies and O'Neill, 2000). This heterodimer remains in combination with IκB in the

cytoplasm and it requires phosphorylation and proteolysis of IκB for activation of NF-κB and its subsequent translocation to the nucleus.

The activity of NF-κB and AP-1 are regulated by redox-sensitive protein kinase- driven pathways. The increase in ROS production inside the cell results in the activation of a family of serine-threonine kinases termed MAPKs (Son et al., 2011). In mammals, three major groups of MAPKs are activated by ROS: ERK1/2, p38 and JNK. ERK1/2 MAPK, also known as p44/42 MAPK, is activated in response to mitogens and growth factors by the phosphorylation of threonine and tyrosine residues by MEK 1/2 via Ras/Raf (Ravingerová et al., 2003). p38 MAPK is selectively activated by MKK3 and MKK6 upon exposure to many forms of cellular stress (Zarubin and Han, 2005). Because JNKs are strongly activated by stress stimuli like inflammatory cytokines, toxins and ultraviolet radiation, they are also referred to as stress-activated protein kinases. In the heart tissue, two isoforms of JNKs are present that are activated by MKK4 and MKK7 (Clerk et al., 1998). While ERK pathway is most commonly linked to the regulation of cell growth and proliferation, JNK and p38 MAPK pathways are strongly involved in cellular responses to stress. After activation, MAPK moves to the nucleus through large pores on the nuclear membrane where it promotes selective phosphorylation of different substrates of many effector proteins, most notably transcription factors (Zhang et al., 2003). For induction of AP-1 activity, while ERK1/2 MAPK or p38 MAPK induces c-Fos synthesis by phosphorylating TCF/ELK-1, JNK phosphorylates the stimulatory site of c-Jun (Karin, 1995). Many studies suggest that p38 and ERK1/2 MAPK pathways can regulate NF-κB transactivation by modulating its DNA binding and oligomerization properties (Jefferies and O'Neill, 2000). For instance, ERK1/2 MAPK-dependent NF-κB activation is known to regulate expression of cell

cycle protein, GADD45β (Wang et al., 2005) and anti-apoptotic protein, cIAP-2 (Philip and Shivakumar, 2013). Likewise, lipopolysaccharide-elicited activation of p38 MAPK contributes to NF-κB activation in macrophages (Chen and Wang, 1999).

II.6.3. Oxidative stress and AT1 gene expression

Although oxidative stress is involved in the pathogenesis of several cardiovascular disorders like hypertension and atherosclerosis (Tsutsui et al., 2011), it is unclear as to how oxidative stress can influence the expression of genes like AT1 under such conditions. Sporadic reports suggest that oxidative stress modulates AT1 expression in macrophages, renal proximal tubules and human aortic smooth muscle cells (Banday and Lokhandwala, 2008; Bhatt et al., 2014; Keidar et al., 2002). Further, in Spontaneously Hypertensive Rats (SHR) and human embryonic kidney cells, oxidative stress is reported to cause an increase in cell surface AT1 receptor aggregation state (Sungkaworn et al., 2011). Surprisingly, however, molecular mechanisms involved in the regulation of AT1 expression under conditions of oxidative stress in any cell type remain inadequately addressed.

III. MATERIALS AND METHODS

III.1. *Materials*

III.1.1. Fine chemicals

Medium199 (M199), albumin (from Bovine serum), collagenase type IA (from *Clostridium histolyticum*), trypsin (from porcine pancreas), pancreatin (from porcine pancreas), DNase I (bovine pancreas), HEPES, EDTA, DMSO, amphotericin B solubilised, D-(+)-dextrose, calcium chloride, disodium hydrogen phosphate, magnesium sulphate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, sodium chloride, sodium dihydrogen phosphate, sodium hydroxide, PD98059, propidium iodide, Hoechst 33342, TRI-reagent, DEPC, DNase amplification kit, protease inhibitor cocktail, SDS, trizma base, agarose, glycine, sodium acetate, acrylamide, bis-acrylamide, mercaptoethanol, TEMED, APS, candesartan cilexetil, Bay 11-7085, SP600125, VAS2870, Angiotensin II EIA Kit, Ang II primers for PCR, DPI, phospho-JNK antibody, enalapril maleate salt, Ponceau S, monoclonal anti-vimentin antibody, anti-human von Willebrand antibody, immunostaining kit for desmin, anti-β-actin primary antibody, anti-rabbit and anti-mouse secondary antibodies and Gen Elute Blood genomic DNA kit were purchased from Sigma-Aldrich, USA. COLA1 (C-18) antibody, AT1 (N-10) antibody and total-JNK (C-17) antibody were from Santa Cruz Biotechnology, USA. Taq DNA polymerase, Dual Luciferase assay kit, *Renilla luciferase* control vector and pGL3 Basic vector, SB203580, fine chemicals for cDNA synthesis including RT buffer, RNase inhibitor, random primers, dNTPs and M-MLV Reverse Transcriptase were purchased from Promega Corporation, Madison, WI, USA. NE-PER nuclear and cytoplasmic extraction reagents, Light shift chemiluminescent EMSA kit, Biotin 3' end DNA labelling kit, Chemiluminescent nucleic acid detection module, Super signal west pico chemiluminescent substrate, BCA protein assay kit, DAB substrate,

West femto trial kit were from Thermo Scientific. Cell lysis buffer (10X), siRNA for p42 MAPK and primary antibodies for phospho ERK1/2 MAPK, total ERK1/2 MAPK, phospho p38 MAPK, and total p38 MAPK were obtained from Cell Signaling Technology. siRNA for p38 MAPK and JNK were from Eurogentec. Lipofectamine 2000, Taqman gene expression master mix and BigDye terminator v3.1 cycle sequencing kit were from Applied Biosystems. GFX PCR DNA and gel band purification kit was from GE health care. SR11302 was from Tocris Bioscience. Taqman gene expression assays like Agtr1A (Assay Id: Rn 02758772_s1), Agt (Assay Id: Rn00593114_m1), Colα1 (Assay Id: Rn01463848_m1), Act b VIC (Assay Id: Rn00667869_m1), 18S rRNA VIC-MGB (Cat#4319413E) and TA Cloning Kit Dual Promoter (pCR II) were from Invitrogen. Nitrocellulose membrane was from Millipore, USA. Opti-Mem and Fetal Bovine Serum (FBS) (US origin) were from Gibco. Cell culture ware was from Nunc or BD Falcon. Low cell number ChIP Kit (kch-maglow-A48) was obtained from Diagenode. Pure link RNA mini kit was from Ambion.

III.1.2. Routine Chemicals

Hydrogen peroxide was from Merck. Methanol, isopropanol and chloroform were from Merck, India. Ethanol absolute was from Commercial Alcohols, Canada. Glycerol was purchased from Sisco Research Laboratories, India. Skim milk was obtained from Himedia, Mumbai, India. Gentamicin and Benzyl Penicillin were from Cadila pharmaceuticals, India and Alembic Limited, India respectively.

III.1.3. Cell culture ware

35 mm and 100 mm cell culture-treated dishes, 12 well plates and 24 well plates were purchased from Becton Dickinson, USA. 15 ml and 50 ml polypropylene

centrifuge tubes were from Tarson. Membrane filter was procured from Millipore, USA. Cell scrapers & T-Flask were from Nunc, USA. 0.6 ml, 1.5 ml/2.0 ml tubes, tips for measuring fine volumes were from Axygen.

III.2. *Equipments used*

Epoch ELISA reader (Bio-Tek instruments, USA), UV-visible spectrophotometer (Shimadzu, Japan), high speed refrigerated centrifuge (Thermo Electron corporation, USA), weighing balance (Sartorius, Germany), water bath (Julabo, India), ice-flaker (Blue star), pH meter (Cyberscan, India), CO_2 incubator (Sanyo, Japan), phase-contrast microscope (Nikon, Japan), fluorescence microscope (Zeiss Axioskop 2 Plus), laminar air flow chamber (Thoshiba, India), magnetic stirrer (Schott, Germany), Gel Doc XR+ System (Bio-Rad, USA), 7500 Real-Time PCR System and PRISM 377 DNA Sequencer (Applied Biosystems, USA), UV-Transilluminator (Bangalore Genei, India), Bioruptor (Diagenode), Mini-PROTEAN Tetra Cell and Powerpac universal (Biorad laboratories, USA), Direct-Q 3 water purification system (EMD Millipore, USA), UP50H ultrasonic processor (Hielscher, Germany), mix mate (Eppendorf, USA), thermo aluminum bath (FINEPCR, Korea), tube rocker (Benchmark scientific, India)

III.3. *Composition of media, reagents and buffers*

III.3.1. Acrylamide 30%

29.2 g of acrylamide and 0.8 g of N, N'-Methylene bisacrylamide were dissolved in 100 ml of deionized water.

III.3.2. Agarose gel (1%) for electrophoresis of DNA or RNA samples

For DNA and RNA – 200 mg agarose was dissolved in 20 ml of 0.5X TBE.

III.3.3. Alkaline lysis solution

6 µl of 10 M NaOH and 15 µl of 20% SDS were mixed and made up to 300 µl with sterile water.

III.3.4. Blocking solution

5% (w/v) skim milk or BSA was dissolved in 1X TBST containing 0.1% Tween-20.

III.3.5. Cardiac fibroblast growth medium (pH 7.4)

M199 with Earle's salts containing FBS (10%), benzyl penicillin (50U/ml) and gentamycin (0.04 mg/ml) was used for maintaining cardiac fibroblast cultures.

III.3.6. Cell Lysis buffer for protein isolation for western blotting (1X)

Cell lysis buffer (10X) was diluted with deionized water to get a 1X solution.

III.3.7. DAB substrate solution

900 µl of A and 100 µl of B were mixed well and added to the membrane.

III.3.8. DEPC-treated deionized water

1 ml of DEPC was added to one litre of deionized water, stirred until the globules were dissolved, kept at 37°C overnight and autoclaved twice.

III.3.9. Dissociation medium for fibroblast isolation

Each 100 ml of dissociation medium was made up of sodium chloride (0.68 g), HEPES (0.555 g), sodium dihydrogen phosphate (0.140 g), glucose (0.100 g), potassium chloride (0.04 g), magnesium sulfate (0.02 g), BSA (100 mg) and 10 µl of 1 mM $CaCl_2$. pH was adjusted to 7.4. Deoxyribonuclease I (100 µl) and amphotericin B (200 µl) were added to 100 ml of the medium under sterile conditions at the time

of isolation. The dissociation medium had collagenase type IA (40 mg), trypsin (70 mg) and pancreatin (2 mg) per 100 ml.

III.3.10. Electrode buffer/ running buffer (pH 8.3) for SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

Tris base (1.5135 g), glycine (7.2 g) and SDS (0.5 g) were dissolved in 500 ml of deionized water.

III.3.11. Ethidium bromide (EtBr) (Stock solution)

1 mg of EtBr was dissolved in 1 ml of de-ionized water. 5 µl of this stock solution was added to 20 ml of 1% agarose for DNA/RNA electrophoresis.

III.3.12. EDTA (0.5 M, pH 8.0)

930 mg EDTA was dissolved in 5 ml DEPC-treated deionized water.

III.3.13. Bis-benzimide H 33324 (Hoechst 33342)

10 mg of Hoechst 33324 powder was dissolved in 5 ml of sterile distilled water to get a final concentration of 10 mM. A working concentration of 10 µM was used for the experiments.

III.3.14. Phosphate-buffered saline (PBS) (pH 7.4)

Sodium chloride (0.8 g), potassium chloride (0.02 g), disodium hydrogen phosphate (0.186 g) and potassium dihydrogen phosphate (0.02 g) were dissolved in 100 ml of deionized water.

III.3.15. Propidium iodide (PI) staining solution

PI (25 µg/ml), RNase (40 µg/ml) and nonidet P-40 (0.3%) were dissolved in PBS.

III.3.16. LB medium

10 g tryptone, 5 g yeast extract and 10 g NaCl were dissolved in 800 ml distilled water. The pH was adjusted to 7.0 with 1 N NaOH. The volume was made up to 1 litre with distilled water and sterilized by autoclaving.

III.3.17. Non-denaturing gel (6%)

5.93 ml water, 2 ml of 30% acrylamide, 2 ml of 5X TBE, 35 µl of 20% APS and 10 µl of TEMED were mixed.

III.3.18. Paraformaldehyde (4%)

4 g paraformaldehyde was dissolved in 100 ml PBS by keeping at 60ºC.

III.3.19. Potassium acetate

To 29.4 g of potassium acetate in 40 ml of water, 50 ml of glacial acetic acid was added, pH was adjusted to 5.2 using glacial acetic acid and made up to 100 ml.

III.3.20. Resolving Gel for SDS – PAGE (10%)

3.3 ml of 30% acrylamide, 2.5 ml of 1.5 M Tris (pH 8.8), 0.1 ml of 10% SDS, 0.1 ml of 10% ammonium persulfate and 4 µl TEMED were added to 4.0 ml of deionized water.

III.3.21. SDS gel-loading buffer (6X)

SDS (9% w/v), bromophenol blue (0.03%), β-mercaptoethanol (9%) and glycerol (50% v/v) were dissolved in 18.75 ml of 1 M Tris HCl- pH 6.8 (375 mM).

III.3.22. Serum-free medium

M199 containing antibiotics (50 U/ml penicillin and 0.04 mg/ml gentamycin).

III.3.23. Stacking gel buffer (pH 6.8)

30 ml from resolving buffer was measured, adjusted the pH to 6.8 using HCl and was made up to 45 ml and stored at room temperature.

III.3.24. Stacking gel for SDS – PAGE (5%)

0.670 ml of 30% acrylamide, 0.500 ml of 1 M Tris (pH 6.8), 0.04 ml of 10% SDS, 0.04 ml of 10% ammonium persulfate and 4 µl TEMED were added to 2.7 ml of deionized water.

III.3.25. TEG buffer

1.25 ml of 1 M Tris (pH 8.0), 1 ml of 0.5 M EDTA and 2.5 ml of 1 M glucose were mixed and made up to 50 ml with water.

III.3.26. Towbin's buffer (Transfer buffer)

1.5135 g of Tris base, 7.2 g of glycine and 100 ml of methanol were mixed and made up to 500 ml with deionized water.

III.3.27. Tris borate EDTA buffer (TBE) (5X, pH 8.3)

27.315 g of Tris base, 13.91 g of boric acid and 1.861 g of disodium EDTA (0.5 M, pH 8.3) were mixed and made up to 1 L with deionized water.

III.3.28. Tris-buffered saline (10X, pH 7.6)

24.2 g of Tris base and 80 g of sodium chloride were dissolved in water. After adjusting the pH to 7.4 using conc.HCl, the solution was made up to 1 L with deionized water.

III.3.29. Tris-buffered saline with Tween-20 (TBST) [1X]

1X TBS containing 0.1% Tween-20.

III.3.30. Trypsin-EDTA solution

25 mg of trypsin and 3.8 mg of EDTA were dissolved in 10 ml of PBS (pH 7.4).

Methods

III.4. *Isolation, culture and characterization of cardiac fibroblasts*

III.4.1. Isolation of cardiac fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague-Dawley rats following the method of Kumaran and Shivakumar, 2002 with some minor modifications. Handling of animals and experimental procedures were in accordance with Institutional Animal Ethics Committee approval (SCT/IAEC-023/June/2012/77). Animals were euthanized (Thiopentone sodium, 5-10 mg/Kg, i.p), the heart excised, atria removed and the ventricles were used for isolation of fibroblasts. The ventricular tissue was washed in PBS, minced into bits of approximately 1-3 mm size and subjected to a series of digestions in dissociation medium (Ref. III.3.9) containing Collagenase type IA (0.4 mg/ml), Trypsin (0.6 mg/ml) and Pancreatin (0.020 mg/ml). Digestion was aided by gentle shaking of the flask containing tissue bits in an orbital shaker maintained at 37°C. The supernatants were centrifuged at 1500 rpm for 5 minutes. The cell pellets were pooled, re-suspended in M199 supplemented with 10% FBS, seeded on two 35 mm cell culture dishes and incubated at 37^{o}C for 2.5 hours in a humidified CO_2 incubator with 95% air-5% CO_2. At the end of this period, the supernatant containing unattached cells and debris was discarded, the dishes with the adherent fibroblasts were rinsed 3-4 times and incubated with M199 containing 10% FBS. The pre-plating step ensured preferential

attachment of cardiac fibroblasts. At 24 hours after isolation, the dishes were washed and incubated with M199 containing 10% FBS (Ref. III.3.5) and maintained in a CO_2 incubator at 37^{o}C.

III.4.2. Sub-culture of cardiac fibroblasts

At confluence, the culture supernatant was removed, cells were washed with PBS (Ref. III.3.14) and trypsinised at 37^{0}C with trypsin-EDTA solution (Ref. III.3.30). Trypsinisation was stopped by addition of M199 containing 10% FBS and the detached cells were collected by centrifugation at 1500 rpm for 5 minutes. The cell pellet was suspended in M199 containing 10% FBS and seeded on fresh culture dishes at a split ratio of 1:3.

III.4.3. Characterization of cardiac fibroblasts in culture

Fibroblastic nature of the cells in culture was ascertained by morphology and immunocytochemistry.

- Analysis of morphology

Sub-confluent and confluent cultures were examined under an inverted phase contrast microscope for morphologic characteristics.

- Immunocytochemical analysis of vimentin, desmin and von Willebrand factor

Cells from passage 2 or 3, grown to about 50% confluence, were washed with PBS and fixed in 4% ice-cold paraformaldehyde (Ref.III.3.18) for 20 minutes. The fixed cells were blocked with PBS containing 5% FBS and 0.2% Triton for 30 minutes. The cells were then incubated overnight in primary antibody diluted in blocking solution. Following PBS wash, cells were incubated in FITC-labelled secondary antibody at room temperature for 90 minutes. After PBS wash to remove unbound stain, nuclei were counterstained with Hoechst 33342 and observed under a

fluorescence microscope. Anti-desmin and anti-von Willebrand factor antibodies were used at 1:500 dilution and immunostaining for anti-vimentin was done using a commercially available kit.

III.5. *Experimental model*

Sub-confluent cultures of cardiac fibroblasts at passage 2 or 3 were used for the experiments. Primary cultures of adult rat cardiac fibroblasts treated with 25 µM H_2O_2 in M199 with or without inhibitors were used as the experimental model.

III.6. *Determination of cell number*

Sub-confluent cardiac fibroblast cultures at passage 2 were synchronized by serum-deprivation for 24 hours. Cell counts were determined before and after 24 hours of treatment using a Neubauer counting chamber.

III.7. *Assessment of cell viability by Hoechst 33342-PI staining*

A stock solution of 10 mM Hoechst 33342 was prepared in deionized distilled water and a working concentration of 10 µM was used for staining. Sub-confluent cardiac fibroblast cultures at passage 2 were synchronized by serum-deprivation for 24 hours. The synchronized cells were treated with or without 0, 10, 25, 50 or 100 µM H_2O_2 in M199 without FBS. After 12 hours of treatment, the cultures were incubated with Hoechst 33342 for 10 minutes at 37^0C in the dark. Immediately after the incubation, PI, 0.25 ug/ml was added and fluorescence was visualized under a fluorescence microscope (Zeiss Axioskop 2 Plus) at excitation wavelengths of 352 nm (UV filter) and 538 nm (Green filter) for Hoechst 3334 and PI, respectively.

III.8. *Intracellular ROS measurement (H_2DCFDA assay)*

Intracellular ROS levels were measured by loading cardiac fibroblasts with 10 µM H_2DCFDA for 10 minutes and incubating the cells with H_2O_2 over a range of concentrations from 10 µM to 100 µM for 15 minutes. Fluorescence was read at excitation and emission wavelengths of 485 nm and 530 nm respectively. Relative fluorescence was calculated as percentage of H_2DCFDA fluorescence.

III.9. *RNA interference*

Cells were seeded on 12 well plates at 80 X 10^3 cells per well. 24 hours after sub-culture, cells were incubated in Opti-MEM containing pre-designed siRNA for p42 MAPK (ERK 2), p38 MAPK or JNK (5 pmol each) and Lipofectamine (2 µl) for 19 hours. The Control and H_2O_2 groups were transfected with scrambled siRNA. Following an additional incubation in M199 with 10% FBS for 12 hours, the cells were serum-deprived for 24 hours. Post serum-deprivation, cells were incubated with or without 25 µM H_2O_2 in M199 for 12 hours. Cell lysate was prepared in 2X SDS lysis buffer and denatured in boiling water bath for 5 minutes and stored at -20ºC until use.

III.10. *Electrophoretic Mobility Shift Assay (EMSA)*

DNA-binding activity of NF-κB or AP-1 in cardiac fibroblasts was assessed by EMSA as described below.

III.10.1. Preparation of nuclear extract

Nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction kit following manufacturer's instructions. Sub-confluent cardiac fibroblast

cultures in M199 were treated with H_2O_2 with and without inhibitors for 30 minutes or 3 hours, harvested by trypsinization, washed in PBS and pelleted. The cell pellet was re-suspended in 75–150 µl (depending on packed cell volume) cytoplasmic extraction reagent I provided with the kit. The mixture was vortexed and incubated on ice for 30 minutes. After addition of cytoplasmic extraction reagent II, the mixture was vortexed, incubated on ice for 5 minutes and centrifuged at 16,000 x g for 5 minutes at 4^0C. The supernatant (the cytoplasmic fraction) was removed and stored at $–80^0$C until use. The pellet was re-suspended in nuclear extraction reagent, incubated on ice for 2 hours with intermittent vortexing and centrifuged at 16,000 x g for 10 minutes at 4^0C. The supernatant (nuclear fraction) was aliquoted and stored at $–80^0$C until use. Protein concentration of the nuclear extracts was determined by Bicinchoninic acid (BCA) assay.

III.10.2. Primers

Single-stranded oligos carrying the consensus sequence for the NF-κB binding site 5'-GGGGACTTTCC-3' and the AP-1 binding site 5'-CGCTTGATGACTCAGCCGGAA-3' were used.

III.10.3. Primer labelling

Single-stranded oligos for NF-κB or AP-1 (forward) were biotin-labelled using Biotin 3' end DNA labelling kit. The reaction components were added in the following order:

Component	Volume in µl	Final concentration
Ultrapure water	25	
5X TdT reaction buffer	10	1X
5 pmol 3'-OH ends of unlabelled DNA	5	1.0

Biotin-11-UTP (5 µM)	5	0.5 µM
Diluted TdT(2U/µl	5	0.2 U/µl

Reaction components were incubated at 37ºC for 30 minutes. Following addition of 2.5 µl of 0.2 M EDTA to stop the reaction, 50 µl of chloroform:isoamyl alcohol (24:1) was added and briefly centrifuged at high speed to separate the phases. The top aqueous phase containing the biotin-labelled oligo was collected.

III.10.4. Annealing of NF-κB or AP-1 oligos

Equal volumes of both complementary oligos (biotinylated forward primer and non-biotinylated reverse primer) were mixed at equimolar concentrations, incubated on thermal block at 90ºC for 2 minutes, allowed to cool and stored at -20ºC until use.

III.10.5. DNA binding

15 µg nuclear protein was incubated with 200 femtomoles of double-stranded biotinylated probes for NF-κB or AP-1 and other components of the Light Shift Chemiluminescent kit at 37ºC for 60 minutes. 2 µl loading dye was added and mixed gently. Samples were loaded on 6% non-denaturing acrylamide gel and electrophoresed in TBE buffer at 100 V until the bromophenol blue moved more than 3/4th the gel. Electrophoretic gel transfer onto a nylon membrane was carried out at 380 mA for 80 minutes, followed by UV cross-linking at 254 nm for 10 minutes.

III.10.6. Detection of biotin-labeled oligos

After blocking for 15 minutes, the membrane was incubated in streptavidin-conjugated-HRP in blocking buffer (1:300) for 1 hour with gentle shaking. The bands were visualized by enhanced chemiluminescence by adding Luminol/Enhancer solution in equal ratio.

III.10.7. Competition assay

Specificity of binding was ascertained by competition with 200-fold excess of unlabelled NF-κB or AP-1 oligonucleotide.

III.11. *Western blot analysis*

Western blot analysis was carried out as described by Wood, 1983 with minor modifications. Cardiac fibroblasts were subjected to the indicated treatments and lysed in cell lysis buffer (Ref.III.3.6). The lysates were sonicated, vortexed vigorously, kept on ice for 30 minutes and centrifuged at 16,000 x g for 20 minutes at 4°C to remove cell debris and the supernatant was stored at –80^{0}C until use. The lysates were denatured by incubation with 6X SDS-gel loading buffer (Ref.III.3.21) at 100ºC for 5 minutes in a water bath. 25 µg of protein was electrophoretically fractionated at 200 V on 10% SDS-PAGE minigels and electroblotted onto nitrocellulose or PVDF membrane for 60 minutes at 100 V. The membrane was blocked for 1 hour with 5% skim milk or BSA (in case of phosphoproteins) (Ref. III.3.4) and incubated overnight with mild shaking at 4^{0}C with the primary antibody prepared in 5% BSA in 1X TBST (Ref. III.3.29) at 1:1000 dilution (or the AT1 antibody at 1:500). Unbound primary antibody was removed by washing (10 minutes x 4 times) with 1X TBST. Immunoblots were exposed for 1 hour to HRP-conjugated anti-mouse/anti-rabbit secondary antibody at 1:10,000 dilution in 5% skim milk

containing TBST, and unbound secondary antibody was removed by washing (10 minutes x 3 times) with TBST. ECL or DAB substrate was used to detect the antigen-antibody complex. The membrane was then stripped overnight by washing with TBST on a rocking platform, re-probed with anti-β actin antibody (1:1000) and was developed as described above. Protein expression was quantified using Bio-Rad Gel Doc XR+ System and normalized to β- actin.

III.12. *Real-time PCR analysis*

III.12.1. Isolation of total RNA

Sub-confluent cultures of cardiac fibroblasts were subjected to the indicated treatments for 3 hours and total RNA was isolated using the Purelink RNA mini kit, according to the manufacturer's instructions. The yield and purity of the isolated RNA were determined spectrophotometrically at A260 and A260/280, respectively. Intactness of RNA was ascertained by 1% agarose gel electrophoresis.

III.12.2. cDNA synthesis

4 µg of isolated RNA was subjected to DNase I (amplification grade) treatment, as per manufacturer's instructions, to remove any genomic DNA contamination. Following DNase treatment, 2 µg of total RNA was reverse transcribed to cDNA. The cDNA synthesizing mixture consisted of:

5X RT buffer	6.0 µl
dNTPs	2.5 µl
Random primers	2.0 µl
RNase inhibitor	0.5 µl
M-MLV RT	1.0 µl
Total RNA	12.0 µl
DEPC-treated water	6.0 µl

2 µg total RNA made up to 12 µl with DEPC-treated water (Ref. III.3.8) and 2.0 µl random primer was initially mixed and heated at 70^0C for 5 minutes. The heated mixture was then snap-cooled on ice and mixed with the remaining constituents listed above. The reaction mixture was incubated at 37^0C for 60 minutes, heated at 90^0C for 5 minutes and then cooled on ice to inactivate M-MLV reverse transcriptase. The cDNA preparations were stored at -20^0C until use.

III.12.3. Real-time PCR analysis

Taqman quantitative Real-time PCR analysis was carried out using 100 ng of cDNA, Taqman master mix, Taqman primers and specific FAM- or VIC-labelled probes. PCR reactions were performed under the following thermal cycling conditions: 95°C for 10 minutes followed by denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute for each of 40 cycles. Gene expression was quantified using $\Delta\Delta C_t$ method with 18S rRNA as reference gene (Philip and Shivakumar, 2013).

$\Delta\Delta C_t$ method to calculate relative fold change in target gene expression with reference to 18S rRNA

- Average C_t of each experimental group is recorded.
- ΔC_t was calculated for each sample using the following formula

  $\Delta C_t = Ct_{target} - Ct_{reference\ gene}$

- $\Delta\Delta C_t$ was calculated as $\Delta\Delta C_t = (Ct_{target} - Ct_{reference})_{sample} - (Ct_{target} - Ct_{reference})_{control}$
- Relative fold of target mRNA levels of treated with respect to control = $2^{-\Delta\Delta Ct}$

III.13. *Promoter binding assay*

III.13.1. Isolation of genomic DNA

DNA was isolated from the blood of young adult male Sprague-Dawley rats using GenElute blood genomic DNA kit according to the manufacturer's protocol. Briefly, to 200 µL of whole blood collected in a heparin-containing tube, 20 µL of the Proteinase K solution and 200 µL of Lysis Solution C were added, vortexed thoroughly and incubated at 55°C for 10 minutes to lyse the cells. After adding 200 µL of 100% ethanol, the lysate was transferred to the prepared column and centrifuged at 6500 x g for 1 minute. After a series of washes, the column was dried by centrifuging at maximum speed and 200 µL of the elution solution was added directly to the center of the column, incubated for 5 minutes and centrifuged for 1 minute at 6500 x g to elute the DNA. The yield and purity of the isolated DNA were determined spectrophotometrically and A260–A320/A280–A320 ratio was determined.

III.13.2. PCR Amplification of AT1 promoter and gel extraction of PCR products

Rat genomic DNA was subjected to PCR using the forward primer, 5'GGTGGTGCTAGCCCTTCCTTCCATCTTTCCTTTCC-3' and reverse primer, 5'GGTGGTCCCGGGGTCCAACCCGCTCCCTCTC-3' to amplify the 500 bp AT1 gene promoter regions carrying the predicted NF-κB and AP-1 binding site. Reaction was set up using the following components in Bio-Rad I cycler under the following conditions: 94°C for 3 minutes followed by 94°C for 30 seconds, 64.5°C for 45 seconds and 72°C for 1 minute repeated over 39 cycles and final extension at 72°C for 7 minutes.

Water	12.5 µl
10X PCR buffer	5.0 µl
25 mM $MgCl_2$	1.5 µl
Forward primer (5 pmol)	1.0 µl
Reverse primer (5 pmol)	1.0 µl
10 mM dNTP	1.5 µl
DNA template	2.0 µl
Taq polymerase	0.5 µl
	25.0 µl

The amplification product was electrophoresed on 1.2% agarose gel with 1 kb DNA ladder.

The single intact band at 500 bp position was excised and DNA was isolated and purified using Illustra GFX PCR DNA and gel band purification kit as per manufacturer's protocol. Briefly, the band of interest was excised from the agarose gel and dissolved in 500 µl of capture buffer at 60°C. The buffer-sample mix was transferred to an assembled GFX MicroSpin™ column and centrifuged at 13,000 x g for 1 minute until all sample was loaded. After washing the column with 500 µl Wash buffer type I, 25 µl of sterile water was added, incubated at room temperature for 5 minutes and centrifuged for 2 minutes at 13,000 x g. The purified sample DNA was collected as flow through and stored at -20°C.

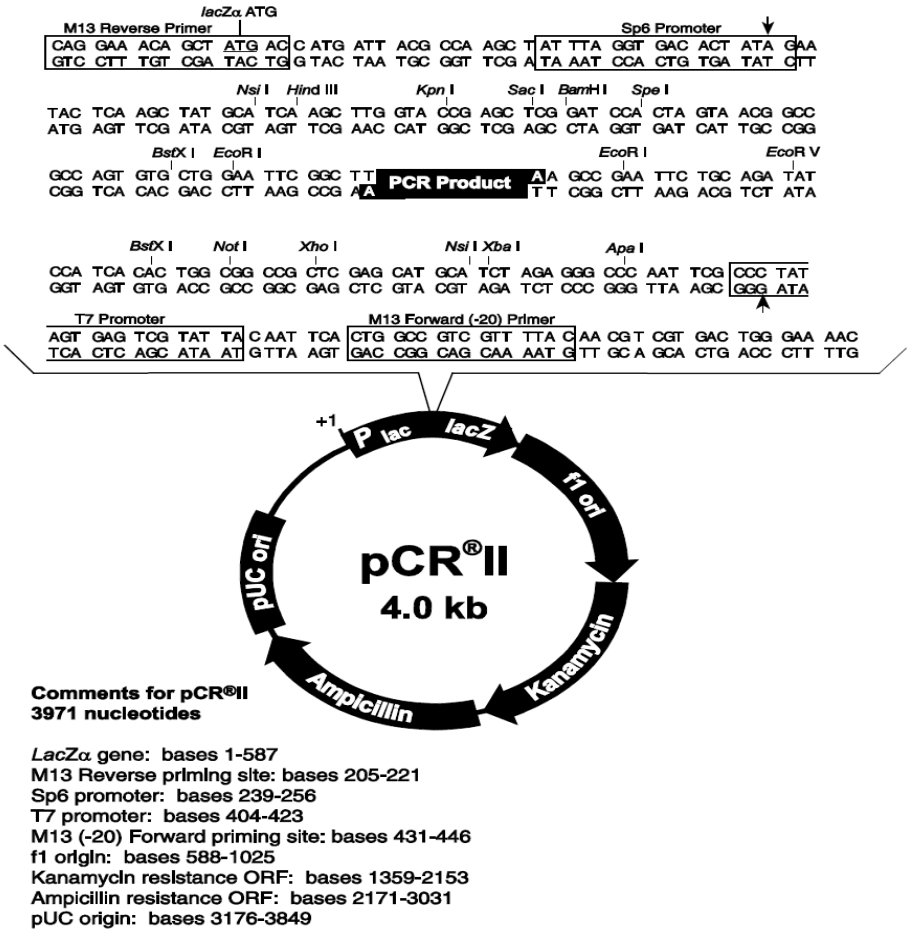
III.13.3. Ligation of the PCR product into pCRII vector

pCRII vector allows a PCR product to ligate efficiently with the plasmid vector. The lac Z promoter allows bacterial expression of the *lac*Zα fragment for α-complementation (blue-white screening). T4 DNA ligase was used to ligate the PCR

product to the pCR II vector in order to produce recombinant DNA constructs. The ligations were carried out with an excess of insert DNA compared to vector DNA (molar ratio of 3:1). The ligation reaction was performed in a total volume of 10 µl as mentioned below:

Reagents used in ligation	Volume in µl
PCR product	7.0
pCR II vector	1.0
10X T4 Ligase buffer	1.0
T4 DNA ligase	1.0
Total volume	10.0

The above mixture was incubated at 4^{o}C for blunt end ligation or at 16^{o}C for cohesive ligation. After incubation, 2 µl of the ligation mix was used for transformation in DH5α competent cells.



(Source: Invitrogen from Life technologies)

Figure 6: Map and features of pCRII

III.13.4. Competent cell preparation

DH5α strain of *E. coli* from glycerol stock were inoculated into 5 ml of LB broth without any antibiotics and incubated overnight in an orbital shaker at 37°C. 1 ml of the overnight culture was transferred to a fresh 100 ml LB broth and incubated for 2 hours with shaking to collect the cells in their early log phase. The two-hour culture was pelleted and re-suspended in 25 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 30-45 minutes. Following incubation, the cells were

centrifuged at 4°C, re-suspended in 2.5 ml 0.1 M $CaCl_2$ containing 15% glycerol (v/v), aliquoted and transferred to -80°C freezer immediately.

III.13.5. Bacterial transformation

To 100 µl of competent cells thawed on ice, 10 µl of ligated pCR II-Agtr1A vector product was added and incubated on ice for 30 minutes. The vials were placed in 42°C water bath for 45 seconds to provide a heat shock and snap cooled. The cells were then incubated in 500 µl of agar broth for 1 hour at 37°C with shaking, centrifuged at 4000 rpm for 4 minutes and the pellet was dissolved in 20 µl of supernatant and plated onto antibiotic-containing agar plates with X-gal (20 µg/ml) and IPTG (1 M). Antibiotic-resistant transformed cells appeared as white colonies within 12 to 16 hours. Blue colonies contained self-ligated pCR II vectors.

III.13.6. Screening of transformed colonies

III.13.6.1. Plasmid DNA isolation and quantification

Plasmid DNA isolation was carried out using a modified protocol of Birnboim and Doly, 1979. The white colony containing the pCR II-Agtr1A plasmid was inoculated in 3-5 ml of LB medium and incubated overnight at 37°C with vigorous shaking. 1.5 ml of culture was centrifuged and the pellet was re-suspended in 200 µl of ice-cold TEG buffer (Ref III.3.25) by vigorous vortexing. After lysing the cells with freshly prepared 300 µl of alkaline lysis solution (Ref III.3.3), 300 µl of ice-cold 3 M potassium acetate (Ref III.3.19) was added, incubated in ice for 3-5 minutes and centrifuged at 13,000 rpm at 4°C for 10 minutes to remove the denatured protein-genomic DNA complex. The supernatant containing plasmid DNA was transferred to a fresh tube and treated with RNase (10 µg/ml) at 45°C for 30 minutes to avoid RNA

contamination. Chloroform extraction was performed twice to remove any remaining traces of proteins and the upper aqueous phase was transferred to a fresh centrifuge tube. The plasmid DNA was further precipitated out with an equal volume of 100% isopropanol followed by 30 minutes incubation at -20°C. Finally, the plasmid DNA was pelleted out by centrifugation at 12,000 rpm for 15 minutes at room temperature and the pellet was washed with 250 µl of 70% ethanol, air-dried, dissolved in 20 µl of 10 mM Tris EDTA (pH 8) and stored at -20°C.

III.13.6.2. Clone confirmation through restriction digestion

The presence of the desired insert in the isolated plasmids was confirmed by restriction digestion. Plasmid was cleaved by EcoRI. Digested products were analysed on 1% agarose gel.

III.13.6.3. DNA Sequencing

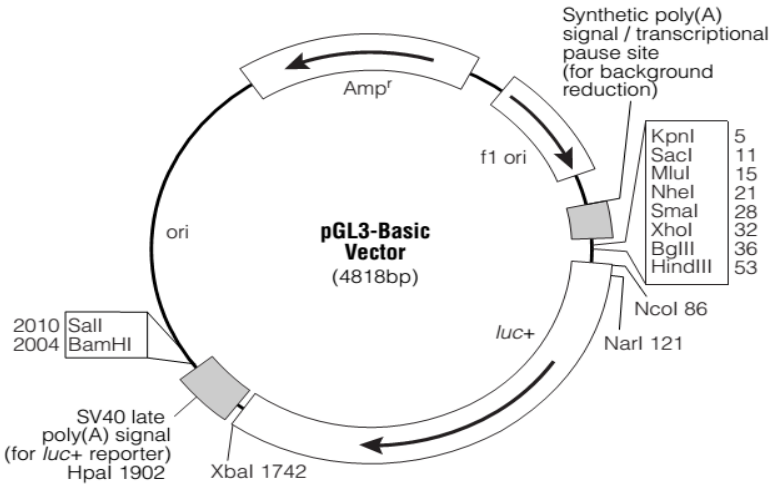
Sequencing PCR: Sequencing PCR was performed using Big Dye Terminator v3.1 cycle sequencing kit and specific primers in ABI 3010 automated sequencer. The reaction mix consisted of 400 ng DNA template, 2 µl of 5X Buffer, 10 picomoles of specific primer and 1 µl of ABI 'ready reaction pre-mix' made up to 10 µl with sterile Milli-Q water. The reaction mix was cycled at 96^0C for 4 minutes (denaturation), 50^0C for 5 seconds (annealing) and 60^0C for 4 minutes (extension). The ramp time of the thermal cycler was set at 1^0C/second.

Post Reaction Clean Up: After sequencing PCR, the products were purified and precipitated by a post reaction clean up protocol. After making up the volume of the PCR product to 100 µl with sterile Milli-Q water, 10 µl of 3 M sodium acetate (pH 4.8) and 250 µl of chilled absolute alcohol were added and centrifuged at 13,000

rpm for 20 minutes at room temperature. The pellet was washed twice with 250 µl of chilled 70% ethanol and the air-dried pellet was subjected to automated DNA sequencing in ABI PRISM 377 DNA Sequencer.

III.13.7. Restriction digestion and ligation of Agtr1A promoter from pCRII into pGL3 basic vector

The backbone of the pGL3-Basic Vector carries a modified coding region for firefly (*Photinus pyralis*) luciferase that was optimized for monitoring transcriptional activity in transfected eukaryotic cells. Expression of luciferase activity in cells transfected with this plasmid depends on the insertion and proper orientation of a functional promoter upstream of *luc*+.



(Source: Promega)

Figure 7: pGL3-Basic Vector circle map

pGL3-Agtr1A plasmid was constructed from pCRII- Agtr1A plasmid and pGL3 basic vector. The pGL3-Agtr1A plasmid was obtained by double digesting pCRII- Agtr1A plasmid and pGL3 basic vector with restriction enzymes in compatible buffer at 37°C.

Reagents used in restriction digestion	Volume in µl
pGL3 basic vector	0.5
Tango	2.0
Kpn I	1.0
Sma I	1.0
Water	15.5
Total volume	10.0

Reagents used in restriction digestion	Volume in µl
pCRII- Agtr1A	0.5
Red buffer	2.0
Kpn I	1.0
EcoR V	1.0
Water	15.5
Total volume	10.0

The digested fragments were visualized by ethidium bromide (EtBr) staining after separating them on 1% agarose gels. The bands corresponding to the Agtr1A promoter and the vector, pGL3 basic vector backbone, were eluted and ligated using T4 DNA ligase to generate pGL3-Agtr1A. The ligations were carried out with an excess of insert DNA compared to vector DNA (molar ratio of 3:1). The ligation mix consisting of vector (0.5 µg), Agtr1A promoter (1.5 µg), T4 DNA ligase buffer (1X) and T4 DNA ligase (30 Weiss Units) was incubated overnight at 4^{o}C for blunt end ligation or for 14 hours at 16^{o}C for cohesive ligation. 2 µl of the ligation mix was used for transformation in DH5α competent cells.

III.13.8. pGL3-Agtr1A plasmid amplification by bacterial transformation

2 µl from ligated plasmid DNA was added to 100 µl of DH5α strain of *E. coli* and incubated on ice for 30 minutes. After incubation at 42ºC for 40 seconds, it was snap cooled on ice and then mixed with 250 µl LB broth. Following incubation at 37ºC for 1 hour with shaking, the broth was plated on petri plates with ampicillin (50 µg/ml)-containing medium and incubated at 37ºC overnight. Antibiotic resistant transformed cells appeared as single colonies after overnight incubation.

III.13.9. Plasmid isolation by gravity column method

Single colonies of DH5α strain of *E. coli* with pGL3-Agtr1A were cultured overnight at 37°C with shaking. The broth was centrifuged, the pellet suspended in 0.4 ml of re-suspension buffer (R3) with RNase, mixed with 0.4 ml of lysis buffer (L7) and incubated at room temperature for 5 minutes. Following incubation, 0.4 ml of precipitation buffer (N3) was added and centrifuged at 13,000 rpm for 10 minutes at room temperature. The supernatant was transferred to the equilibrated column and the solution in the column was allowed to drain by gravity flow. After a series of washing and elution steps, 0.63 ml of isopropanol was added to the elution tube, mixed well and centrifuged. DNA pellet was washed with 1 ml of 70% ethanol and centrifuged again at 13,000 rpm for 5 minutes at 4°C. The pellet was air dried for 10 minutes and re-suspended in 25 µL TE Buffer.

III.13.10. Clone confirmation through restriction digestion

Presence of the desired insert was further confirmed by restriction digestion. The plasmid was cleaved by BamH I and the digested products were

checked on 1% agarose gel. The bands were viewed under UV transilluminator and documented using Bio-Rad gel documentation system.

III.13.11. Transfection of cardiac fibroblasts with pGL3-Agtr1A plasmid

Cardiac fibroblasts, plated on 24 well plates, were co-transfected with pGL3-Agtr1A and phRL-TK plasmid using Lipofectamine according to manufacturer's protocol. 1 µg of pGL3-Agtr1A plasmid, 50 ng of Renilla and 2 µl of Lipofectamine 2000 were mixed with 100 µl of Opti-MEM, incubated for 20 minutes at room temperature and added to 24 well plates. 8-10 hours after transfection, the cells were revived for 12 hours in M199 + 10% FBS, serum deprived for 12 hours, treated with 25 µM of H_2O_2 for 3 hours and assessed for transgene expression.

III.13.12. Dual luciferase assay

Standard dual luciferase analysis was carried out using a Luminometer (TD20/20, Promega). Briefly, 3 hours after treatment of transfected cells with H_2O_2, cells were incubated with 1X passive lysis buffer for 20 minutes at room temperature on a rocking platform and the lysate was collected for luciferase assay. 30 µl of cell lysate was mixed with 100 µl of substrate A (LAR II) and Firefly Luciferase activity was measured, followed by the addition of 100 µl of substrate B (Stop and glow reagent) for the measurement of Renilla luciferase activity. Values were normalized and plotted.

III.14. *Chromatin immunoprecipitation assay*

ChIP assay was performed using LowCell number ChIP kit from Diagenode according to the manufacturer's protocol.

III.14.1. Binding antibodies to magnetic beads

10 µl of the pre-washed Protein A-beads was mixed with 90 µl of Buffer A and 3 µg of the specific antibody (p65, c-Fos or c-Jun) or normal human IgG, 'centrifuged' at 40 rpm for 2 hours at 4˚C and the pellet was used for immunoprecipitation.

III.14.2. Cell collection and DNA-protein cross-linking

Cardiac fibroblasts were treated with H_2O_2 for the indicated durations. The cells were trypsinised and the cell pellet, suspended in 500 µl of PBS, was mixed with 13.5 µl of 36.5% formaldehyde and 57 µl of 1.25 M glycine and incubated for 8-10 minutes at room temperature for fixing the protein-DNA interaction. The mixture was centrifuged and the pellet was washed twice with 0.5 ml ice-cold PBS.

III.14.3. Cell lysis and chromatin shearing with Bioruptor

The cell pellet was suspended in 130 µl of Buffer B containing protease inhibitor and sodium butyrate (NaBu) and the samples were sonicated using Bioruptor for 10 cycles (30 second ON / 30 seconds OFF) to shear the chromatin into fragments of size between 500 and 600 bp that is suitable for ChIP. 130 µl of sheared chromatin was mixed with 870 µl of Buffer A and the shearing efficiency was assessed by electrophoresis on 1% agarose gel.

III.14.4. Magnetic Immunoprecipitation and washes

To the antibody-coated beads (from III.14.1), 100 µl of diluted sheared chromatin was added and incubated overnight at 4°C on a rotator. 10 µl of sheared chromatin was stored as input sample at 4°C. After overnight incubation, the immunoprecipitated samples were washed using 100 µl each of ice cold Buffer A (X 3 times) and Buffer C and the beads were captured in a magnetic rack. Input

samples were centrifuged and both the input and IP samples were processed for the remaining steps.

III.14.5. DNA isolation

The tubes were removed from the magnetic rack and 100 µl of complete DNA isolation buffer (DIB) was added to re-suspend the beads. 99 µl of complete DIB was added to 1 µl of input DNA sample. The tubes were incubated at 55°C for 15 minutes followed by 100°C for 15 minutes and centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was aliquoted and stored at -20°C until use.

III.14.6. Quantitative PCR & Data analysis

The final step involved amplifying and analyzing the immunoprecipitated DNA. Amplification of immunoprecipitated DNA was carried out by PCR using AT1 primers, 5'CCTTCCTTCCATCCTTTCCTTTCC-3' and 5'-GTCCAACCCGCTCCCTC TC-3', which corresponded to 500 bp of the AT1 promoter region, including the predicted NF-κB and AP-1 binding sites. DNA isolated from an aliquot of the total sheared chromatin was used as loading control for PCR (input control). Chromatin immunoprecipitation using a non-specific antibody (normal human IgG) served as negative control. Reaction was set up using the following components under the following conditions: 94°C for 3 minutes followed by 94°C for 30 seconds, 64.5°C for 45 seconds and 72°C for 1 minute repeated over 39 cycles and final extension at 72°C for 7 minutes.

Water	12.5 µl
10X buffer	5.0 µl
$MgCl_2$	1.5 µl
Forward primer (5 pmol)	1.0 µl

Reverse primer (5 pmol)	1.0 µl
dNTP	1.5 µl
DNA template	2.0 µl
Sigma Taq polymerase	0.5 µl
	25 µl

The amplification product was electrophoresed on 1.2% agarose gel.

III.15. *Enzyme-linked immunosorbent assay*

Ang II, secreted into the culture medium, was detected using an in vitro quantitative assay, according to the manufacturer's protocol. Cells were seeded on 96 well plates and exposed to H_2O_2 for 12 hours in M199. 100 µl of anti-Angiotensin II antibody was added to each well of the 96-well plate (coated with secondary antibody) and incubated overnight at 4°C. Following incubation, the wells were washed thrice with 200 µl of 1X Wash Buffer, and 100 µl each of standard, positive control and culture medium were added to appropriate wells. The wells were covered and incubated for 2.5 hours at room temperature with gentle shaking. After incubation, the solution was discarded and the wells were washed thrice with wash buffer. 100 µl of prepared HRP-Streptavidin solution was added to each well and incubated for 45 minutes with gentle shaking at room temperature. After washing the wells thrice with wash buffer, 100 µl of tetramethylbenzidine (TMB) one-step substrate reagent was added to each well and the plate was incubated for 30 minutes at room temperature in the dark with gentle shaking. Following incubation, 50 µl of stop solution was added to each well and absorbance was read immediately at 450 nm.

III.16. *Statistical analysis*

Data were expressed as Mean±SD and analysed by one-way ANOVA. Student's t-test was used to ascertain differences between experimental groups. $p \leq 0.05$ was considered statistically significant.

IV. RESULTS

IV.1. Characterization of adult rat cardiac fibroblasts

IV.1.1. Morphological analysis

Cardiac fibroblasts isolated from adult rat ventricular tissue were grown in culture as described under 'Methods'. Incubation for 2.5 hours following isolation ensured that greater than 99% of the cells in the cultures were fibroblasts. Morphological analysis and immunocytochemical staining established the fibroblastic nature of the cells. At 2.5 hours after isolation, the cells had a dense nest-like morphology (Figure 8) and, by 24 hours, the cells attained spindle-like appearance (Figure 9). At confluence, the cultures exhibited a monolayer pattern (Figure 10). Absence of 'cobblestone' or 'hill and valley' pattern of morphology ruled out contamination of cultures by endothelial cells and VSMCs, respectively. Cells at passages 2 and 3 were used for the experiments.

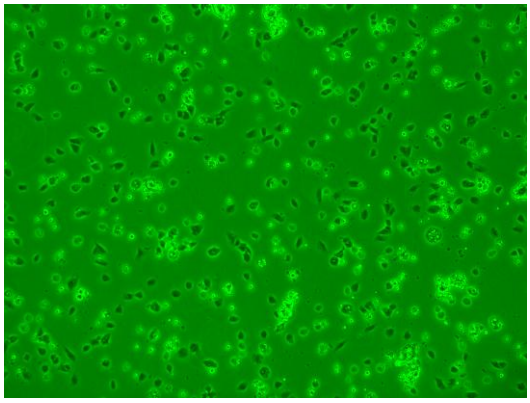


Figure 8: Phase contrast micrograph of cardiac fibroblasts at 2.5 hours after plating (10X magnification)

Cardiac fibroblasts were isolated as described under 'Methods' and seeded on 35 mm dishes in M199 containing 10% FBS. At 2.5 hours of seeding, cultures enriched with adherent fibroblasts were rinsed and incubated with M199 containing 10% FBS.

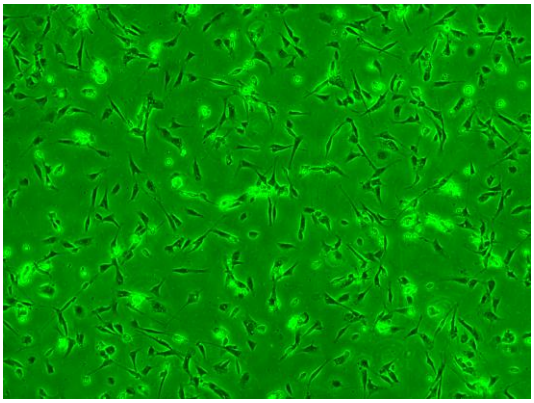


Figure 9: Phase contrast micrograph of adult rat cardiac fibroblasts 24 hours after isolation (10X magnification)

Cardiac fibroblasts grown in M199 containing 10% FBS for 24 hours showing spindle-shaped morphology.

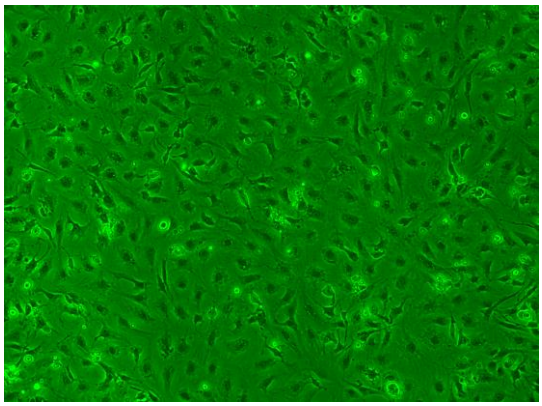


Figure 10: Phase contrast micrograph of cardiac fibroblasts at confluence (10X magnification)

Cardiac fibroblasts grown to confluence in M199 containing 10% FBS formed a monolayer with spindle-shaped morphology.

IV.1.2. Immunocytochemical staining

Cardiac fibroblasts were examined for immunoreactivity with antibodies against the cytoskeletal proteins, vimentin, desmin and von Willebrand Factor. Cells were positive for vimentin (Figure 11) but negative for desmin and von Willebrand

factor (Figures 12 & 13), confirming their fibroblastic nature and ruling out the presence of VSMCs and endothelial cells, respectively, in the culture.

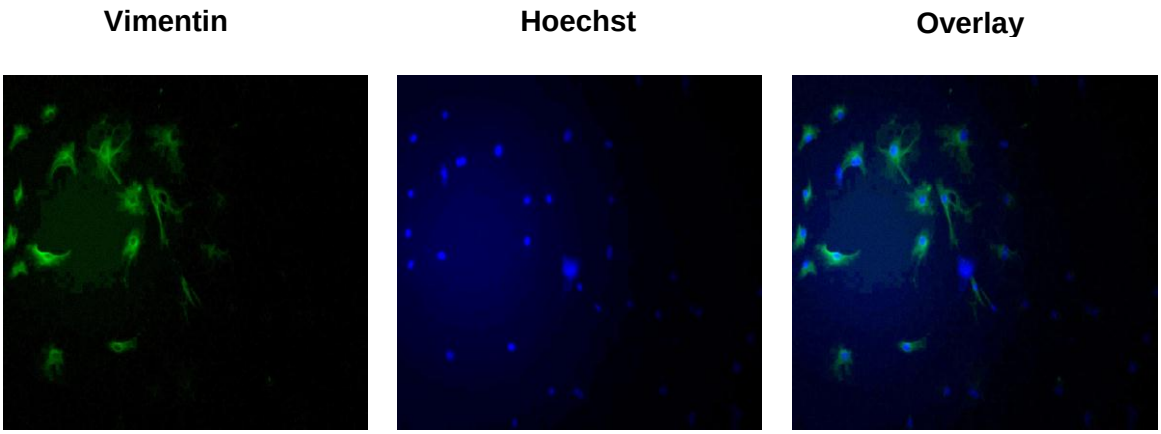


Figure 11: Fluorescent micrographs of vimentin-positive adult rat cardiac fibroblasts (10X magnification)

Sub-confluent cultures of cardiac fibroblasts were immunostained with anti-vimentin antibody. Nuclei were counter-stained with Hoechst 33342.

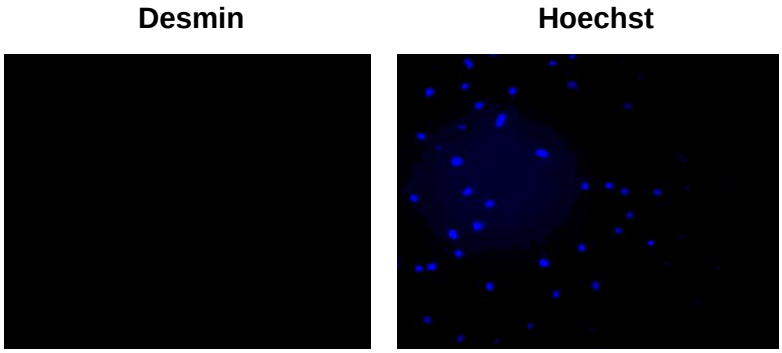


Figure 12: Fluorescent micrographs of adult rat cardiac fibroblasts stained negative for desmin (10X magnification)

Sub-confluent cultures of cardiac fibroblasts were immunostained with anti-desmin antibody. Nuclei were counter-stained with Hoechst 33342.

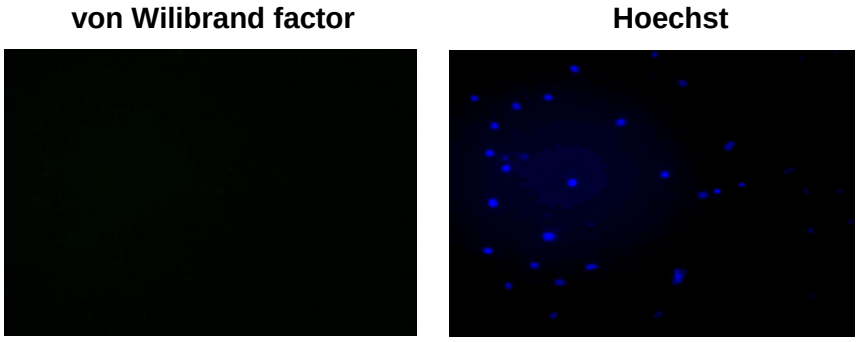


Figure 13: Fluorescent micrographs of von Willebrand factor-negative adult rat cardiac fibroblasts (10X magnification)

Sub-confluent cultures of cardiac fibroblasts were immunostained with anti-von Willebrand factor antibody. Nuclei were counter-stained with Hoechst 33342.

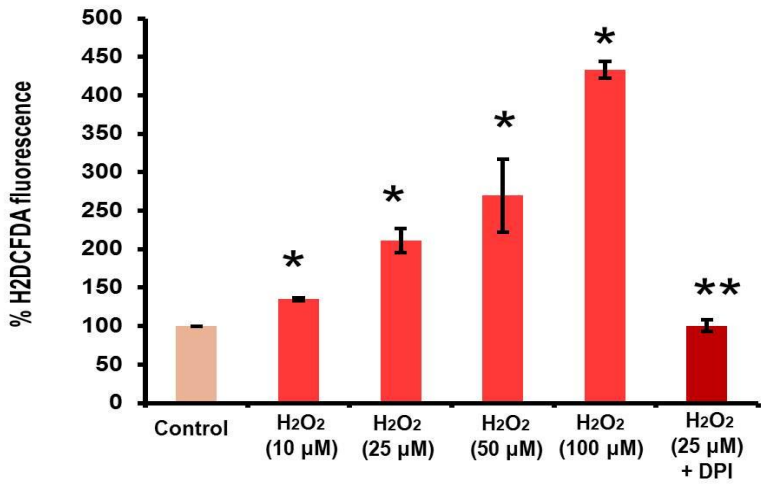
IV.2. *Experimental model*

Cardiac fibroblasts from passage 2 or 3 exposed to H_2O_2 served as the experimental model.

IV.3. *Validation of experimental model*

IV.3.1. ROS generation in cardiac fibroblasts exposed to H_2O_2

To examine whether exposure to H_2O_2 results in generation of intracellular ROS, cells were loaded with H_2DCFDA and exposed to various concentrations of H_2O_2 for 15 minutes. Significant increase in intracellular ROS levels was observed in cardiac fibroblasts treated with 25 µM, 50 µM and 100 µM H_2O_2, which was inhibited by 10 µM of diphenyleneiodonium (DPI).



*p<0.02 vs. Control. **p<0.02 vs H_2O_2

Figure 14: Intracellular ROS generation in cardiac fibroblasts treated with H_2O_2

Intracellular ROS in cardiac fibroblasts exposed to H_2O_2 was measured by pre-treatment of cells with 10 µM H_2DCFDA followed by treatment with the indicated concentrations of H_2O_2 for 15 minutes. Fluorescence was measured at excitation and emission wavelengths of 485 nm and 530 nm, respectively.

IV.3.2. Assessment of cell viability when exposed to H_2O_2

The effect of H_2O_2 on the viability of cardiac fibroblasts was determined in terms of Hoechst 33342 and propidium iodide (PI) uptake. Cardiac fibroblasts were exposed to a range of H_2O_2 concentrations and viability was checked at 3 hours of treatment. There was no significant loss of viability with 10 or 25 µM H_2O_2 in cardiac fibroblasts. However, exposure to 50 and 100 µM H_2O_2 caused significant cell death.

Based on these observations, a concentration of 25 µM H_2O_2 was chosen for subsequent experiments since 25 µM significantly increased intracellular ROS without compromising cell viability.

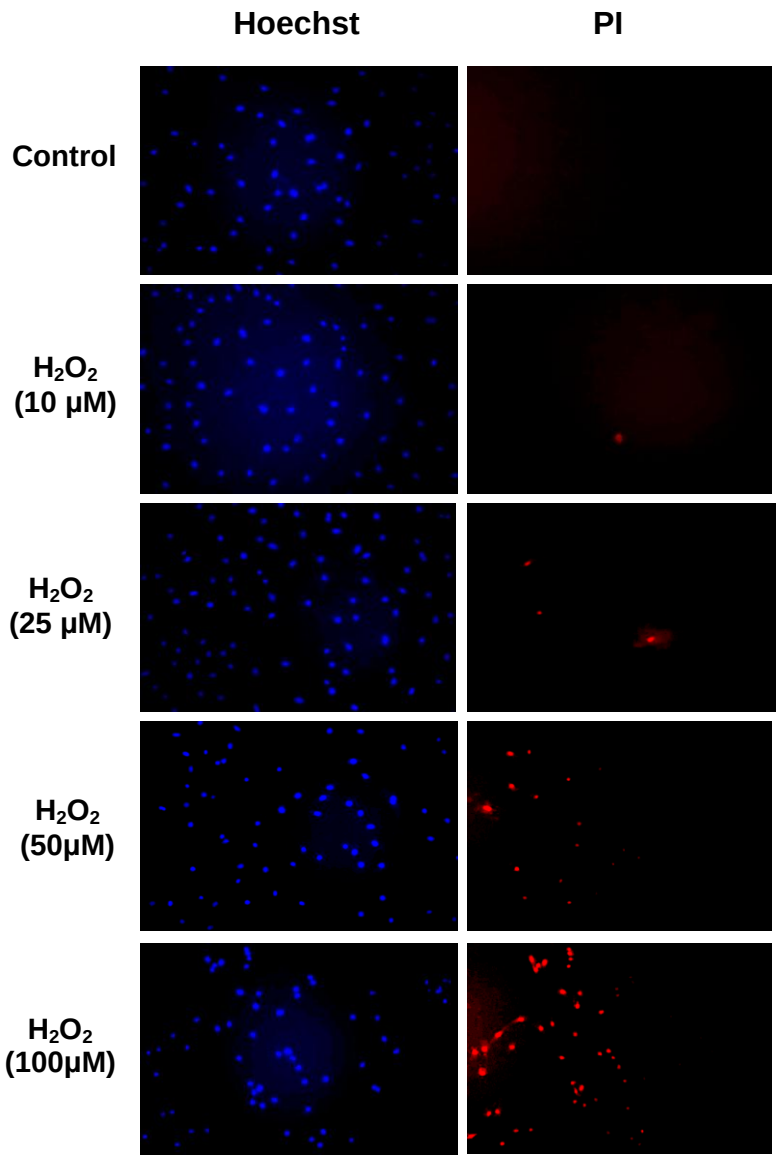


Figure 15: Effect of H_2O_2 on viability of cardiac fibroblasts (10X magnification)

Sub-confluent cultures of cardiac fibroblasts in M199 were exposed to a range of concentrations of H_2O_2 for 3 hours and then stained with Hoechst 33342 and PI as described under 'Methods'.

IV.4. *Effect of H_2O_2 on AT1 gene expression in cardiac fibroblasts*

The influence of ROS on transcriptional regulation of AT1 in cardiac fibroblasts was assessed. Sub-confluent cultures of cardiac fibroblasts were exposed to H_2O_2 for 3 or 12 hours. Total RNA was isolated using Purelink RNA Mini Kit and its purity was ascertained by 1% agarose gel electrophoresis. Appearance of intact 18S and 28S rRNA bands confirmed the intactness of the isolated RNA.

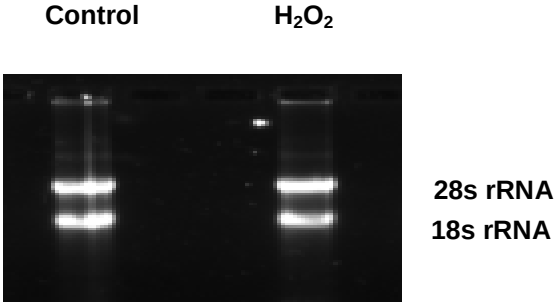
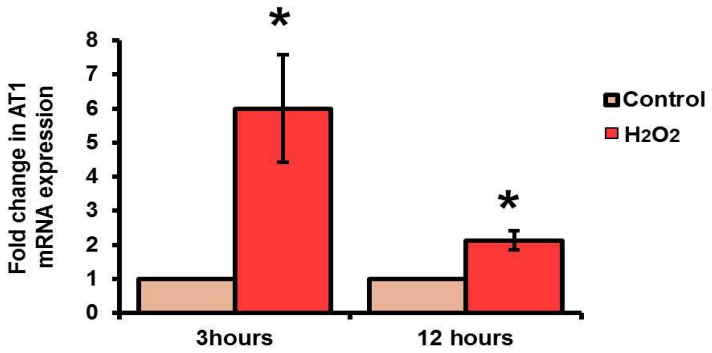


Figure 16: Agarose gel electrophoresis of RNA

RNA, isolated from control and H_2O_2-treated cells, was subjected to 1% agarose gel electrophoresis. The presence of intact 28S and 18S rRNA bands was documented using Bio-Rad Imaging system.

To get rid of any genomic DNA contamination, total RNA was subjected to DNase I treatment as per manufacturer's instructions. 2 µg of DNase I-treated RNA was reverse transcribed to cDNA with random hexamer primers and M-MLV reverse transcriptase. The cDNA was co-amplified over 40 cycles with primers specific for rat AT1 and 18S rRNA using Real-time PCR analysis. Figure 17 shows that 3 hours of H_2O_2 treatment caused a 6-fold increase in AT1 mRNA levels, which reduced to 2-fold at the end of 12 hours.

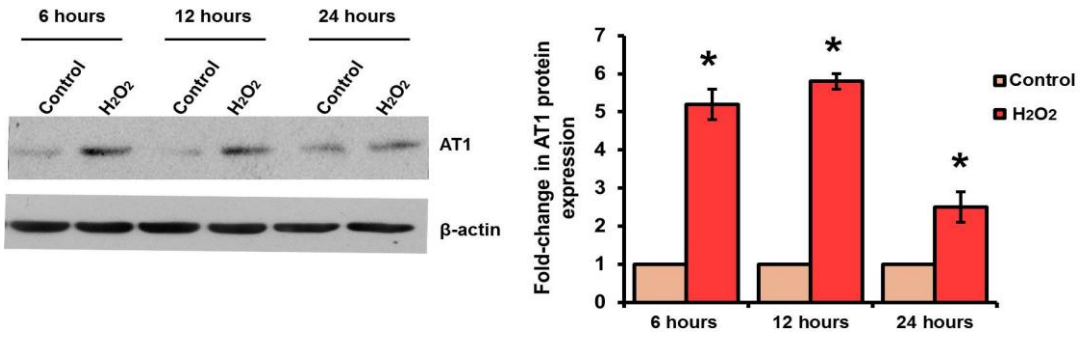


* p< 0.05 vs Control, n=3

Figure 17: Effect of H_2O_2 on AT1 mRNA expression

Serum-deprived sub-confluent cultures of cardiac fibroblasts in M199 were treated with H_2O_2 for 3 or 12 hours. AT1 mRNA expression was examined by Taqman quantitative Real-time PCR analysis. 18S rRNA served as endogenous control.

To determine whether the increase in AT1 mRNA expression translates into comparable changes in AT1 protein levels, cardiac fibroblasts were incubated for 6, 12 or 24 hours with 25 µM of H_2O_2, total protein was isolated and AT1 levels were determined by western blot analysis, using β-actin as loading control. A significant up-regulation of AT1 protein was found at 6 and 12 hours, which reduced significantly at 24 hours of treatment (Figure 18).



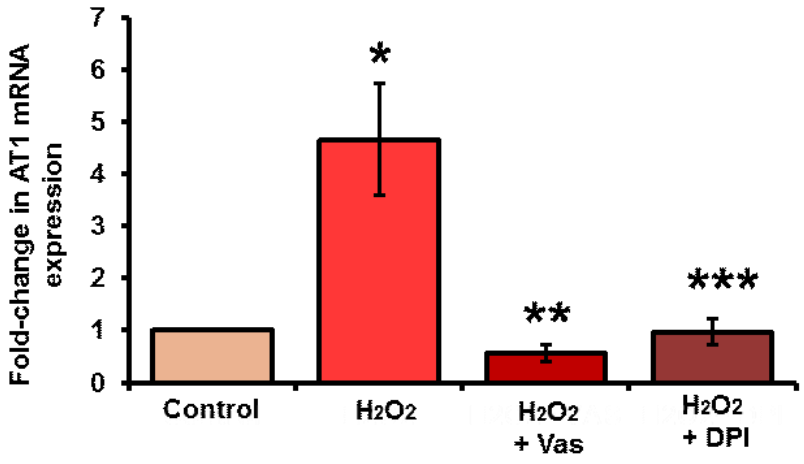
* p< 0.05 vs Control , n=3

Figure 18: Effect of H_2O_2 on AT1 protein expression

Western blot analysis was performed as described under 'Methods' using polyclonal anti-AT1 antibody. β-actin served as loading control.

IV.5. *H_2O_2 up-regulates AT1 expression via NOX-dependent ROS*

H_2O_2 is known to trigger NOX-mediated ROS release in cardiac fibroblasts (Colston et al., 2005). To ascertain the source of ROS that modulates AT1 expression, cardiac fibroblasts were pre-incubated with specific pharmacological inhibitors of NOX, DPI (10 µM) or VAS2870 (5 µM), 1 hour before treating them with H_2O_2 and the effect of H_2O_2 on AT1 mRNA and protein levels was assessed. NOX inhibition completely abolished the effects of H_2O_2 on AT1 mRNA and protein, indicating that NOX-dependent ROS generation augments AT1 expression in H_2O_2-treated cells.

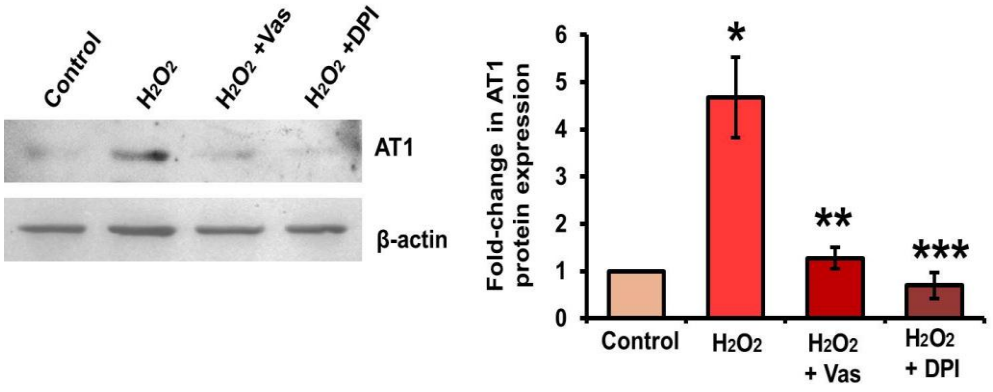


*p<0.05 vs Control. **p<0.001 vs H_2O_2. ***p<0.01 vs H_2O_2; n=5

Figure 19: NOX inhibition attenuates H_2O_2-mediated AT1 mRNA expression

Following pre-treatment of cells with Vas (5 µM) or DPI (10 µM) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA expression was examined by

Taqman quantitative Real-time PCR analysis. 18S rRNA was used as endogenous control.



*p<0.05 vs Control. **p<0.05 vs H_2O_2. ***p<0.001 vs H_2O_2; n=5

Figure 20: NOX inhibition attenuates H_2O_2-mediated AT1 protein expression

Following pre-treatment of cells with Vas (5 μM) or DPI (10 μM) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined by western blot analysis with β-actin as endogenous control.

IV.6. *Transcriptional Regulation of AT1 expression by NF-κB and AP-1*

Subsequent experiments addressed the molecular mechanisms involved in ROS-induced enhancement of AT1 gene expression, focusing on the involvement of the redox-sensitive transcription factors, NF-κB and AP-1. The regulatory role of NF-κB and AP-1 in AT1 gene expression was evaluated in terms of

- their activation status in cells exposed to H_2O_2 (EMSA),
- the effect of their inhibition on AT1 expression and
- their association with the AT1 gene promoter (Luciferase assay and ChIP).

IV.6.1. Nuclear translocation of NF-κB in response to H_2O_2

To determine whether H_2O_2 activates NF-κB in cardiac fibroblasts, its activation status was first assessed by EMSA using nuclear extracts from cells treated with H_2O_2 for 30 minutes or 3 hours. H_2O_2 induced nuclear translocation of NF-κB within 30 minutes, which was sustained at 3 hours of treatment. Nuclear import of NF-κB in cells exposed to oxidative stress was inhibited when the cells were pre-treated with its specific pharmacological inhibitor, Bay 11-7085 (2 µM), for 1 hour prior to H_2O_2 treatment. Specificity of the binding was confirmed using a 200-fold excess of the unlabelled double stranded NF-κB probe, which competitively inhibited the binding of the transcription factor to the biotinylated primer sequence.

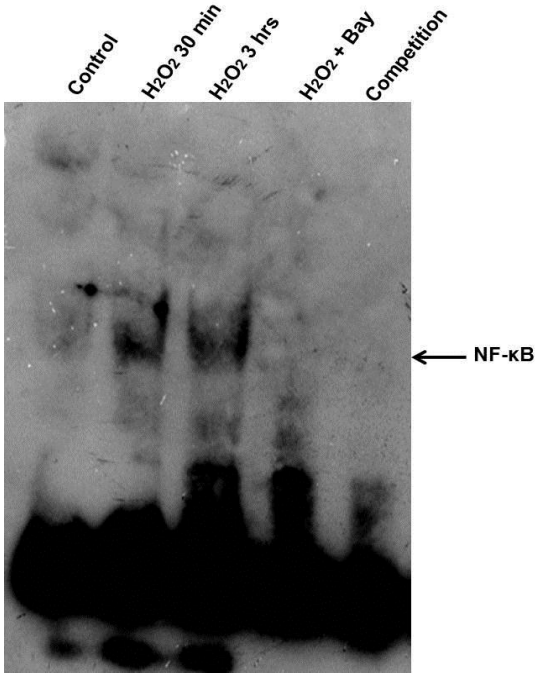


Figure 21: H_2O_2 induces NF-κB activation

Sub-confluent cultures of quiescent cardiac fibroblasts were subjected to H_2O_2 treatment for 30 minutes or 3 hours in serum-free M199. Electrophoretic mobility shift of NF-κB was observed in response to H_2O_2 at both 30 minutes and 3 hours,

which was significantly reduced upon pre-treatment with Bay (2 µM) and in the presence of a 200-fold excess of unlabelled oligos. A representative profile from one of three experiments is shown.

IV.6.2. H_2O_2 promotes of AP-1 activation

Since NF-κB and AP-1 are reported to act in concert to regulate multiple genes involved in oxidative stress in various cell types (Fujioka et al., 2004), the possibility that AP-1 may be involved in oxidative stress-mediated AT1 expression in cardiac fibroblasts was examined. To begin with, the activation status of AP-1 was assessed by EMSA using nuclear extracts from cells treated with H_2O_2 for 30 minutes or 3 hours. ROS induced nuclear translocation of AP-1 at both time points, which was inhibited in the presence of the AP-1 trans-repressing-retinoid, SR11302 (1 µM), as shown in Figure 22.

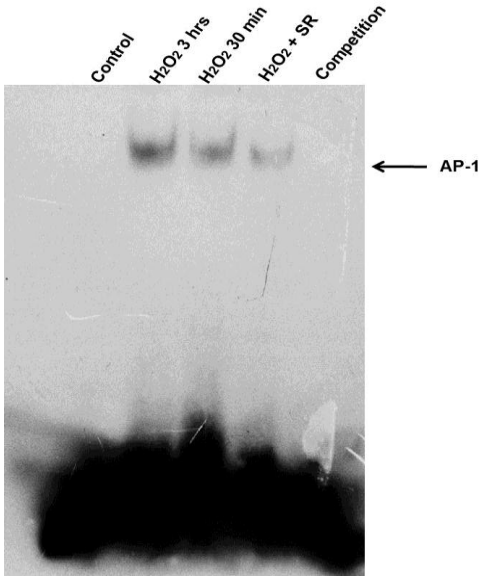


Figure 22: H_2O_2 induces AP-1 activation

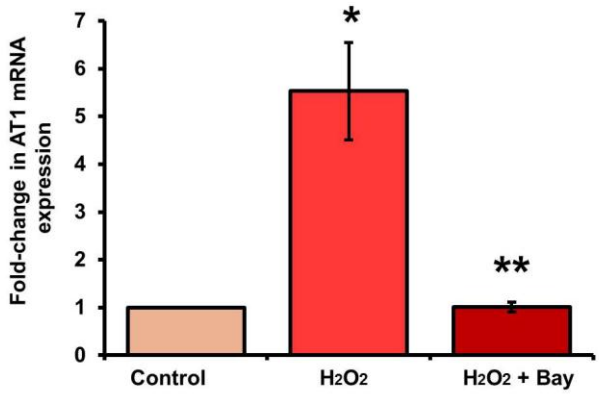
Sub-confluent cultures of quiescent cardiac fibroblasts were subjected to H_2O_2 treatment for 30 minutes or 3 hours in serum-free M199. Electrophoretic mobility shift of AP-1 was observed in response to H_2O_2 at both 30 minutes and 3 hours, which was significantly reduced upon pre-treatment with SR (1 µM) and in the

presence of a 200-fold excess of unlabelled oligos. A representative profile from one of three experiments is shown.

Having demonstrated the nuclear localization of NF-κB and AP-1 in H_2O_2-treated cardiac fibroblasts, further experiments were performed to evaluate whether these transcription factors play a role in the regulation of AT1 gene expression.

IV.6.3. NF-κB or AP-1 inhibition attenuates H_2O_2-induced AT1 gene expression

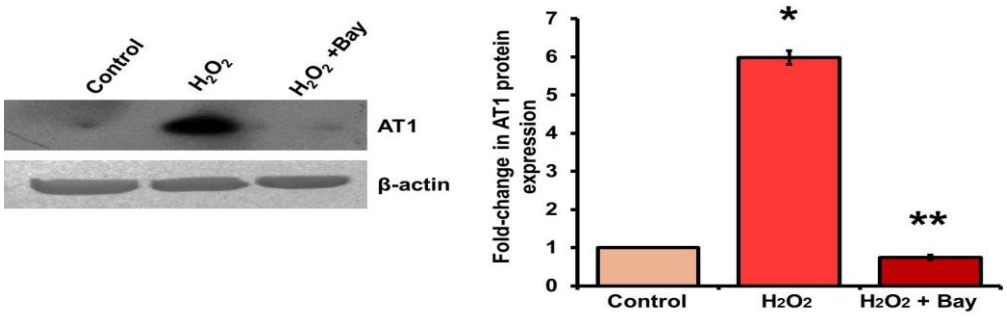
Cardiac fibroblasts were pre-incubated with Bay 11-7085 for 1 hour prior to the addition of 25 μM of H_2O_2. A significant 6-fold increase in the AT1 mRNA and protein levels, observed in response to H_2O_2 treatment, was attenuated upon inhibiting NF-κB activation, suggesting that NF-κB transcriptionally up-regulates AT1 in H_2O_2-treated cells.



*p<0.01 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 23: NF-κB inhibition by Bay down-regulates H_2O_2-mediated AT1 mRNA levels

Following pre-treatment of cells with Bay (2 μM) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA expression was examined, with 18S rRNA as endogenous control.

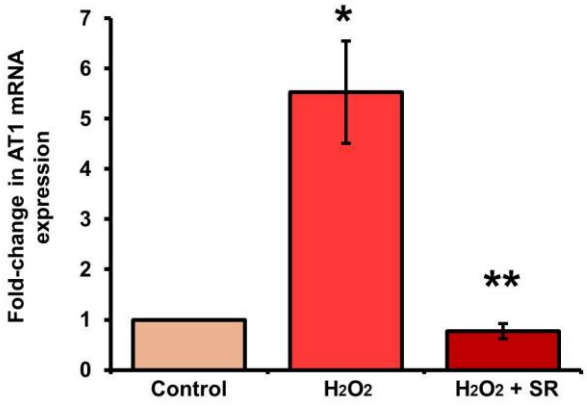


*p<0.05 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 24: NF-κB inhibition by Bay down-regulates H_2O_2-mediated AT1 protein levels

Following pre-treatment of cells with Bay (2 μM) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined, with β-actin as endogenous control.

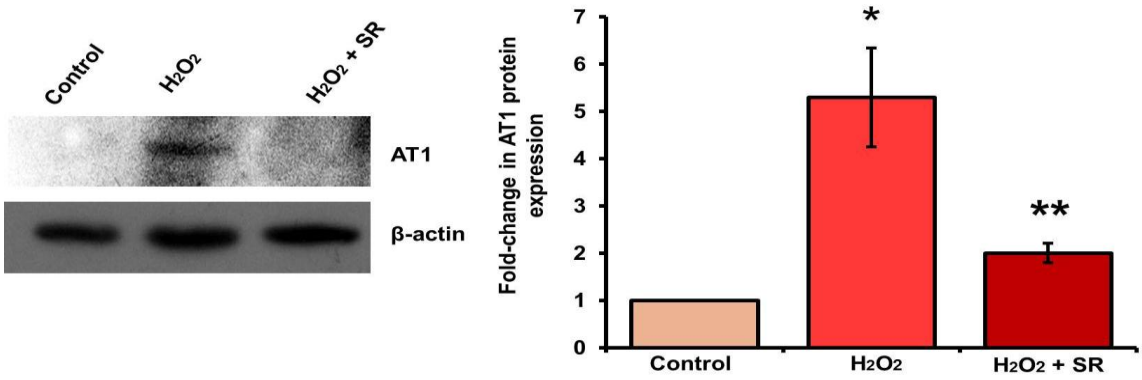
The possibility that AP-1 may be involved in ROS-mediated AT1 expression in cardiac fibroblasts was checked following its inhibition using SR11302. Pre-incubation of cells for 1 hour with SR11302 before H_2O_2 treatment attenuated AT1 mRNA and protein expression.



*p<0.05 vs Control. **p<0.05 vs H_2O_2; n=3

Figure 25: AP-1 inhibition by SR down-regulates H_2O_2-mediated AT1 mRNA levels

Following pre-treatment of cells with SR (1 µM) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA levels were examined with 18S rRNA as endogenous control.



*p<0.05 vs Control. **p<0.01 vs H_2O_2; n=3

Figure 26: AP-1 inhibition by SR down-regulates H_2O_2-mediated AT1 protein levels

Following pre-treatment of cells with SR (1 µM) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined, with β-actin as endogenous control.

IV.7. *AT1 Promoter binding ability of NF-κB and AP-1*

Subsequent experiments probed the AT1 gene promoter-binding activity of NF-κB and AP-1 in H_2O_2-treated cardiac fibroblasts.

IV.7.1. Dual luciferase assay

IV.7.1.1. Amplification of AT1 promoter sequence using PCR

The promoter sequence for AT1 receptor in *Rattus Norvegicus* was identified using USSC software and NF-κB and AP-1 binding regions were identified using TF Search or ALGGEN software. Using Beacon Designer PREMIER Biosoft

International, a set of primers was designed that would amplify a 500 bp segment of AT1 promoter carrying the NF-κB and AP-1 binding sites. The whole genomic DNA isolated from Sprague Dawley rat blood was subjected to PCR using forward primer, 5'GGTGGTGCTAGCCCTTCCTTCCATCTTTCCTTTCC-3' and reverse primer, 5'GGTGGTCCCGGGGTCCAACCCGCTCCCTCTC-3' to amplify a 500 bp region in the AT1 promoter. The PCR product was run on 1.2% agarose gel to confirm the size of the amplicon and the specificity of the primers.

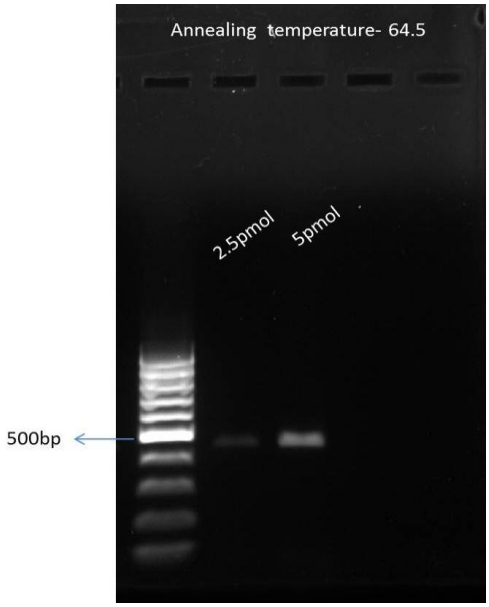


Figure 27: Amplification of AT1 promoter sequence using PCR

Using forward and reverse primers, the AT1 promoter region carrying NF-κB and AP-1 binding sites was amplified by PCR as described under 'Methods' and size (500 bp) was determined on 1.2% agarose gel.

IV.7.1.2. Amplification of AT1 promoter by cloning into pCR II vector

The agarose gel band was excised, purified and the AT1 promoter was ligated to pCR II vector. pCR II-Agtr1A plasmid was amplified by transformation in

DH5α strain of *E. coli*. The transformed cells were plated on antibiotic-coated plates containing X-gal and IPTG, which would facilitate blue-white selection. Transformed cells appeared as white colonies within 12 to 16 hours. Transformed clones were selected and amplified further in LB medium. pCR II-Agtr1A plasmid was isolated as described under 'Methods' and was subjected to verification steps by restriction digestion and DNA sequencing.

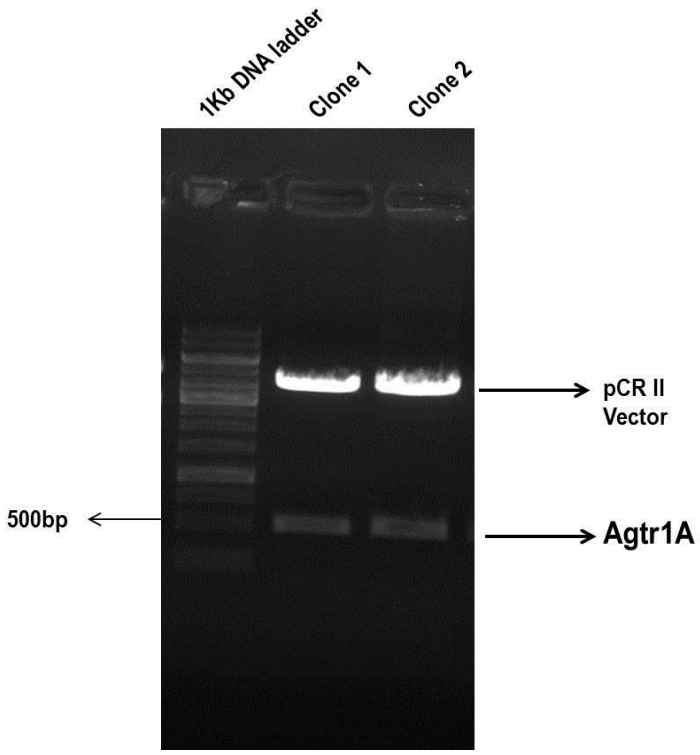


Figure 28: EcoRI digestion for confirming the presence of the desired insert in isolated pCR II-Agtr1A plasmid

Transformed clones of pCR II-Agtr1A plasmid, isolated and subjected to single site restriction digestion with EcoR1 to yield the linearized form of pCR II vector (4.0 kb) and Agtr1A promoter (500 bp), which were analyzed on 1.2% agarose gel.

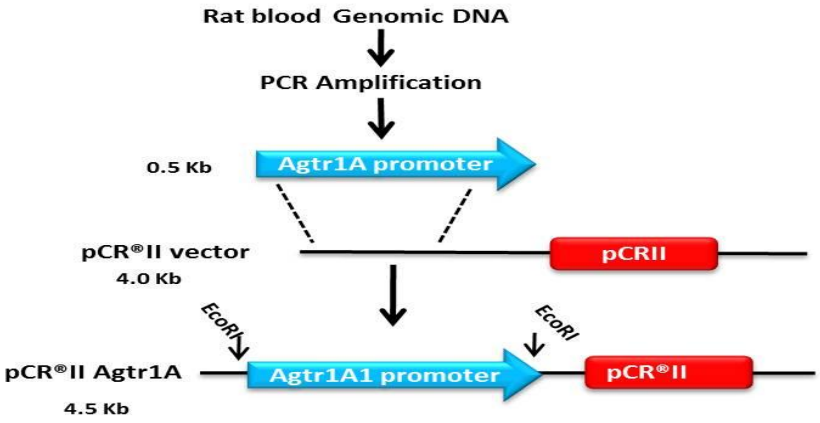


Figure 29: Schematic representation of pCR II-Agtr1A cloning

IV.7.1.3. Generation of pGL3-Agtr1A plasmid

The pGL3-Agtr1A plasmid was obtained by double digestion of pCRII-Agtr1A plasmid and pGL3 basic vector with restriction enzymes.

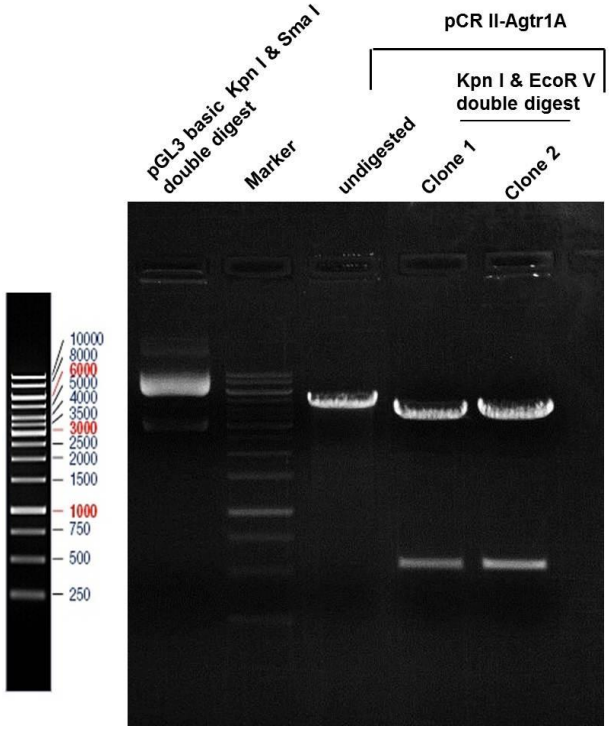


Figure 30: Double digestion of pCRII-Agtr1A plasmid and pGL3 basic vector

Transformed clones of pCR II-Agtr1A plasmid were isolated and subjected to double site restriction digestion using EcoRV and KpnI. pGL3 basic vector was subjected to double site restriction digestion using SmaI and KpnI. The enzyme-digested products were analyzed on 1.2% agarose gel.

The bands corresponding to the Agtr1A promoter and pGL3 basic vector backbone were eluted and ligated to generate pGL3-Agtr1A which was amplified by transformation in competent DH5α strain of *E. coli*. Kanamycin-resistant clones were selected and amplified further in LB medium. pGL3-Agtr1A plasmid was isolated and subjected to single restriction site digestion at BamH1 site to yield the linearized form of the plasmid (5310 bp), which was further analysed on 1.2% agarose gel.

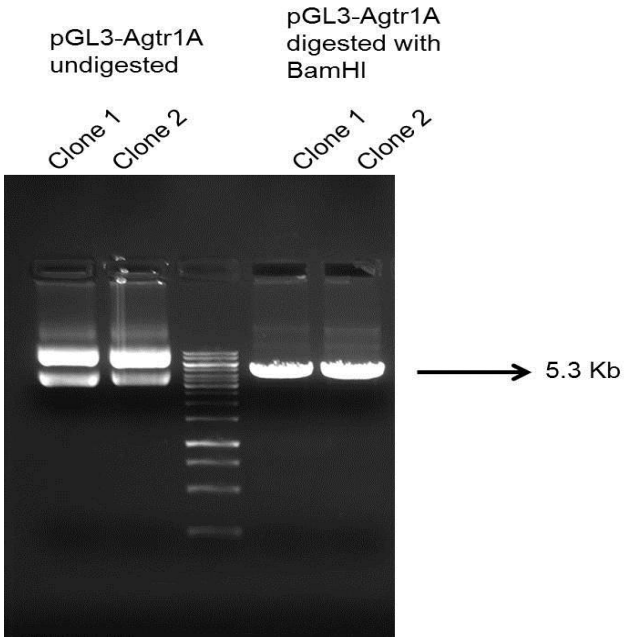


Figure 31: Confirmation of pGL3-Agtr1A clone using BamH1 digestion

Transformed clones of pGL3-Agtr1A plasmid, isolated and subjected to single restriction site digestion with BamH1, yielded the linearized form of pGL3-Agtr1A plasmid (5.3 kb) which were analysed on 1.2% agarose gel.

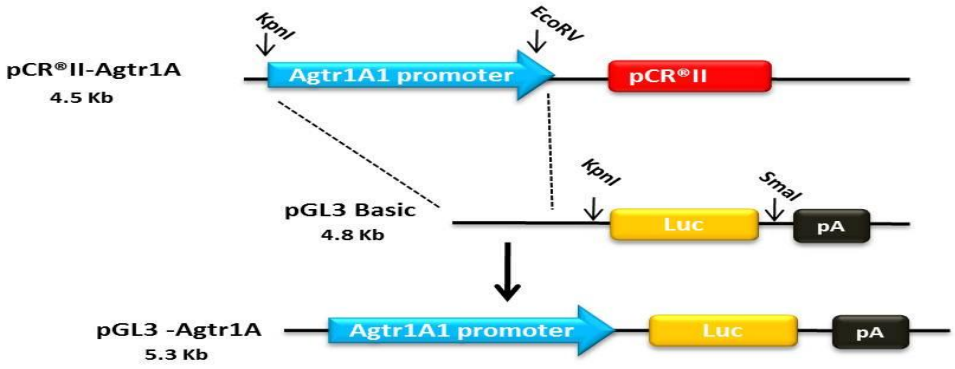
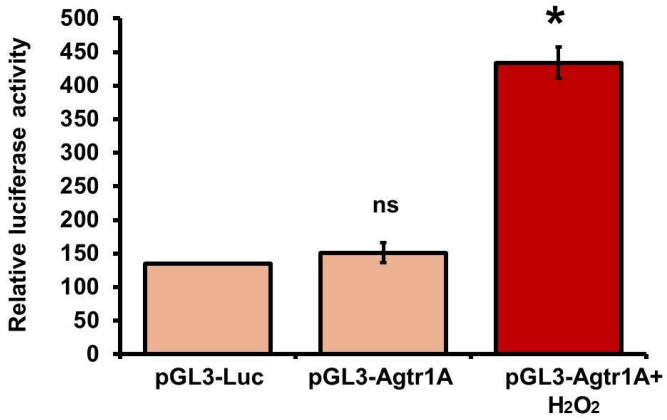


Figure 32: Schematic representation of pGL3-Agtr1A cloning

IV.7.1.4. Transfection of cardiac fibroblasts and dual luciferase assay

Cardiac fibroblasts were co-transfected with pGL3-Agtr1A plasmid and phRL-TK plasmid (Renilla luciferase). After treatment with 25 µM of H_2O_2 for 3 hours, cell lysate was assayed for Firefly Luciferase activity (which is an index of the promoter binding activity) followed by measurement of Renilla luciferase activity. H_2O_2 induced a significant increase in luciferase activity over the control group, showing H_2O_2-dependent activation of the AT1 promoter.



ns, not significant vs pGL3-Luc. *p<0.01 vs pGL3-Agtr1A; n=3

Figure 33: Dual luciferase assay

Luciferase activity was assayed in control and H_2O_2-treated cells 48 hours after transfection with pGL3-Agtr1a in the presence of phRL-TK plasmid.

IV.7.2. Chromatin immunoprecipitation assay

Cell lysate obtained from cardiac fibroblasts treated with and without H_2O_2 for 3 hours was lysed and then sheared to yield chromatin fragments of size between 500 and 600 bp.

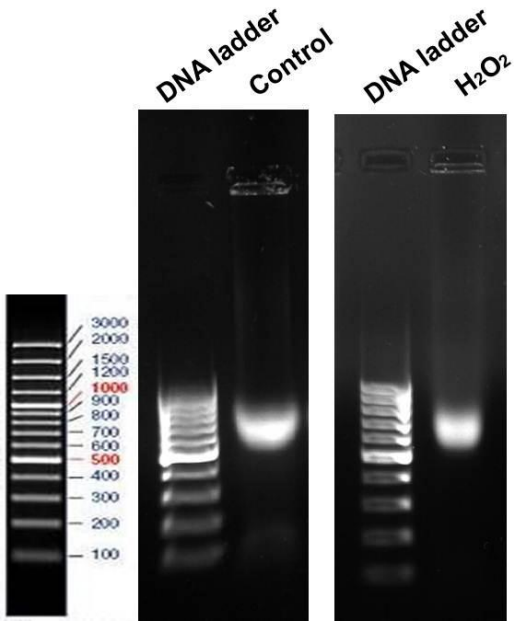
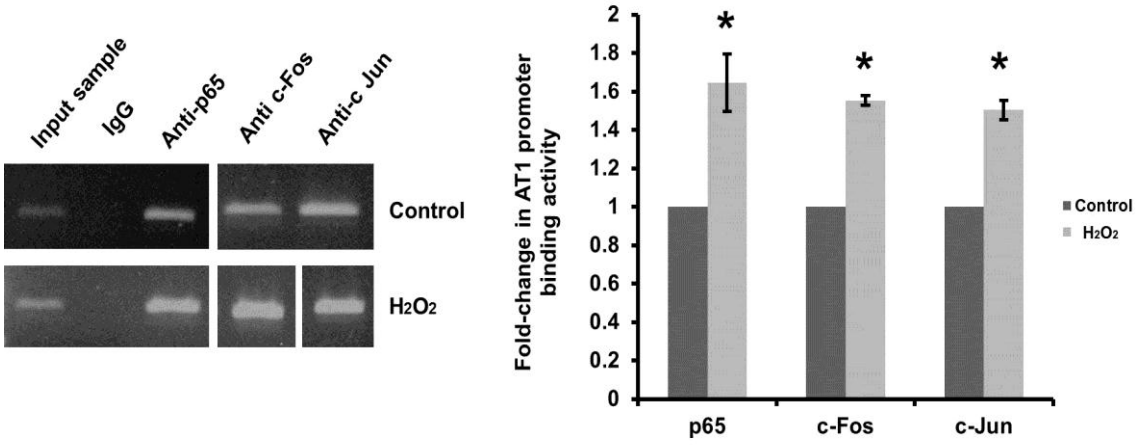


Figure 34: Assessment of shearing efficiency

Chromatin isolated from cardiac fibroblast was sheared in a Bioruptor to yield fragments of 500-600 bp and analysed on a 1.2% agarose gel.

The chromatin fragments were immunoprecipitated with p65, c-Fos or c-Jun antibody and the immunoprecipitated DNA was subjected to PCR analysis using primers that corresponded to 500 bp of the AT1 promoter region, including the predicted NF-κB and AP-1 binding sites. Amplification of input chromatin prior to

immunoprecipitation served as positive control and normal human IgG served as negative control. There was significantly higher NF-κB p65, c-Fos and c-Jun cross-linking with the AT1 promoter sequence in H_2O_2-treated cells, compared to the control group.



*p<0.05 vs control; n=3

Figure 35: Binding of NF-κB and AP-1 to the AT1 gene promoter

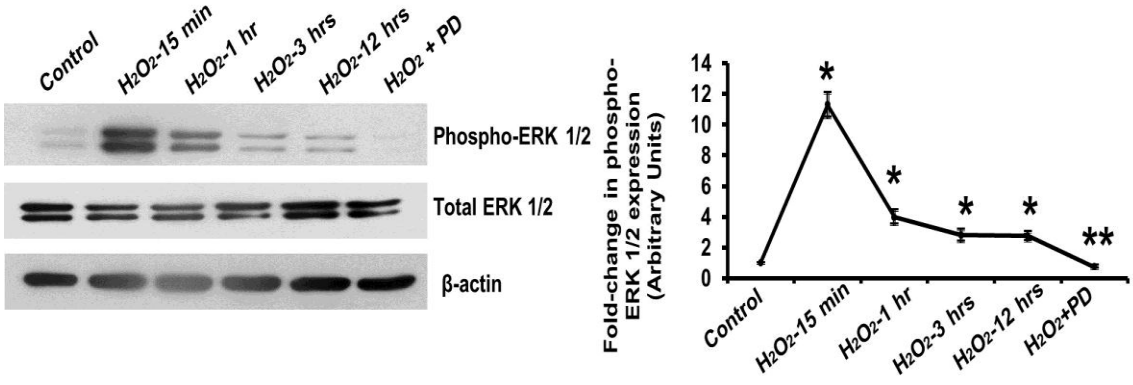
Following immunoprecipitation with anti-p65, c-Fos or c-Jun antibodies, chromatin segment was subjected to PCR analysis and the PCR products were analysed on 1.2% agarose gel. Relevant treatment groups from within the same gel have been juxtaposed for ease of comparison.

IV.8. *NF-κB or AP-1 activation is mediated by ERK1/2 MAPK, p38 MAPK and JNK*

Redox-sensitive protein kinase-driven pathways are known to regulate NF-κB and AP-1 activation in a variety of cell types (Guo et al., 2005; Philip and Shivakumar, 2013; Slomiany and Slomiany, 2013). In the present study, the activation status of ERK1/2 MAPK, p38 MAPK and JNK was assessed in H_2O_2-treated cardiac fibroblasts.

IV.8.1. H_2O_2 activates ERK1/2 MAPK, p38 MAPK and JNK

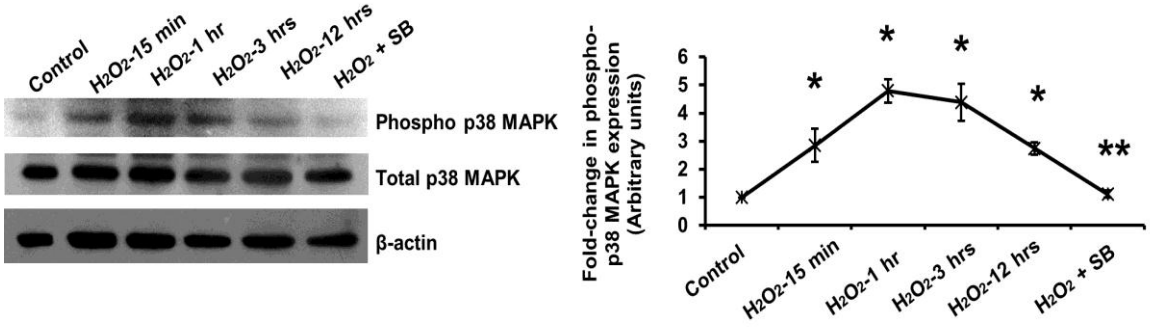
Western blot analysis revealed significantly higher levels of the phosphorylated forms of ERK1/2 MAPK, p38 MAPK and JNK, indicating their activation in cardiac fibroblasts treated with H_2O_2. Activation of ERK1/2 MAPK and JNK peaked as early as 15 minutes whereas p38 MAPK peaked at 1 hour and was sustained upto 12 hours. The inhibition of H_2O_2-induced ERK1/2 MAPK, p38 MAPK and JNK activation by their respective pharmacological inhibitors was also ascertained.



*p<0.05 vs Control. **p<0.001 vs H_2O_2-15'; n=3

Figure 36: H_2O_2 activates ERK1/2 MAPK

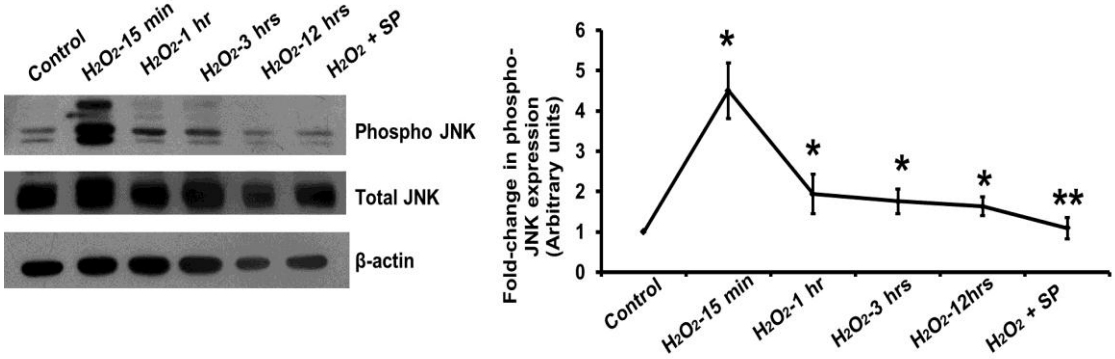
Sub-confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 36 hours followed by exposure to H_2O_2 for the indicated durations. Western blot analysis performed using monoclonal anti-phospho-ERK1/2 MAPK antibody showed that phospho(p)-ERK1/2 MAPK levels, normalized to total ERK1/2 MAPK, peaked at 15 minutes in response to H_2O_2 treatment, which was sustained upto 12 hours. The ERK1/2 inhibitor, PD (10 μM), was found to abolish the H_2O_2-induced ERK1/2 activation. A representative profile from one of three experiments is shown.



*p<0.01 vs Control. **p<0.001 vs H_2O_2-1 hr ; n=3

Figure 37: H_2O_2 activates p38 MAPK

Sub-confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 36 hours followed by exposure to H_2O_2 for the indicated durations. Western blot analysis performed using monoclonal anti-phospho-p38 MAPK antibody showed that phospho p38 levels, normalized to total p38 levels, peaked at 1 hour in response to H_2O_2 treatment, which was sustained upto 12 hours. The p38 inhibitor, SB (10 µM), was found to abolish the H_2O_2-induced P38 activation. A representative profile from one of three experiments is shown.



* p<0.05 vs Control. **p<0.01 vs H_2O_2-15'; n=3

Figure 38: H_2O_2 activates JNK

Sub-confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 36 hours followed by exposure to H_2O_2 for the indicated durations. Western blot analysis performed using anti-phospho-JNK antibody showed that p-JNK levels, normalized to total JNK levels, peaked at 15 minutes in response to H_2O_2 treatment,

.which was sustained upto 12 hours. The JNK inhibitor, SP (10 μM), was found to abolish the H_2O_2-induced JNK activation. A representative profile from one of three experiments is shown.

IV.8.2. ERK1/2 MAPK and p38 MAPK mediate H_2O_2-induced NF-κB activation

To determine whether activation of these MAPKs is a pre-requisite for NF-κB activation, cells were pre-incubated for 1 hour with 10 μM each of ERK1/2 MAPK inhibitor (PD98059), p38 MAPK inhibitor (SB203580) or JNK inhibitor (SP600125) prior to treatment with H_2O_2. ERK1/2 MAPK and p38 MAPK inhibitors exerted significant inhibitory effects on H_2O_2-induced NF-κB activation (Figure 39 and 40). However, JNK inhibition did not exert any effect on ROS-induced NF-κB activation (Figure 41).

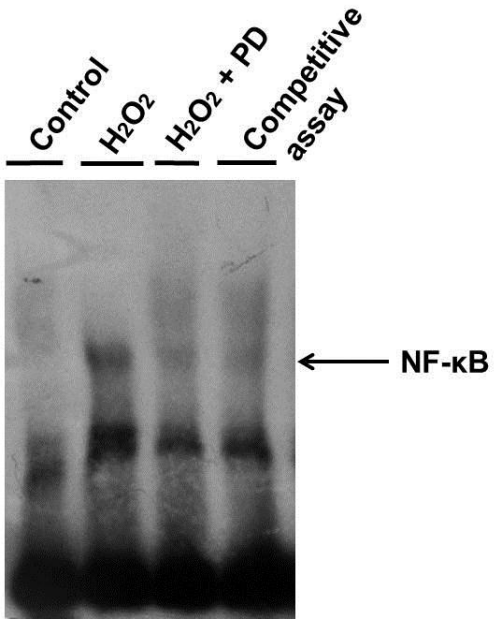


Figure 39: ERK1/2 MAPK regulates NF-κB activation in cardiac fibroblasts

Sub-Confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 24 hours followed by H_2O_2 treatment for 3 hours in the presence/absence of PD (10 μM). EMSA showed that the nuclear translocation of NF-κB observed in response to

H_2O_2 was significantly reduced upon ERK1/2 MAPK inhibition. A representative profile from one of three experiments is shown.

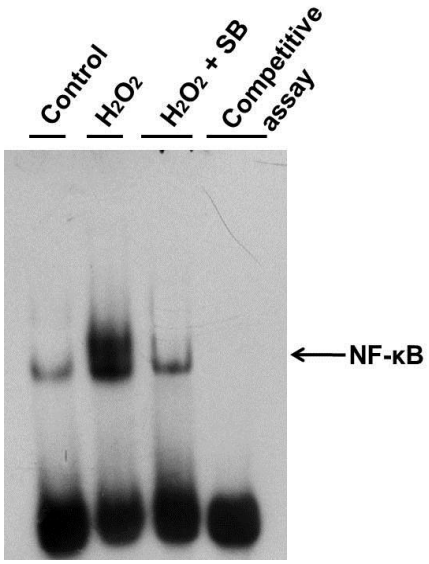


Figure 40: p38 MAPK regulates NF-κB activation in cardiac fibroblasts

Sub-Confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 24 hours followed by H_2O_2 treatment for 3 hours in the presence/absence of SB (10 µM). EMSA showed that the nuclear translocation of NF-κB observed in response to H_2O_2 was significantly reduced upon p38 MAPK inhibition. A representative profile from one of three experiments is shown.

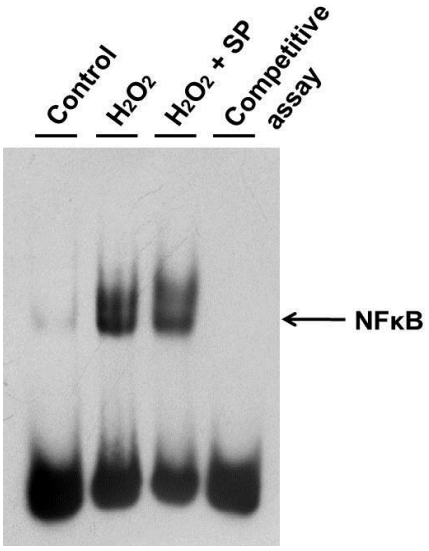


Figure 41: NF-κB activation in cardiac fibroblasts is unaffected by JNK inhibition

Sub-Confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 24 hours followed by H_2O_2 treatment for 3 hours in the presence/ absence of SP (10 µM). EMSA showed that the nuclear translocation of NF-κB observed in response to H_2O_2 was unaffected upon JNK inhibition. A representative profile from one of three experiments is shown.

IV.8.3. Attenuation of H_2O_2-induced AP-1 activity upon ERK1/2 MAPK, p38 MAPK or JNK inhibition

MAPKs are reported to induce AP-1 activity in response to a diverse array of extracellular stimuli (Karin, 1995). In the present study, inhibition of any of the three MAPKs was found to attenuate AP-1 activation.

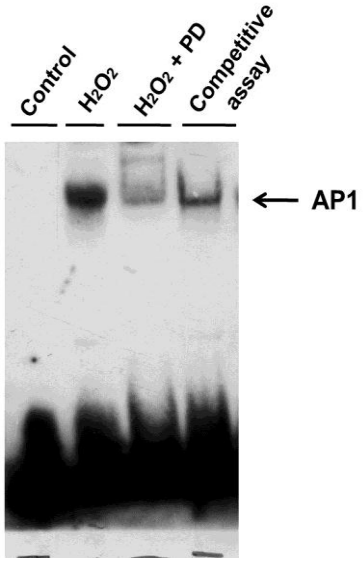


Figure 42: ERK1/2 MAPK regulates AP-1 activation in cardiac fibroblasts

Sub-Confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 24 hours followed by H_2O_2 treatment for 3 hours in the presence/ absence of PD (10 µM). EMSA showed that the nuclear translocation of AP-1 observed in response to H_2O_2 was significantly reduced upon ERK1/2 MAPK inhibition. A representative profile from one of three experiments is shown.

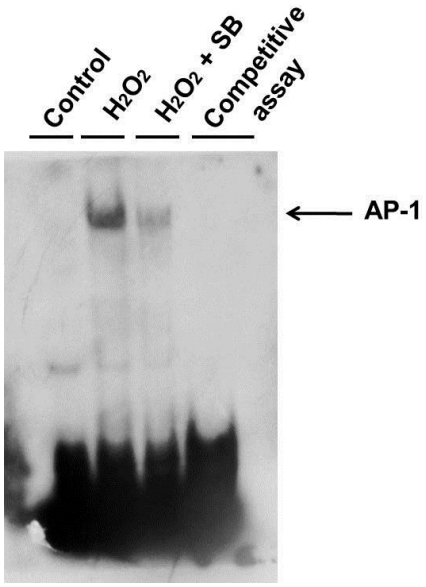


Figure 43: p38 MAPK regulates AP-1 activation in cardiac fibroblasts

Sub-Confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 24 hours followed by H_2O_2 treatment for 3 hours in the presence/ absence of SB (10 µM). EMSA showed that the nuclear translocation of AP-1 observed in response to H_2O_2 was significantly reduced upon p38 MAPK inhibition. A representative profile from one of three experiments is shown.

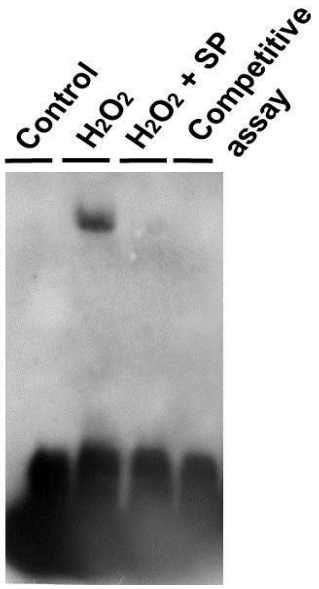


Figure 44: JNK regulates AP-1 activation in cardiac fibroblasts

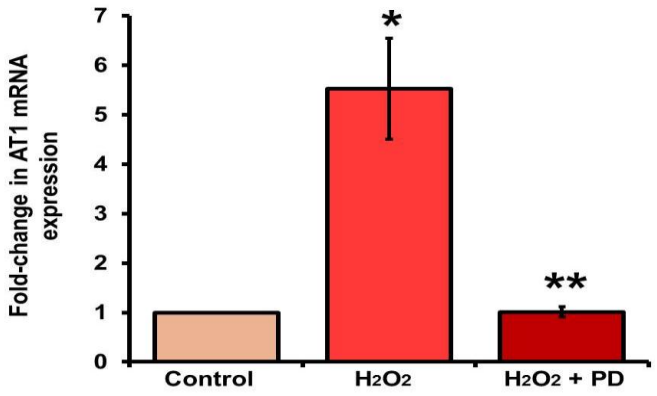
Sub-Confluent cultures of cardiac fibroblasts in M199 were serum-deprived for 24 hours followed by H_2O_2 treatment for 3 hours in the presence/absence of SP (10 µM). EMSA showed that the nuclear translocation of AP-1 observed in response to H_2O_2 was significantly reduced upon JNK inhibition. A representative profile from one of three experiments is shown.

IV.9. *MAPKs mediate H_2O_2-induced increase in AT1 expression*

To determine the role of MAPKs in H_2O_2-induced AT1 up-regulation, expression levels of AT1 were checked in MAPK-inhibited cells exposed to H_2O_2.

IV.9.1. Attenuation of H_2O_2-induced AT1 expression upon ERK1/2 MAPK inhibition

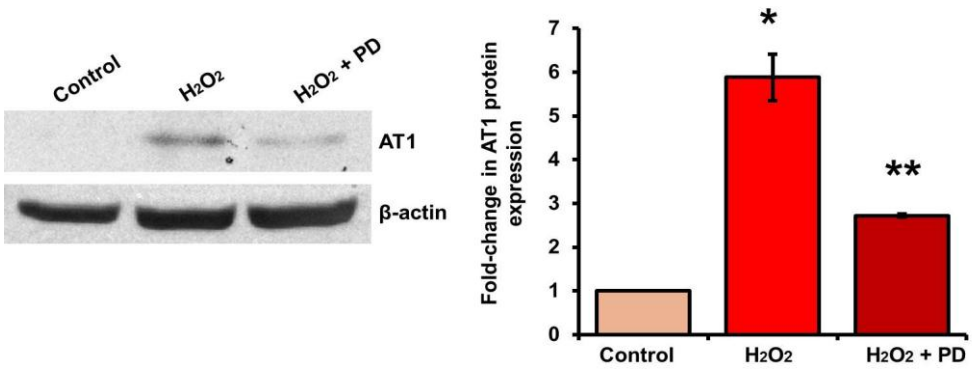
Significant reduction in both AT1 mRNA and protein expression levels was observed in ERK1/2 MAPK-inhibited cardiac fibroblasts exposed to H_2O_2. Since PD98059 inhibits MAPKK, RNA interference-based ERK1/2 MAPK knockdown was performed to exclude the possible involvement of other ERKs acting downstream of MAPKK to enhance AT1. Consistent with the PD effect, ERK1/2 MAPK knockdown attenuated H_2O_2-induced AT1 protein expression.



*p<0.05 vs. Control, ** p<0.001 vs. H_2O_2; n=3

Figure 45: ERK1/2 MAPK inhibition by PD down-regulates H_2O_2-mediated AT1 mRNA levels

Following pre-treatment of cells with PD (10 μM) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA expression was examined, with 18S rRNA as endogenous control.



*p<0.05 vs. Control, ** p<0.001 vs. H_2O_2; n=3

Figure 46: ERK1/2 MAPK inhibition by PD down-regulates H_2O_2-mediated AT1 protein expression

Following pre-treatment of cells with PD (10 μM) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined, with β-actin as endogenous control.

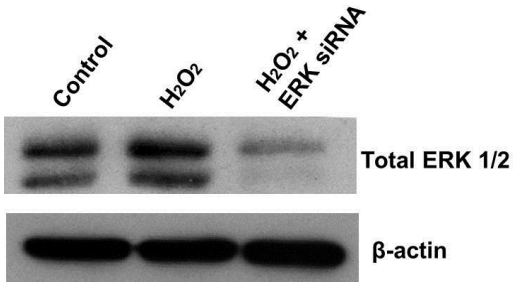
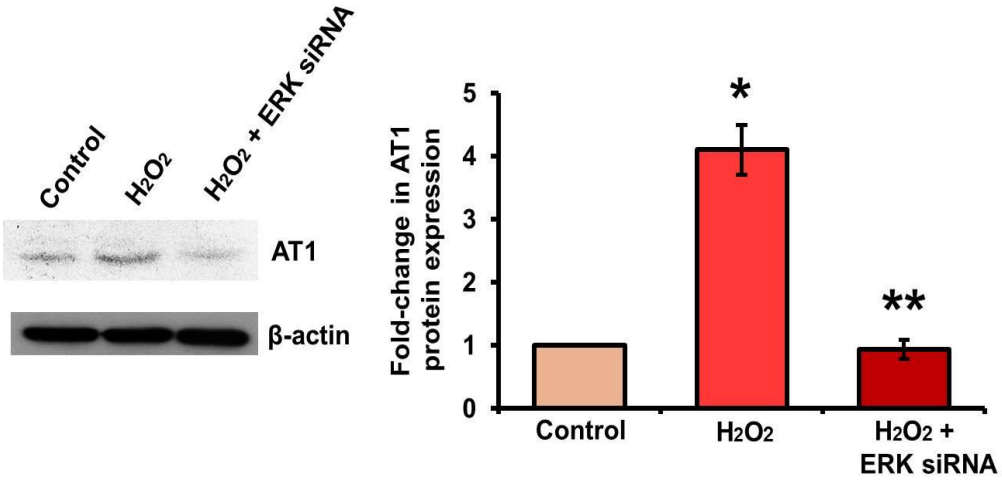


Figure 47: Validation of ERK1/2 MAPK knockdown

Cardiac fibroblasts were transiently transfected with ERK 2 siRNA (5 pmol) or scrambled siRNA (Control groups), as described under 'Methods'. Total ERK1/2 MAPK levels were assessed by western blot analysis using β-actin as loading control.



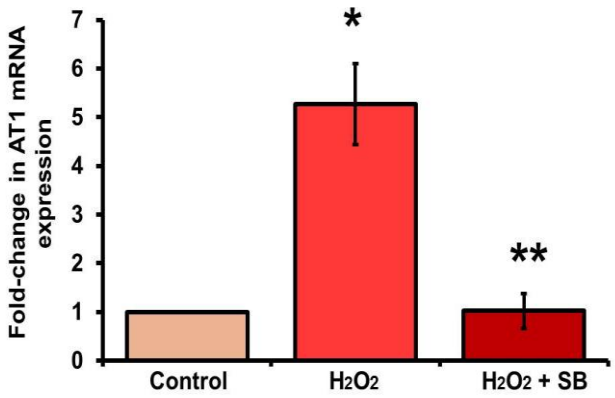
*p<0.01 vs. Control, ** p<0.05 vs. H_2O_2; n=3

Figure 48: siRNA-mediated ERK MAPK inhibition down-regulates H_2O_2-induced AT1 protein expression

Cardiac fibroblasts were transiently transfected with ERK 2 siRNA (5 pmol) or scrambled siRNA (Control groups) prior to treatment with H_2O_2. AT1 protein expression was examined, with β-actin as endogenous control.

IV.9.2. Attenuation of H_2O_2-induced AT1 expression upon p38 MAPK inhibition

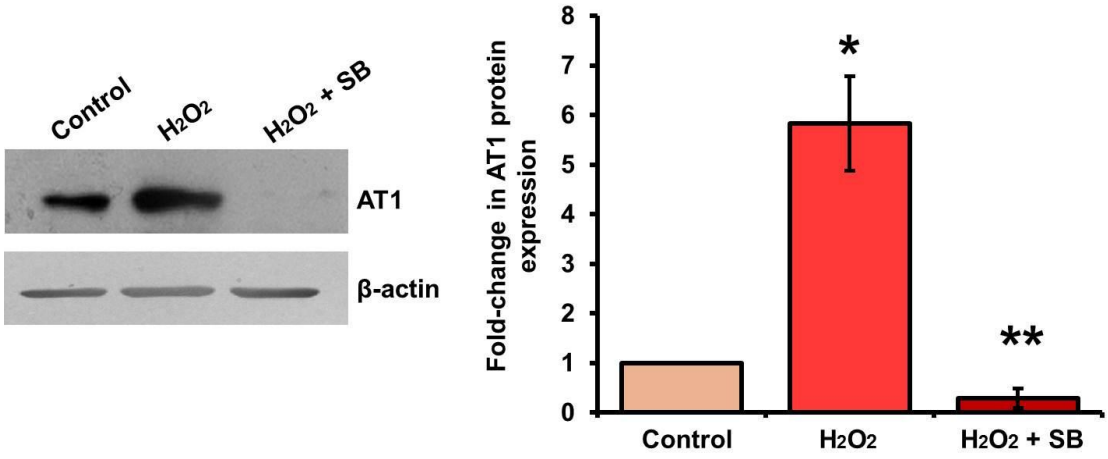
Significant reduction in both AT1 mRNA and protein expression levels was observed in p38 MAPK-inhibited cardiac fibroblasts exposed to H_2O_2. Consistent with the SB effect, p38 MAPK knockdown by RNA interference attenuated H_2O_2-induced AT1 protein expression in cardiac fibroblasts.



*p<0.05 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 49: p38 MAPK inhibition by SB down-regulates H_2O_2-mediated AT1 mRNA levels

Following pre-treatment of cells with SB (10 μM) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA expression was examined, with 18S rRNA as endogenous control.



*p<0.01 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 50: p38 MAPK inhibition by SB down-regulates H_2O_2-induced increase in AT1 protein expression

Following pre-treatment of cells with SB (10 μM) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined, with β-actin as endogenous control.

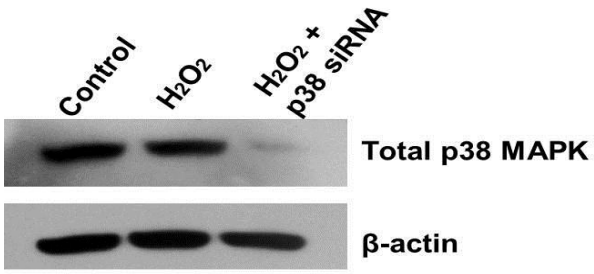
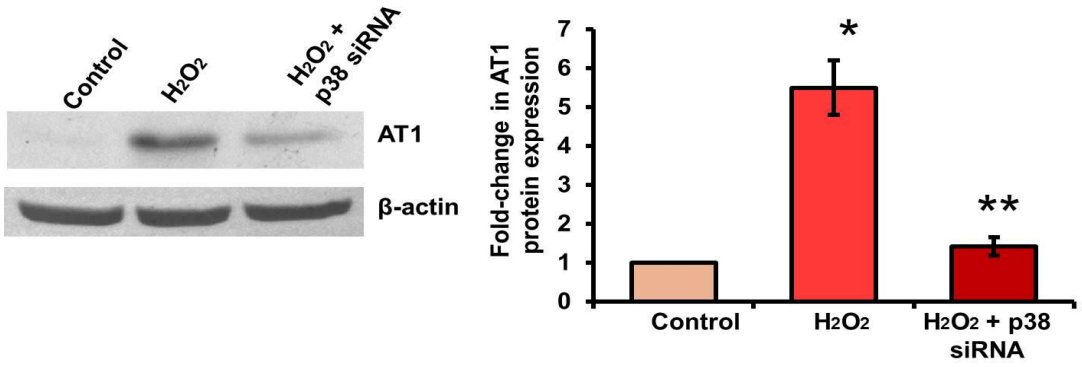


Figure 51: Validation of p38 MAPK knockdown

Cardiac fibroblasts were transiently transfected with p38 MAPK siRNA (5 pmol) or scrambled siRNA (Control groups), as described under 'Methods'. Total p38 MAPK levels were assessed by western blot analysis using β-actin as loading control.



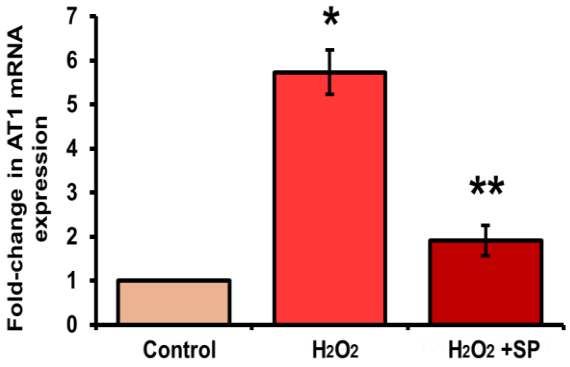
*p<0.01 vs. Control, **p<0.01 vs. H_2O_2; n=3

Figure 52: siRNA-mediated p38 MAPK inhibition prevents H_2O_2-induced AT1 protein expression

Cardiac fibroblasts were transiently transfected with p38 MAPK siRNA (5 pmol) or scrambled siRNA (Control groups) prior to treatment with H_2O_2. AT1 protein expression was examined, with β-actin as endogenous control.

IV.9.3. Attenuation of H_2O_2-induced AT1 expression upon JNK inhibition

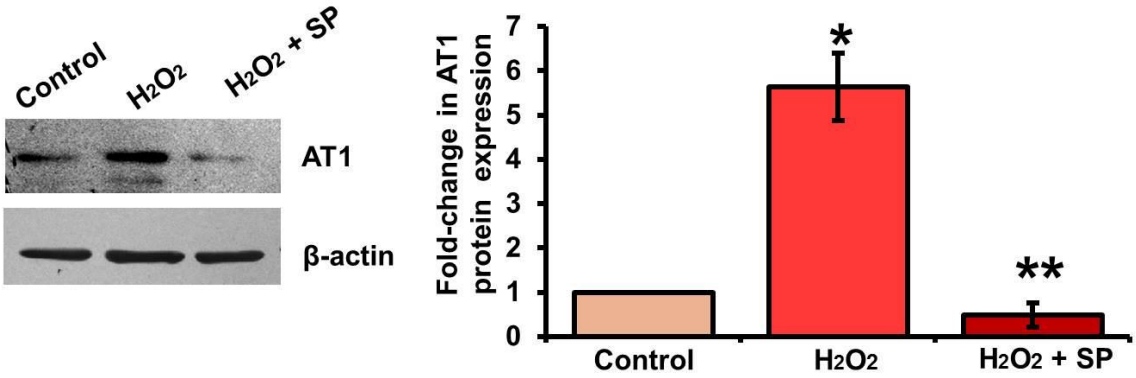
Significant reduction in both AT1 mRNA and protein expression levels was observed in JNK-inhibited cells exposed to H_2O_2. Consistent with the SP effect, siRNA-mediated JNK silencing attenuated H_2O_2-induced AT1 protein expression.



*p<0.05 vs Control. **p<0.01 vs H_2O_2; n=3

Figure 53: JNK inhibition by SP inhibits H_2O_2-induced increase in AT1 mRNA levels

Following pre-treatment of cells with SP (10 μM) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA expression was examined, with 18S rRNA as endogenous control.



*p<0.05 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 54: JNK inhibition by SP abolishes H_2O_2-stimulated AT1 protein expression

Following pre-treatment of cells with SP (10 μM) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined, with β-actin as endogenous control.

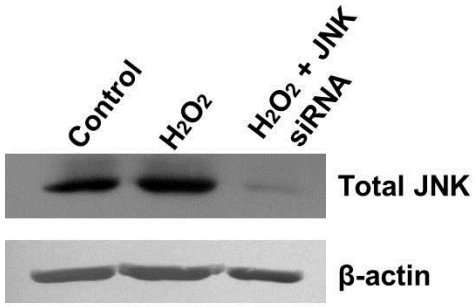
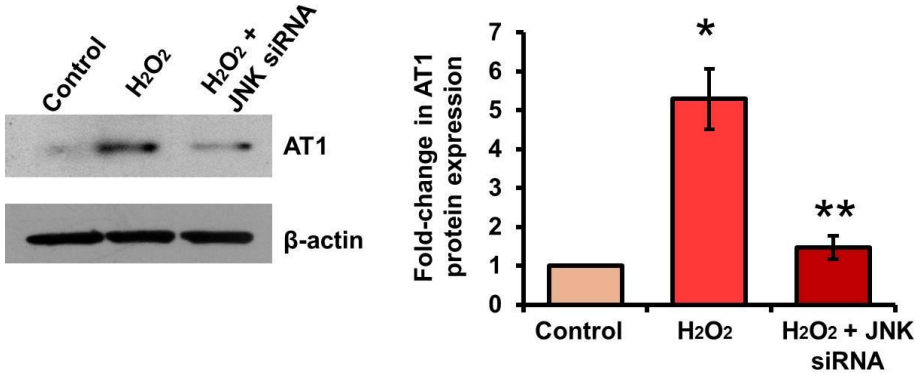


Figure 55: Validation of JNK knockdown

Cardiac fibroblasts were transiently transfected with JNK siRNA (5 pmol) or scrambled siRNA (Control groups), as described under 'Methods'. Total JNK levels were assessed by western blot analysis, using β-actin as loading control.



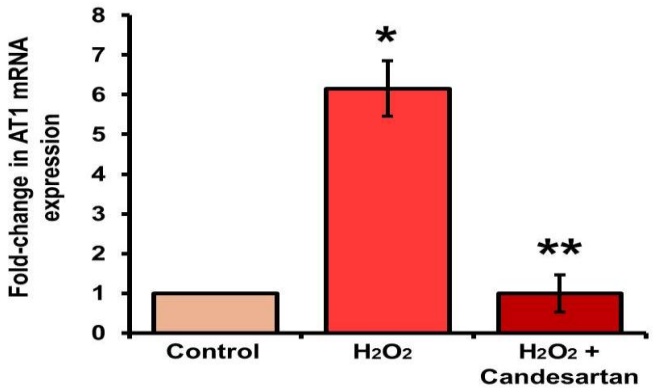
*p<0.01 vs. Control, **p<0.01 vs. H_2O_2; n=3

Figure 56: siRNA-mediated JNK inhibition attenuates H_2O_2-induced increase in AT1 protein expression

Cardiac fibroblasts were transiently transfected with of JNK siRNA (5 pmol) or scrambled siRNA (Control groups) prior to treatment with H_2O_2. AT1 protein expression was examined, with β-actin as endogenous control.

IV.10. *A role for endogenously generated Ang II in H_2O_2-induced increase in AT1 expression*

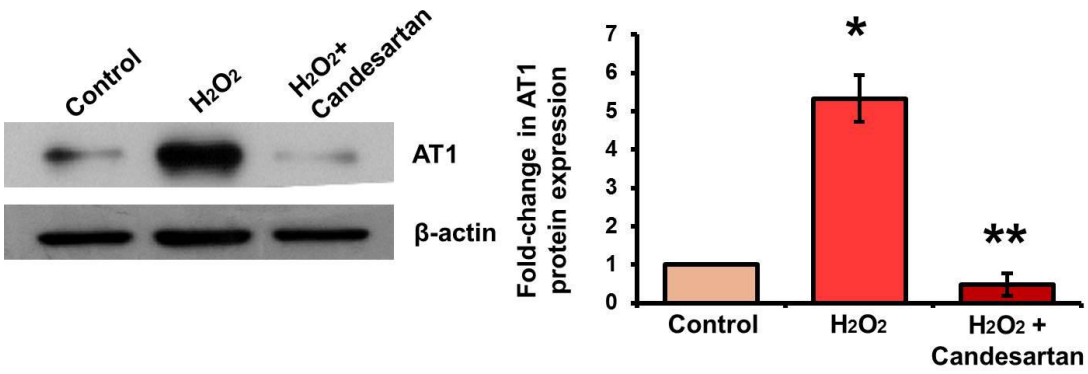
Candesartan, the AT1 receptor antagonist, and enalapril, the ACE inhibitor that prevents Ang I conversion to Ang II, were found to abolish the H_2O_2-induced increase in AT1 mRNA and protein expression, indicating that H_2O_2 triggers local Ang II production that in turn results in the up-regulation of its receptor in these cells.



*p<0.05 vs Control. **p<0.01 vs H_2O_2; n=3

Figure 57: Candesartan abolishes H_2O_2-induced increase in AT1 mRNA expression

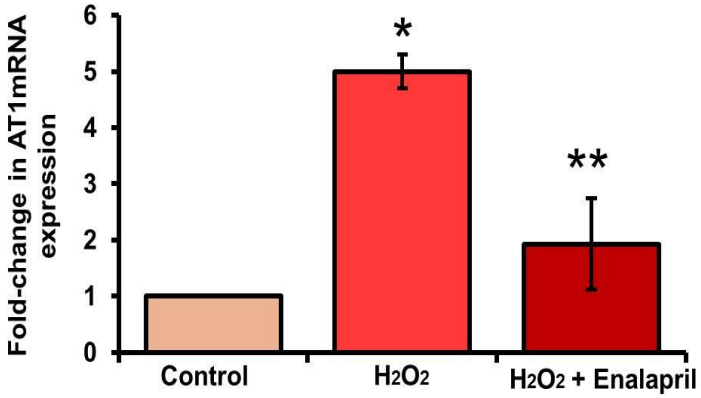
Following pre-treatment of cells with candesartan (100 nm) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA expression was examined, with 18S rRNA as endogenous control.



*p<0.05 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 58: Candesartan abolishes H_2O_2-induced increase in AT1 protein expression

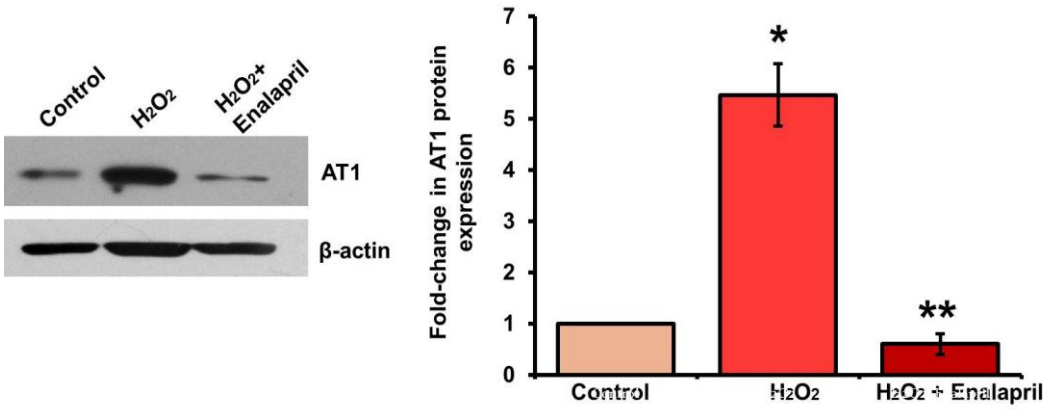
Following pre-treatment of cells with candesartan (100 nm) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined, with β-actin as endogenous control.



*p<0.01 vs Control. ** p< 0.05 vs H_2O_2; n=3

Figure 59: Enalapril abolishes H_2O_2-induced increase in AT1 mRNA expression

Following pre-treatment of cells with enalapril (60 μM) for 1 hour and subsequently with H_2O_2 for 3 hours, AT1 mRNA expression was examined, with 18S rRNA as endogenous control.

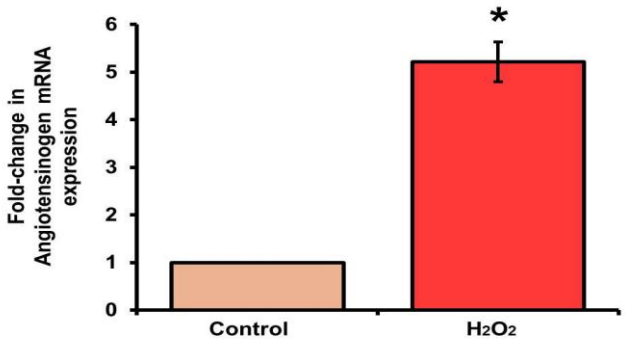


*p<0.05 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 60: Enalapril abolishes H_2O_2-induced increase in AT1 protein expression

Following pre-treatment of cells with enalapril (60 μM) for 1 hour and subsequently with H_2O_2 for 12 hours, AT1 protein expression was examined, with β-actin as endogenous control.

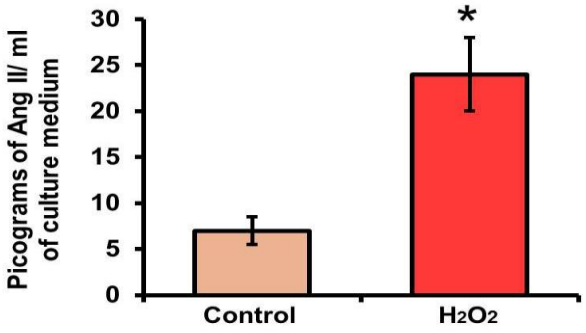
Consistent with these observations, H_2O_2 was found to cause a 5-fold increase in Angiotensinogen mRNA expression and a 3-fold increase in secreted Ang II levels.



*p<0.05 vs Control; n=3

Figure 61: H_2O_2 enhances Angiotensinogen mRNA expression in cardiac fibroblasts

Serum-deprived sub-confluent cultures of cardiac fibroblasts in M199 were treated with H_2O_2 for 3 hours. Angiotensinogen mRNA expression was examined by Taqman quantitative Real-time PCR analysis. 18S rRNA served as endogenous control.

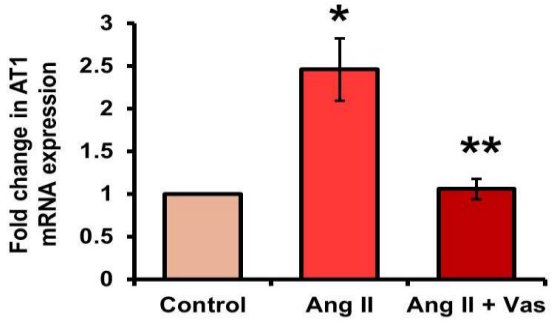


*p<0.05 vs Control; n=3

Figure 62: H_2O_2 enhances Ang II production in cardiac fibroblasts

Enzyme immunoassay revealed significantly elevated levels of secreted Ang II in H_2O_2- treated cells

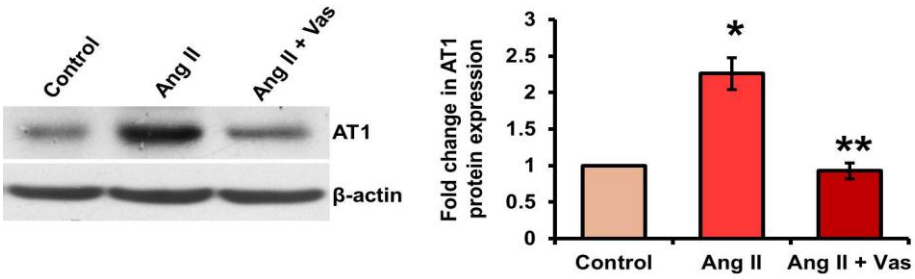
Consistent with a role for endogenously generated Ang II in mediating the H_2O_2 effect on AT1 expression, exogenously added Ang II (100 nM) was found to cause a significant increase in AT1 mRNA and protein expression, which was attenuated upon NOX inhibition.



*p<0.01 vs Control. **p<0.01 vs H_2O_2; n=3

Figure 63: NOX inhibition attenuates Ang II-mediated AT1 mRNA levels

Following pre-treatment of cells with Vas (5 μM) for 1 hour and subsequently with Ang II for 3 hours, AT1 mRNA expression was examined by Taqman quantitative Real-time PCR, with 18S rRNA as endogenous control.



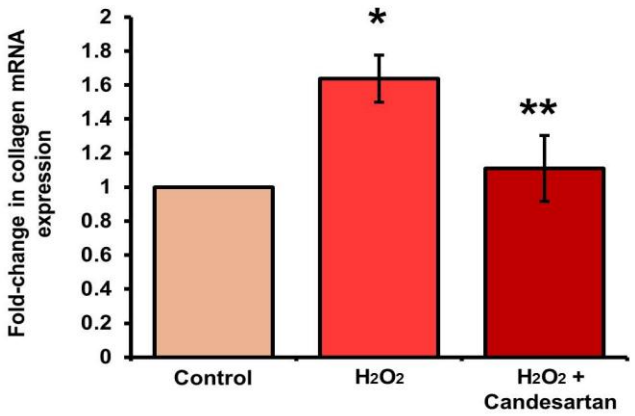
*p<0.001 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 64: NOX inhibition attenuates Ang II-mediated AT1 protein expression

Following pre-treatment of cells with Vas (5 μM) for 1 hour and subsequently with Ang II for 12 hours, AT1 protein expression was examined by western blot analysis, with β-actin as endogenous control.

IV.11. *Functional implication of H_2O_2-induced Ang II*

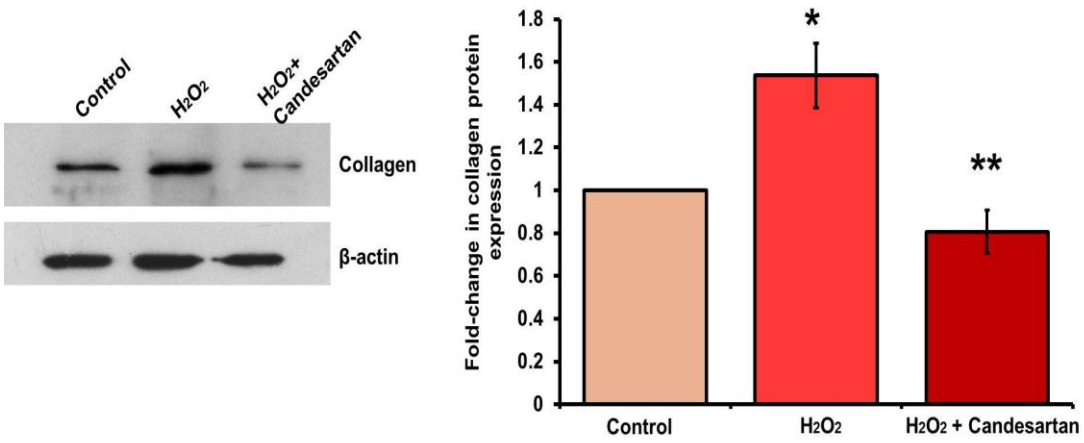
Importantly, H_2O_2 was found to significantly increased collagen mRNA and protein expression in these cells, which was attenuated by candesartan.



*p<0.01 vs Control. **p<0.01 vs H_2O_2; n=3

Figure 65: Candesartan abolishes H_2O_2-induced increase in collagen mRNA levels

Following pre-treatment of cells with candesartan (100 nm) for 1 hour and subsequently with H_2O_2 for 3 hours, collagen mRNA expression was examined, with 18S rRNA as endogenous control.



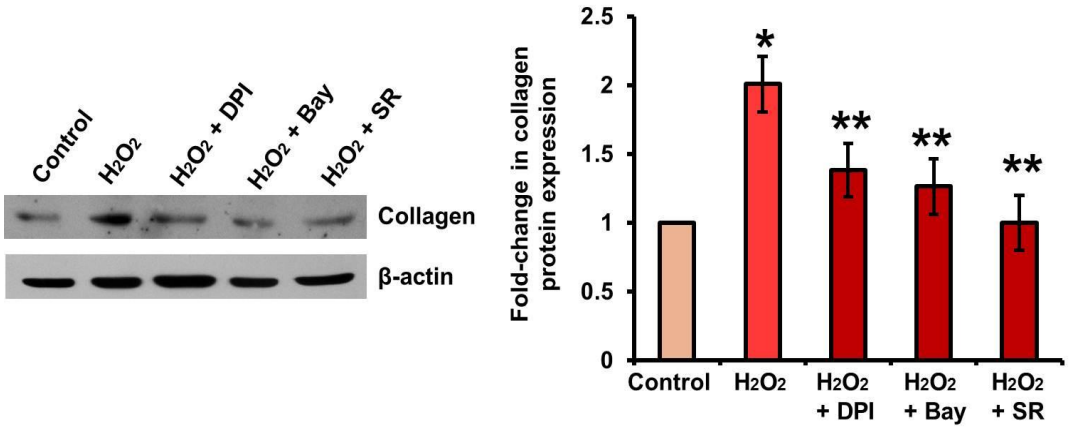
*p<0.05 vs Control. **p<0.001 vs H_2O_2; n=3

Figure 66: Candesartan abolishes H_2O_2-induced increase in collagen protein expression

Following pre-treatment of cells with candesartan (100 nm) for 1 hour and subsequently with H_2O_2 for 12 hours, collagen protein expression was examined, with β-actin as endogenous control.

IV.11.1. Ang II mediates H_2O_2-induced increase in collagen expression via NOX, MAPKs, NF-κB and AP-1

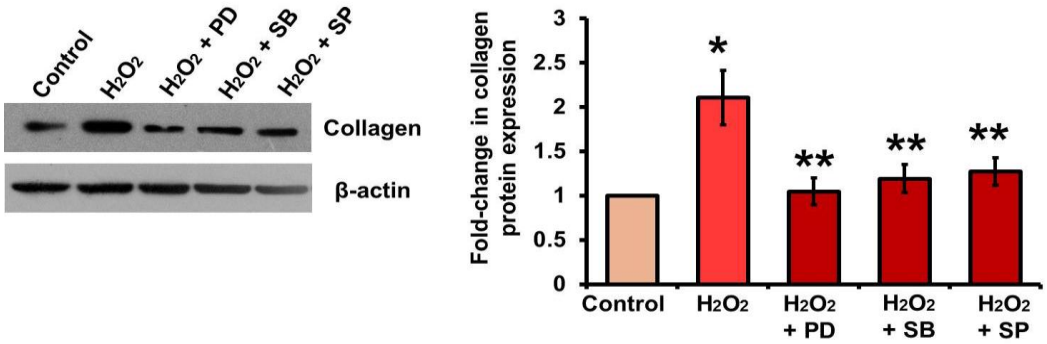
Further, inhibition of NOX, NF-κB, AP-1, ERK1/2 MAPK, p38 MAPK and JNK attenuated H_2O_2-induced collagen protein expression, suggesting that the same pathway that mediates H_2O_2-induced AT1 expression regulates collagen expression as well in response to oxidative stress.



*p<0.05 vs Control. **p<0.05 vs H_2O_2; n=3

Figure 67: Effect of NOX, NFκB and AP-1 inhibition on H_2O_2-induced collagen expression

Following pre-treatment of cells with DPI (10 μM), Bay (2 μM) or SR (1 μM) for 1 hour and subsequently with H_2O_2 for 12 hours, collagen protein expression was examined, with β-actin as endogenous control.



*p<0.05 vs Control. **p<0.05 vs H_2O_2; n=3

Figure 68: Effect of ERK1/2 MAPK, p38 MAPK or JNK inhibition on H_2O_2-induced collagen expression

Following pre-treatment of cells with PD, SB or SP (10 μM each) for 1 hour and subsequently with H_2O_2 for 12 hours, collagen protein expression was examined, with β-actin as endogenous control.

V. DISCUSSION

Cardiovascular diseases have emerged as one of the major causes of mortality globally (Pagidipati and Gaziano, 2013). Any severe myocardial injury triggers a sequence of events that results in phenotypic changes in the constituent cell types of the heart, culminating in myocardial remodelling (Mitchell et al., 2007). These changes mainly include myocyte hypertrophy and cardiac fibroblast hyperplasia. While myocyte hypertrophy can be physiological, resulting from normal growth and exercise, or pathological as in chronic hypertension, valvular disease and ischemia/infarction (Shimizu and Minamino, 2016), cardiac fibroblast hyperplasia is always pathological (Dostal et al., 2015). Importantly, fibroblasts being the principal myocardial cell type responsible for producing ECM components, cardiac fibroblast hyperplasia accelerates the synthesis of ECM proteins, leading to structural remodelling of the interstitium and fibrosis (Eckhouse and Spinale, 2012). Central to the development of fibrosis is the activation of cardiac fibroblasts in the myocardium, which makes them proliferate, migrate and differentiate into the collagen-expressing, hyper-secretory, wound healing myofibroblast phenotype (Dostal et al., 2015; Porter and Turner, 2009; Souders et al., 2009). During infarction and injury, many pro-inflammatory cytokines like Ang II, IL-1, TNF-α, IL-6 and stimulatory humoral factors like Adipokines and ET-1 are reported to be elevated in the myocardium, and cardiac fibroblasts represent the main effector cell target for the actions of these cytokines (Palmer et al., 1995; Regitz-Zagrosek et al., 1998; Wang et al., 2015). Importantly, with its pleotropic effects on cardiac fibroblasts, Ang II is a critical regulator of cardiac fibroblast function (Kawano et al., 2000). Of particular interest in the context of cardiac fibrogenesis is the stimulatory effect of Ang II on collagen synthesis in cardiac fibroblasts and the underlying mechanisms (Chen et al., 2004; Murphy et al., 2015). Ang II exerts its manifold actions on cardiac

fibroblasts via the GPCR pathway downstream of its AT1 receptor (Dinh et al., 2001). A major goal of the present study was to explore the regulation of AT1 receptor gene expression in cardiac fibroblasts exposed to oxidative stress that has been implicated in several disease states such as ischemia/reperfusion injury, hypertension and congestive heart failure (Tsutsui et al., 2011).

V.1. *In vitro oxidative stress model*

H_2O_2-induced oxidative stress, under culture conditions, is a widely employed experimental model since exogenously added H_2O_2 triggers similar signalling pathways as H_2O_2 produced endogenously (Schroder and Eaton, 2008). Further, H_2O_2 is also implicated in cardiac dysfunction attributable to ischemia-reperfusion (Wang et al., 1999). In the present study, cardiac fibroblasts were exposed to a concentration of 25 μM H_2O_2 as it significantly increased intracellular ROS without compromising cell viability.

V.2. *H_2O_2 induces AT1 gene expression*

In the current study, cardiac fibroblasts were subjected to H_2O_2 treatment over different durations and an evaluation of the time-dependence of the oxidative stress effect on AT1 expression demonstrated an up-regulation of AT1 gene expression in H_2O_2-treated cardiac fibroblasts as early as 3 hours for RNA and 6 and 12 hours for protein, which reduced subsequently (Figures 17 and 18). Although the available literature on the effect of ROS on AT1 expression is sparse, the findings presented here are consistent with sporadic reports, which show that AT1 receptor expression is oxidant-sensitive in some cell types (Banday and Lokhandwala, 2008; Bhatt et al., 2014; Keidar et al., 2002).

Over-expression of the AT1 receptor is closely associated with the pathogenesis of many cardiovascular events. Further, there is growing evidence of a direct relationship between increasing AT1 receptor density and agonists like insulin, glucose and LDL that are actively involved in metabolic disorders (Jufang and Hangyuan, 2012; Nickenig et al., 1997, 1998), which underscores the association between AT1 and metabolic disorders. Cytokines like IL-1β and TNF-α act co-ordinately via Ang II to increase AT1 receptor expression on cardiac fibroblasts in post-MI heart to facilitate ECM remodelling and fibrosis (Gurantz et al., 2005).

V.3. *Molecular mechanisms involved in the regulation of oxidative stress-induced AT1 expression*

V.3.1. H_2O_2-activated NF-κB and AP-1 up-regulate AT1 expression

In looking for the regulation of AT1 receptor expression under conditions of oxidative stress, this study focussed on the involvement of two redox-sensitive, stress-activated transcription factors, NF-κB and AP-1. In the cardiovascular system, a role for NF-kB has been suggested in many disease states, including atherosclerosis, myocardial ischemia/reperfusion injury, ischemic pre-conditioning, cardiac hypertrophy and heart failure (Gordon et al., 2011). AP-1 is known to regulate the expression and function of several molecules involved in cell proliferation, neoplastic transformation and apoptosis (Shaulian and Karin, 2001). Further, these two transcription factors are reported to act in concert and regulate many genes involved in myocardial pathophysiology, thereby facilitating maximal target gene expression (Fujioka et al., 2004). The present study demonstrated that H_2O_2 promotes nuclear translocation and activation of NF-κB and AP-1 (Figures 21 and 22), the inhibition of which caused significant down-regulation of AT1 protein

and mRNA levels, pointing to NF-κB and AP-1-dependent transcriptional regulation of AT1 under conditions of oxidative stress.

Many previous studies have sought to establish a link between NF-κB/ AP-1 and the AT1 gene. In the RVLM of Spontaneously Hypertensive Rats, while c-Fos (a subunit of AP-1) was found to be an important contributor to AT1 up-regulation (Chan et al., 2002), in the RVLM of rabbits with congestive heart failure, activation of c-Jun (a subunit of AP-1) was found to mediate up-regulation of AT1 (Zucker et al., 2009). Further, it was reported that pressure overload by aortic coarctation increased the expression of AT1 promoter luciferase reporter gene injected into the apex and left ventricular free wall of the heart and that this response was mediated partly by the functional interaction between AP-1 and GATA-4 transcription factors (Herzig et al., 1997). In another study that probed the effect of a combination of oxidants, L-buthionine sulfoximine (BSO) and H_2O_2, on AT1 mRNA levels in human aortic smooth muscle cells, the oxidant-mediated increase in AT1 transcript levels was attenuated upon NFκB inhibition (Bhatt et al., 2014). While investigating the regulation of AT1 in neuronal cell lines in response to treatment with Ang II, Zucker and colleagues (Haack et al., 2013; Mitra et al., 2010) have reported the involvement of NF-κB via intermediates like CREB and Elk-1 rather than through direct binding to the AT1 promoter. In contrast to these findings, the present study demonstrated NF-κB and AP-1-mediated activation of the AT1 promoter in response to H_2O_2 as shown by dual luciferase assay. Furthermore, this study also demonstrated, through ChIP assay, the direct binding of these two transcription factors to the chromatin segment corresponding to the AT1 promoter *in vivo*, confirming the presence of binding sites for these transcription factors on the AT1

gene promoter. To the best of our knowledge, this is the first demonstration of the direct interaction of NF-κB and AP-1 with the AT1 promoter.

V.3.2. H_2O_2-induced MAPK activation up-regulates NF-κB and AP-1-mediated AT1 expression

The activation of redox-sensitive transcription factors is reported to be driven by several pathways involving the redox-sensitive protein kinases, p38 MAPK, ERK1/2 MAPK, JNK, tyrosine kinase and PI3K/Akt (Haddad, 2003; Surh et al., 2005). In the present study, extracellular administration of H_2O_2 was found to activate p38 MAPK, ERK1/2 MAPK and JNK. Further, attenuation of NF-κB activation under ERK1/2 MAPK or p38 MAPK-inhibited conditions underscored the relationship between ERK1/2 MAPK, p38 MAPK and NF-κB. However, inhibition of JNK did not significantly alter NF-κB activation upon exposure to oxidative stress (Figure 41), suggesting that JNK may not be an upstream regulator of NF-κB under these conditions. ERK1/2 MAPK activates NF-κB via two different mechanisms. pp90rsk, a downstream target of ERK1/2 MAPK-activated signaling pathway, phosphorylates IκBα on Ser-32, triggers its degradation by ubiquitination and activates NF-κB (Ghoda et al., 1997). Secondly, phosphorylation of p65 by ERK1/2 MAPK can also mediate NF-κB activation (Jefferies and O'Neill, 2000). Similarly, p38 MAPK can activate NF-κB in two different ways: firstly, by reducing IκBα levels and triggering NF-κB-DNA binding activity and, secondly, by potentiating the transactivation of the p65 subunit of NF-κB (Baeza-Raja and Muñoz-Cánoves, 2004).

This study also attempted to examine upstream regulators of AP-1 and identified that all the three MAPKs, p38 MAPK, ERK1/2 MAPK and JNK, are important in the activation of AP-1 since inhibition of any one of them prevented the

nuclear translocation of AP-1. The mechanism by which MAPKs modulate AP-1 activity involves differential induction of c-Fos and c-Jun. Activation of p38 MAPK or ERK1/2 MAPK phosphorylates Elk-1, a ternary complex factor (TCF), which results in the induction and increased synthesis of c-Fos. Activated c-Fos translocates to the nucleus where it associates with c-Jun, which is activated by JNK, resulting in the formation of an active and stable AP-1 dimer (Karin, 1995). The present study also demonstrated that expression of AT1 receptor was down-regulated upon ERK1/2 MAPK, p38 MAPK or JNK inhibition, confirming that MAPK-activated, NF-κB- and AP-1-mediated redox signaling is involved in oxidative stress-dependent up-regulation of AT1 expression in cardiac fibroblasts.

It is, however, pertinent to note that the involvement of these MAPKs in the activation of NF-κB and AP-1 is cell type- and stimulus-specific. For instance, while recent studies from our laboratory had reported that ERK1/2 MAPK-mediated NF-κB activation enhances cIAP-2 expression in H_2O_2-treated cardiac fibroblasts (Philip and Shivakumar, 2013), p38 MAPK-mediated NF-κB activation was found to be involved in doxorubicin-induced pro-inflammatory cytokine expression in H9c2 cardiac cells (Guo et al., 2013). Similarly, although p38 MAPK was reported to induce IL-8 gene transcription via AP-1 activation in human VSMCs (Jung et al., 2002), there have been contrasting reports in cardiomyocytes where ERK1/2 MAPK but not p38 MAPK was found to activate AP-1 (Tu et al., 2003). Interestingly, in the regulation of AT1 expression in neuronal cells, JNK is reported to be the sole regulator of AP-1 with no role for ERK1/2 MAPK or p38 MAPK (Liu et al., 2008). Admittedly, the complexity and heterogeneity inherent in MAPK-mediated transcription factor regulation may potentially determine cellular responses to a

plethora of stimuli to achieve specific endpoints, which calls for further investigations on different cell types exposed to diverse stimuli.

V.4. *H_2O_2 triggers local Ang II production to augment AT1 and collagen expression*

Abolition of H_2O_2-induced increase in AT1 mRNA and protein expression in cardiac fibroblasts upon AT1 receptor blockade and ACE inhibition, and the significant increase in Angiotensinogen mRNA expression and secreted Ang II levels in H_2O_2-treated cells, indicated a role for locally-generated Ang II in the up-regulation of AT1 expression in cardiac fibroblasts exposed to H_2O_2. Cardiac fibroblasts are known to express all components of RAS (Shivakumar et al., 2003) and the existence of a functional intracellular RAS stimulated under high glucose conditions has been demonstrated in these cells (Singh, Le, et al., 2008). But, to the best of our knowledge, oxidative stress-dependent increase in Ang II production has not been reported previously. Further, H_2O_2 significantly increased collagen mRNA and protein expression in these cells, which was decreased by candesartan, suggesting a role for Ang II. Importantly, many earlier studies have demonstrated that both ROS and Ang II exert stimulatory effects on collagen expression independently (Chen et al., 2004; Siwik et al., 2001). However, the present study attempts to link these findings and suggest, for the first time, that oxidative stress enhances collagen expression by inducing Ang II. The finding indicates that oxidative stress may act via Ang II to induce a phenotypic shift in cardiac fibroblasts marked by augmented matrix production.

Interestingly, the study also demonstrated that exogenously added H_2O_2 triggers endogenous ROS production by activating NOX, which is consistent with an

earlier report on the existence of an H_2O_2-inducible oxidant generating system in cardiac fibroblasts that is inhibited by DPI and antioxidants (Colston et al., 2005). Since Ang II *per se* is reported to exert several of its effects via NOX-dependent ROS generation, including its stimulatory effect on AT1 (Liu et al., 2008), the finding from this study that ROS triggers Ang II production suggests the existence of a regulatory feedback loop between oxidative stress and Ang II in cardiac fibroblasts. Accordingly, within this loop, ROS and Ang II-AT1 may potentially trigger a cascade of reciprocal regulation that may serve to sustain Ang II signalling via NOX-dependent ROS generation after its initial trigger by exogenous H_2O_2. In turn, such a cascade of reciprocal regulation by ROS and Ang II-AT1 would perturb the myocardial redox milieu and promote AT1 up-regulation in these cells. The observations are significant because oxidative stress and Ang II are both known to be independently involved in the pathogenesis of many cardiovascular diseases (Fanelli and Zatz, 2011; Shah and Channon, 2004) and are reported to co-exist in conditions such as obesity, diabetes and hypertension (Ritz and Haxsen, 2003; Romero and Reckelhoff, 1999; Sowers, 2002). The findings are in line with the possibility that RAS blockade may confer collateral benefits in a setting of oxidative stress.

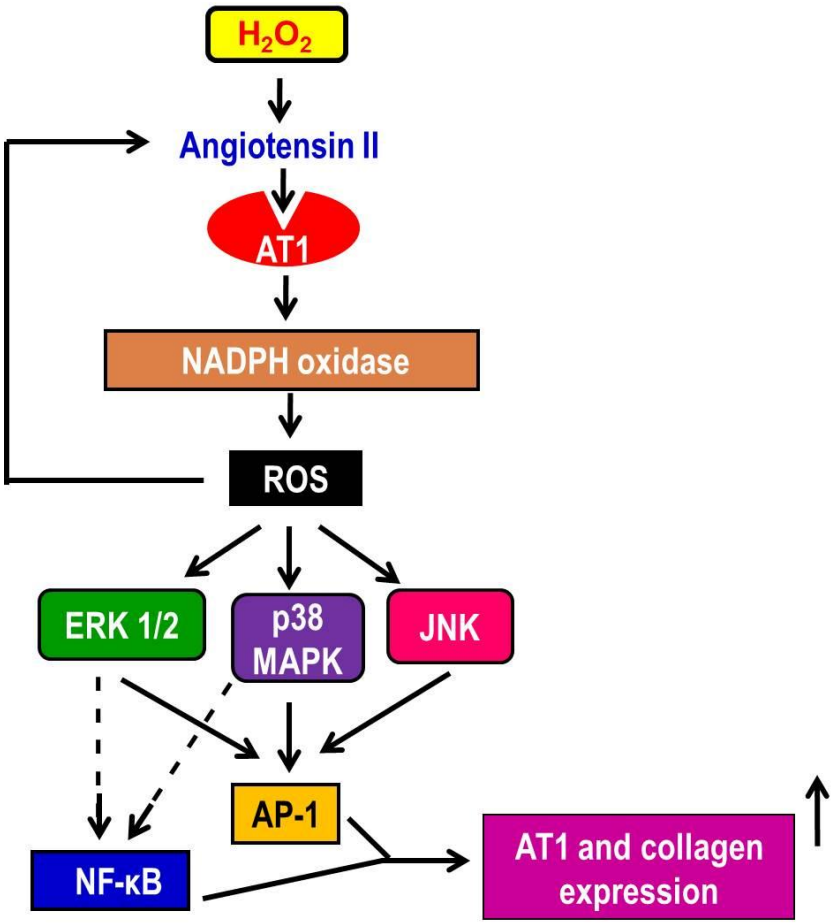
To conclude, the findings presented in this study indicate a complex mechanism of AT1 regulation involving the three MAPKs acting in tandem to activate the two transcription factors and enhance AT1 expression in cardiac fibroblasts. Further, oxidative stress triggers local Ang II production that in turn increases AT1 and collagen gene expression in cardiac fibroblasts.

V.5. *Significance of the study*

By linking oxidative stress to augmented expression of Ang II, the Ang II receptor and collagen in cardiac fibroblasts, this study expands our understanding of the implications of oxidative stress in cardiac pathobiology and provides a new paradigm to appreciate the pathogenesis of cardiovascular conditions associated with oxidative stress.

V.6. *Limitations of the study and future directions*

Although the present study succeeded in establishing the molecular basis of oxidative stress-induced up-regulation of AT1 gene expression in cardiac fibroblasts, the findings are from an *in vitro* cell culture model. Future investigations must probe similar mechanisms in an *in vivo* model of myocardial injury to ascertain the involvement of a redox-sensitive intracardiac RAS in matrix production.



(Anupama et al., 2016)

Figure 69: Proposed mechanism for oxidative stress-mediated increase in AT1 receptor gene expression in cardiac fibroblasts.

The existence of a positive feedback loop between ROS and Ang II may serve to sustain Ang II signalling via NOX-dependent ROS generation to augment AT1 and collagen expression after its initial trigger by exogenous H_2O_2.

VI. SUMMARY

The major findings from this study are:

- H_2O_2 up-regulates AT1 receptor gene expression in cardiac fibroblasts.
- AT1 mRNA and protein levels are down-regulated in NOX-inhibited cells exposed to H_2O_2.
- NF-κB and AP-1 are activated in response to H_2O_2 and their inhibition using pharmacological inhibitors attenuates AT1 gene expression.
- NF-κB and AP-1 directly bind to and activate the AT1 promoter site in response to oxidative stress.
- H_2O_2 induces ERK1/2 MAPK, p38 MAPK and JNK activation in cardiac fibroblasts.
- ERK1/2 MAPK and p38 MAPK inhibition but not JNK inhibition attenuates NF-κB activation in H_2O_2-treated cells.
- Inhibition of ERK1/2, p38 MAPK and JNK attenuates AP-1 activation.
- AT1 is down-regulated in ERK1/2 MAPK p38 MAPK or JNK-inhibited cells exposed to H_2O_2.
- H_2O_2 triggers local RAS in cardiac fibroblasts.
- H_2O_2-induced Ang II production enhances AT1 and collagen expression in cardiac fibroblasts.
- Inhibition of NF-κB, AP-1, NOX, ERK1/2 MAPK, p38 MAPK or JNK attenuates H_2O_2-induced increase in collagen expression in cardiac fibroblasts.

In conclusion, this study uncovers molecular mechanisms that modulate AT1 receptor gene expression in cardiac fibroblasts and, importantly, underscores the ROS - RAS link in these cells.

VII.PUBLICATIONS

[1] **Anupama V**, M. George, S.B. Dhanesh, A. Chandran, J. James, K. Shivakumar, Molecular mechanisms in H_2O_2-induced increase in AT1 receptor gene expression in cardiac fibroblasts: A role for endogenously generated angiotensin II, J Mol Cell Cardiol 97(2016) 295–305.

[2] M. George, **Anupama V**, S.B. Dhanesh, J. James, K. Shivakumar, Molecular basis and functional significance of Angiotensin II-induced increase in Discoidin Domain Receptor 2 gene expression in cardiac fibroblasts, J Mol Cell Cardiol 90 (2016) 59–69.

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IX. ANNEXURES

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Molecular mechanisms in H_2O_2-induced increase in AT1 receptor gene expression in cardiac fibroblasts: A role for endogenously generated Angiotensin II

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ABSTRACT

The AT1 receptor (AT1R) mediates the manifold actions of angiotensin II in the cardiovascular system. This study probed the molecular mechanisms that link altered redox status to AT1R expression in cardiac fibroblasts. Real-time PCR and western blot analysis showed that H_2O_2 enhances AT1R mRNA and protein expression via NADPH oxidase-dependent reactive oxygen species induction. Activation of NF-κB and AP-1, demonstrated by electrophoretic mobility shift assay, abolition of AT1R expression by their inhibitors, Bay-11-7085 and SR11302, respectively, and luciferase and chromatin immunoprecipitation assays confirmed transcriptional control of AT1R by NF-κB and AP-1 in H_2O_2-treated cells. Further, inhibition of ERK1/2, p38 MAPK and c-Jun N-terminal kinase (JNK) using chemical inhibitors or by RNA interference attenuated AT1R expression. Inhibition of the MAPKs showed that while ERK1/2 and p38 MAPK suffice for NF-κB activation, all three kinases are required for AP-1 activation. H_2O_2 also increased collagen type I mRNA and protein expression. Interestingly, the AT1R antagonist, candesartan, attenuated H_2O_2-stimulated AT1R and collagen mRNA and protein expression, suggesting that H_2O_2 up-regulates AT1R and collagen expression via local Angiotensin II generation, which was confirmed by real-time PCR and ELISA. To conclude, oxidative stress enhances AT1R gene expression in cardiac fibroblasts by a complex mechanism involving the redox-sensitive transcription factors NF-κB and AP-1 that are activated by the co-ordinated action of ERK1/2, p38 MAPK and JNK. Importantly, by causally linking oxidative stress to Angiotensin II and AT1R up-regulation in cardiac fibroblasts, this study offers a novel perspective on the pathogenesis of cardiovascular diseases associated with oxidative stress.

1. Introduction

Angiotensin II (Ang II) is an important determinant of cardiac and vascular pathology associated with hypertension, cardiac hypertrophy, coronary heart disease and congestive heart failure. While activation of systemic renin-angiotensin system (RAS) and excessive sympathetic outflow may contribute substantially to these clinical conditions, there also exists an independent functional cardiac RAS whose effects on the myocardium can be much broader and consequential than initially perceived [1]. All components of RAS, including Ang II, are found in the heart where they constitute an autocrine/paracrine cardiac tissue RAS. Locally generated Ang II can directly modulate cardiac and coronary vascular function, inflammation, myocyte apoptosis and myocardial growth and remodelling [2,3].

Since the physiological effects of Ang II are mediated by the AT1 receptor (AT1R), which is a G protein-coupled receptor [4], AT1R abundance determines the biological efficacy of Ang II [5]. AT1R expression is highly variable and many cardiovascular disease states are associated with significant up-regulation of AT1R expression [6,7], which exacerbates Ang II action [8]. The beneficial effects of AT1R antagonists in patients with myocardial infarction and congestive heart failure emphasize its emergence as an important drug target [9] and provide robust rationale for investigations on the molecular basis of AT1R gene regulation in the cardiovascular system. Surprisingly, however, molecular mechanisms involved in AT1R gene regulation in cardiac cells remain inadequately addressed despite the widely appreciated role of intracardiac RAS in myocardial pathophysiology [10,11].

As a major source of extracellular matrix proteins, matrix metalloproteinases and several growth factors and cytokines, cardiac fibroblasts impact multiple aspects of myocardial pathophysiology [12,13]. Further, the regulatory role of Ang II in collagen turnover in cardiac

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fibroblasts is a major determinant of the structural and functional integrity of the myocardium following injury [14]. The pro-fibrogenic effects of Ang II are mediated by AT1R whose expression in cardiac fibroblasts surpasses that in cardiac myocytes [15]. In this context, apart from regulation by Ang II, 'heterologous receptor regulation' of AT1R expression [16] by factors such as estrogen [17], insulin [18] and LDL [19] is reported in different cell types. Sporadic reports suggest that oxidative stress modulates AT1R expression in macrophages, renal cells and vascular smooth muscle cells [20–22]. In the heart, reactive oxygen species (ROS) signalling has been implicated in the activation of a variety of signalling kinases and transcription factors that mediate diverse cellular events such as myocyte growth and apoptosis, cardiac fibroblast proliferation and activation of matrix metalloproteinases that in turn promote tissue remodelling and contribute to the development and progression of heart failure [23–25]. In view of the cardinal role of fibroblasts in myocardial growth, the importance of AT1R as the mediator of Ang II actions on cardiac fibroblasts and the strong association between oxidative stress and several pathological states of the heart [25], delineation of molecular mechanisms underlying regulation of AT1R expression in these cells in a setting of altered redox status is of obvious importance.

Against this backdrop, this study probed the mechanisms involved in the regulation of AT1R gene expression in cardiac fibroblasts exposed to H_2O_2, which has been implicated in cardiac dysfunction attributable to ischemia-reperfusion [26]. The study focused on molecular events and mechanisms that link altered redox status to altered AT1R expression in cardiac fibroblasts. The findings demonstrate that oxidative stress enhances AT1R expression by a mechanism involving two redox-sensitive transcription factors, AP-1 and NF-κB, which are activated by the co-ordinated action of ERK1/2, p38 MAPK and JNK. Importantly, we also provide, for the first time, preliminary evidence of oxidative stress-induced transcriptional up-regulation of Ang II expression that in turn can potentially perpetuate a vicious cycle of ROS-dependent RAS effects in cardiac fibroblasts. Admittedly, generation of such mechanistic insights is a desirable clinical goal as it may eventually direct therapeutic strategies aimed at regulating AT1R expression and, in turn, fibroblast function in the heart post injury.

2. Material and methods

2.1. Materials

NE-PER nuclear and cytoplasmic extraction reagents, Light shift chemiluminescent EMSA kit, Biotin 3′ end DNA labelling kit, Chemiluminescent nucleic acid detection module, SuperSignal West Pico Chemiluminescent Substrate, BCA protein assay kit, DAB substrate, SuperSignal West Femto Substrate Trial kit were from Thermo Scientific. Pure link RNA mini kit was from Ambion. Opti-Mem and fetal bovine serum (US) were from Gibco. Random primers, Taq DNA polymerase, Dual Luciferase assay kit, RNase inhibitor, dNTPs, SB203580, MMLV-reverse transcriptase, *Renilla* luciferase control vector and pGL3 Basic vector were from Promega Corporation. Cell lysis buffer (10×), siRNA for p42 MAPK and primary antibodies for total ERK1/2, and total p38 MAPK were obtained from Cell Signalling Technology. siRNA for p38 MAPK and JNK were from Eurogentec. Lipofectamine 2000, Taqman gene expression master mix and BigDye terminator v3.1 cycle sequencing kit were from Applied Biosystems. GFX PCR DNA and gel band purification kit was from GE health care. SR11302 was from Tocris Bioscience. Taqman gene expression assays like Agtr1A (Assay Id: Rn 02758772_s1), Agt (Assay Id: Rn00593114_m1), Colα1 (Assay Id: Rn01463848_m1), Act b VIC (Assay Id: Rn00667869_m1), 18S rRNA VIC-MGB (Cat#4319413E) and TA Cloning Kit Dual Promoter (pCR II) were from Invitrogen. COLA1 (C-18) antibody, AT1 (N-10) antibody and total-JNK (C-17) antibody were from Santa Cruz Biotechnology. Low cell number ChIP Kit (kch-maglow-A48) was obtained from Diagenode. Hydrogen peroxide was from Merck. DNase amplification kit, Angiotensin II EIA kit, Angiotensin II, primers for PCR, DPI, enalapril maleate salt, candesartan cilexetil, Bay-11-7085, PD98059, VAS2870, fine chemicals for cell culture and analytical experiments were from Sigma-Aldrich. Cell culture ware was from Nunc or BD Falcon.

2.2. Isolation of cardiac fibroblasts

Cardiac fibroblasts were prepared from young adult (2–3 months) male Sprague Dawley rats as described earlier [27]. Handling of animals and experimental procedures were in accordance with the US National Institutes of Health guidelines for animal care and use (No. 85-23, revised 2011) with Institutional Animal Ethics Committee approval (SCT/IAEC-023/June/2012/77). Animals were euthanized (Thiopentone sodium, 5–10 mg/kg, i.p), the heart excised, atria removed and the ventricles were used for isolation of fibroblasts. Sub-confluent (60–70%) cultures from passage 3, serum-starved in M199 for 24–36 h, were used for the experiments. H_2O_2 was used at a concentration of 25 μM in all the experiments.

2.3. Measurement of intracellular ROS

Intracellular ROS production in sub-confluent cultures of cardiac fibroblasts was determined following standard protocols [28]. Cells were incubated for 30 min with 10 μM 2′, 7′-dichloro-dihydro-fluorescein-diacetate (H_2DCF-DA) and then treated with H_2O_2 at the indicated concentrations. H_2DCFDA fluorescence was determined at excitation and emission wavelengths of 485 nm and 530 nm, respectively, and expressed as percentage of control.

2.4. Western blot analysis

Cardiac fibroblasts in M199 were subjected to the indicated treatments and western blot analysis was carried out by standard protocols [29]. Expression levels of target proteins were determined using specific antibodies, with β-actin as loading control. All antibodies were used at 1:1000 dilution, except the AT1R antibody (1:500). ECL or DAB substrate was used to detect the proteins. Protein expression was quantified using Bio-Rad Gel Doc XR + System.

2.5. Real-time polymerase chain reaction (real-time PCR)

Total RNA was isolated from sub-confluent cultures of cardiac fibroblasts using the Pure link RNA mini kit, according to the manufacturer's instructions. Following DNase I treatment, 2 μg of total RNA was reverse transcribed to cDNA with random primers and M-MLV reverse transcriptase. Real-time PCR analysis was carried out, as described previously by us [30], using the Applied Biosystems 7500 real-time PCR system with Taqman primers and specific FAM- or VIC-labelled probes over 40 cycles under the following thermal cycling conditions: 95 °C for 10 min followed by denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min for each of 40 cycles. Gene expression was quantified using C_T values. 18S rRNA or β-actin was the endogenous control. Relative fold change in target mRNA levels of treated versus control = $2^{-\Delta\Delta CT}$.

2.6. RNA interference

Cells were seeded on 12-well plates at 80,000 cells/well. After 24 h, cells were incubated in Opti-MEM containing p42 MAPK (ERK), p38 MAPK or JNK siRNA (5 pmol) and Lipofectamine (2 μl) for 19 h. Following incubation in 10% serum-containing M199 for 15 h, cells were exposed to H_2O_2 for 12 h in M199. Cell lysate was prepared in 2× SDS lysis buffer, denatured and stored at −20 °C until use.

2.7. Electrophoretic mobility shift assay (EMSA)

Cells in M199 were treated with H_2O_2 for the indicated durations, in the presence or absence of inhibitors, and nuclear extracts were prepared using the NE-PER nuclear extraction kit. Single-stranded oligos containing the consensus sequence for the NF-κB binding site 5′-GGGGACTTTCC-3′ and oligos containing the AP-1 binding site 5′-CGCT TGATGACTCAGCCGGAA-3′ were biotinylated and annealed with their complementary strand at 90 °C. Following determination of protein concentration using the BCA kit, 25 μg of the nuclear extract was incubated with the biotinylated probes and components of the Light shift chemiluminescent kit at 37 °C for 60 min and electrophoresed on a 6% non-denaturing gel containing 0.5× TBE, transferred to nylon membrane and immobilized by UV cross-linking at 254 nm for 10 min. The bands were visualized by enhanced chemiluminescence using streptavidin-conjugated HRP. Specificity of binding was confirmed by competition with excess unlabeled NF-κB or AP-1 oligonucleotides (200-fold).

2.8. Dual luciferase assay

Rat genomic DNA was subjected to PCR to amplify the 500 bp AT1R gene promoter regions with forward primer, 5′GGTGGTGCTAGCCCTTCC TTCCATCTTTCCTTTCC-3′ and reverse primer, 5′GGTGGTCCCGGGGTCC AACCCGCTCCCTCTC-3′, and cloned into TA cloning vector, pCRII. AT1R promoter luciferase (pAgtr1A-Luc) plasmid was constructed by directionally cloning *Nhe* I and *Sma* I-digested 500 bp promoter fragment from pCRII-Agtr1A into pGL3 basic vector. The orientation and fidelity of the sequence were confirmed by restriction enzyme digestion and gene sequencing using ABI PRISM 377 DNA Sequencer. 0.8 μg of pGL3-Agtr1A plasmid and 50 ng of *Renilla* luciferase control vector were co-transfected into cardiac fibroblasts in triplicate by lipotransfection. After 45 h of transfection, cardiac fibroblasts were treated with 25 μM of H_2O_2 for 3 h and total cell lysate was prepared and luciferase activity was determined by the Dual Luciferase reporter assay and normalized to *Renilla*.

2.9. Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed with a Low cell number ChIP kit, according to the manufacturer's protocol. Briefly, after treatment of cardiac fibroblasts with 25 μM of H_2O_2 for 3 h, the cells were cross-linked with 1% formaldehyde, lysed and sonicated in a Diagenode Bioruptor to generate 500 bp DNA fragments. The lysates were incubated with anti-p65 NF-κB or c-Fos or c-Jun antibody overnight at 4 °C with rotation. Immune complexes were precipitated with protein A-coated magnetic beads. After digestion with proteinase K to revert the DNA-protein cross-links of the immune complexes, DNA was isolated and subjected to PCR using AT1R primers, 5′ CCTTCCTTCCATCCTTTCCTTTCC-3′ and 5′-GTCCAACC CGCTCCCTCTC-3′, which corresponded to 500 bp of the AT1R promoter region, including the predicted NF-κB and AP-1 binding sites. DNA isolated from an aliquot of the total sheared chromatin was used as loading control for PCR (input control). Chromatin immunoprecipitation using a non-specific antibody (normal human IgG) served as negative control. The PCR products were subjected to electrophoresis on 1% agarose gel.

2.10. Measurement of Ang II levels

Levels of Ang II in the culture medium were measured by quantitative, competitive ELISA, as per the manufacturer's protocol. Briefly, after incubation of the secondary antibody pre-coated 96-well ELISA plate with anti-Angiotensin II antibody, 100 μl of culture medium from control and H_2O_2-treated dishes along with known concentrations of biotinylated Ang II were added. After incubation, streptavidin-horseradish peroxidase solution was applied followed by tetramethylbenzidine substrate solution. Concentrations of Ang II were determined from absorbance at 450 nm.

2.11. Statistical analysis

Data were expressed as mean ± SD and analyzed by one-way ANOVA. Student's *t*-test was used to ascertain differences between experimental groups. $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. H_2O_2 enhances AT1R expression in cardiac fibroblasts

To evaluate the effect of H_2O_2 on AT1R gene expression in cardiac fibroblasts, cells were incubated with H_2O_2 at 25 μM for different durations and AT1R mRNA and protein levels were determined by real-time PCR and western blot analyses, respectively. H_2O_2 produced a 6-fold increase in AT1R mRNA levels at 3 h of treatment, which dropped to 2-fold at the end of 12 h (Fig. 1a). Consistent with the increase in transcript levels, western blot analysis showed a significant increase in AT1R protein expression as well, which was maximum at 6 and 12 h of H_2O_2 treatment (Fig. 1b).

Subsequent experiments examined the effect of pathway-specific inhibitors on AT1R expression in cells exposed to H_2O_2. The inhibitors had no effect on H_2O_2-untreated cells (Supplementary Fig. 1).

3.2. H_2O_2 increases AT1R expression via NADPH oxidase-dependent ROS

H_2O_2 is reported to trigger NADPH oxidase-mediated ROS release in cardiac fibroblasts [31]. In the present study, cardiac fibroblasts were pre-incubated with specific pharmacological inhibitors of NADPH oxidase, DPI (10 μM) or VAS2870 (5 μM), and the effect of H_2O_2 on AT1R mRNA and protein levels was assessed. NADPH oxidase inhibition abolished the effects of H_2O_2 on AT1R mRNA and protein (Fig. 1c & d), indicating that NADPH oxidase-dependent ROS generation augments AT1R expression in H_2O_2-treated cells. Consistent with these observations, a significant increase in intracellular ROS levels was observed in H_2DCFDA-loaded cardiac fibroblasts exposed to H_2O_2, which was inhibited by DPI (Fig. 1e).

3.3. ROS signalling activates NF-κB and AP-1

Since ROS mediates H_2O_2-induced AT1R expression, the involvement of redox-sensitive transcription factors NF-κB and AP-1 in the regulation of AT1R expression was probed next. Activation status of NF-κB and AP-1 was assessed by EMSA using nuclear extracts from cardiac fibroblasts treated with H_2O_2 for 30 min or 3 h. H_2O_2-induced activation and nuclear translocation of NF-κB (Fig. 2a) and AP-1 (Fig. 2b) was observed within 30 min of exposure to H_2O_2, which was inhibited in the presence of their specific pharmacological inhibitors, BAY-11-7085 (2 μM) and the AP-1 trans-repressing retinoid, SR11302 (10 μM). Specificity of binding was confirmed with 200-fold excess of wild unlabelled double stranded NF-κB or AP-1 probe that inhibited the binding of the transcription factor to the biotinylated primer sequence.

3.4. NF-κB inhibition attenuates H_2O_2-mediated AP-1 activation

NF-κB activity is reported to be involved in H_2O_2- and serum-induced AP-1 activation in a human pancreatic tumor cell line [32]. To determine whether NF-κB activation and AP-1 activation are coupled in H_2O_2-treated cardiac fibroblasts, nuclear lysates from NF-κB-inhibited cells exposed to H_2O_2 were subjected to gel shift assay after incubating them with biotin-labelled AP-1 sequence. Inhibition of AP-1 activation in NF-κB-inhibited cells suggested that NF-κB is required for AP-1 activation in response to H_2O_2 (Fig. 2c).

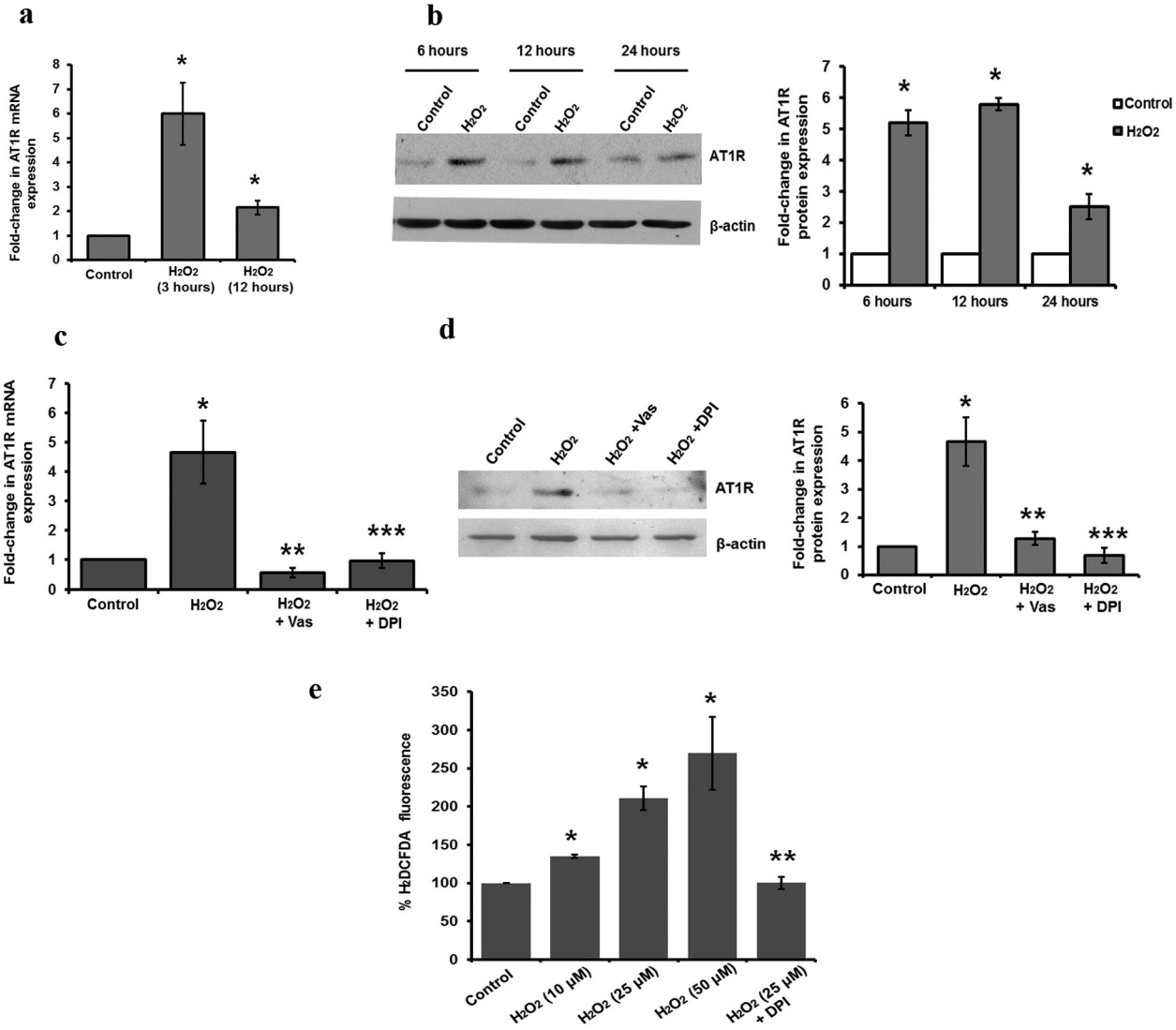


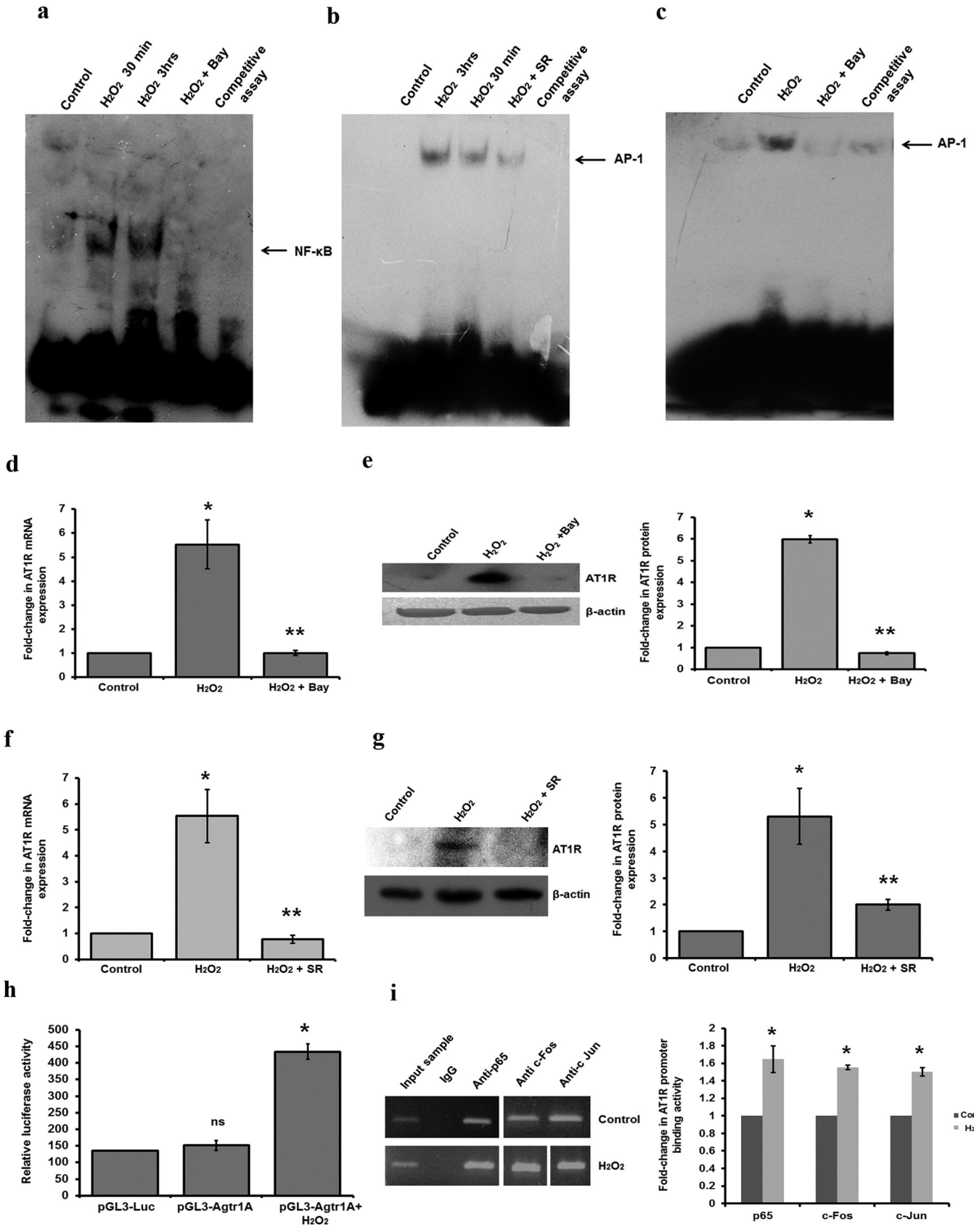
Fig. 1. Oxidative stress enhances AT1R expression in cardiac fibroblasts via NADPH oxidase-dependent ROS. (a) Effect of H_2O_2 on AT1R mRNA expression was examined by Taqman quantitative real-time PCR analysis with 18S rRNA as endogenous control. *$p < 0.03$ vs control. (b) Western blot analysis showing H_2O_2-induced increase in AT1R protein expression. *$p < 0.05$ vs control. (c and d) Inhibition of NADPH oxidase by Vas or DPI attenuated H_2O_2-induced increase in AT1R expression. c, *$p < 0.05$ vs control. **$p < 0.001$ vs H_2O_2. ***$p < 0.01$ vs H_2O_2. d, *$p < 0.05$ vs control. **$p < 0.05$ vs H_2O_2. ***$p < 0.001$ vs H_2O_2. (e) Intracellular ROS in cardiac fibroblasts exposed to H_2O_2 was measured by pre-treatment of cells with 10 μM H_2DCFDA followed by treatment with the indicated concentrations of H_2O_2 for 15 min. *$p < 0.02$ vs control. **$p < 0.02$ vs H_2O_2. H_2O_2 was used at a concentration of 25 μM in all the experiments. Values in all the graphs represent mean ± SD of 3 independent experiments. Representative blots from 1 of 3 independent experiments are shown. (ANOVA $p \leq 0.05$).

3.5. Transcriptional regulation of AT1R by NF-κB and AP-1

Further experiments were performed to evaluate the role of NF-κB and AP-1 in the regulation of the AT1R gene. Pre-incubation with BAY-11-7085 for 60 min prior to the addition of H_2O_2 prevented H_2O_2-induced AT1R mRNA (Fig. 2d) and protein (Fig. 2e) up-regulation, suggesting that NF-κB activation is required for AT1R up-regulation by H_2O_2. The possibility that AP-1 may also be involved in H_2O_2-mediated AT1R expression in cardiac fibroblasts was examined following its inhibition. Pre-incubation of cells for 60 min with SR11302 before H_2O_2 treatment attenuated AT1R mRNA (Fig. 2f) and protein expression (Fig. 2g).

Subsequent experiments probed the AT1R gene promoter-binding activity of NF-κB and AP-1 in H_2O_2-treated cardiac fibroblasts. Cells were transiently co-transfected with 0.8 μg of AT1R promoter-luciferase DNA construct (pGL3-AgTr1A) carrying the NF-κB and AP-1 binding sites and 50 ng of *Renilla* plasmid. Following transfection, the cells were treated with H_2O_2 for 3 h and luciferase activity was assayed

Fig. 2. Transcriptional regulation of AT1R expression by NF-κB and AP-1. (a and b) DNA binding activity of NF-κB and AP-1, determined by EMSA, was observed in response to H_2O_2, which was attenuated upon pre-treatment with Bay and SR, respectively. (c) AP-1 activation was not observed in NF-κB-inhibited cells treated with H_2O_2. (d & e) NF-κB inhibition by Bay attenuated H_2O_2-induced AT1R mRNA and protein expression, determined by Taqman quantitative real-time PCR analysis and western blot analysis, respectively. d, *$p < 0.01$ vs control. **$p < 0.001$ vs H_2O_2. e, *$p < 0.02$ vs control. **$p < 0.001$ vs H_2O_2. (f and g) AP-1 inhibition by SR attenuated H_2O_2-induced increase in AT1R mRNA and protein expression. f, *$p < 0.03$ vs control. **$p < 0.02$ vs H_2O_2. g, *$p < 0.003$ vs control. **$p < 0.006$ vs H_2O_2. (h) Luciferase activity was assayed in control and H_2O_2-treated cells 48 h after transfection with pGL3-Agtr1a in the presence of *Renilla* expressing vector. ns, not significant vs pGL3-Luc. *$p < 0.01$ vs pGL3-Agtr1A. (i) Binding of NF-κB and AP-1 to the AT1R gene promoter was confirmed by ChIP. Relevant treatment groups from within the same gel have been juxtaposed for ease of comparison. A representative image showing the PCR amplification product is given. *$p < 0.05$ vs control. H_2O_2 was used at a concentration of 25 μM in all the experiments. Values in all the graphs represent mean ± SD of 3 independent experiments. Representative profiles from one of three experiments are shown. (ANOVA $p \leq 0.05$).


a
Control
H2O2 30 min
H2O2 3hrs
H2O2 + Bay
Competitive assay
NF-κB
b
Control
H2O2 3hrs
H2O2 30 min
H2O2 + SR
Competitive assay
AP-1
c
Control
H2O2
H2O2 + Bay
Competitive assay
AP-1
d
Fold-change in AT1R mRNA expression
Control
H2O2
H2O2 + Bay
e
Control
H2O2
H2O2 +Bay
AT1R
β-actin
Fold-change in AT1R protein expression
Control
H2O2
H2O2 + Bay
f
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AT1R
β-actin
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Input sample
IgG
Anti-p65
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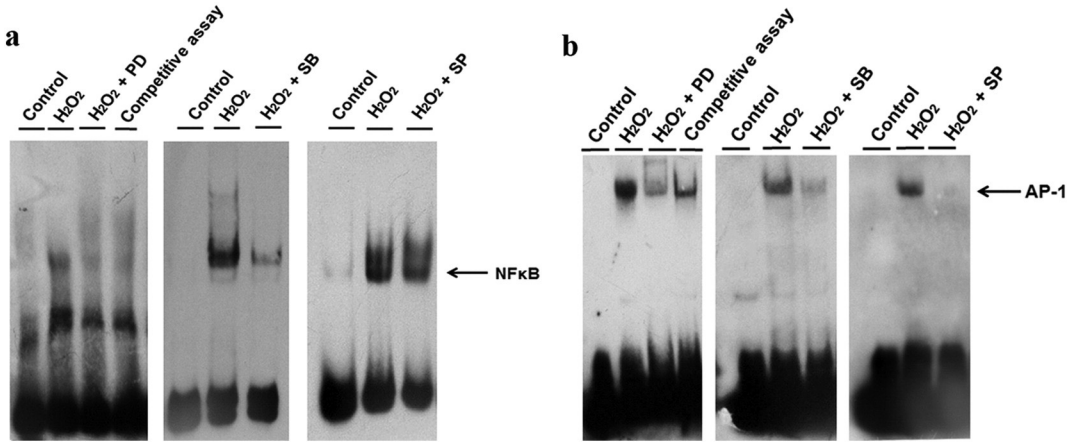

Fig. 3. Regulation of NF-κB and AP-1 activation by MAPKs. Activation of NF-κB and AP-1 in response to H_2O_2 (25 μM) was examined by EMSA. (a) NF-κB activation was significantly reduced upon ERK1/2 and p38 MAPK inhibition using PD and SB, respectively, but was unaffected upon JNK inhibition using SP (b) AP-1 activation was significantly reduced upon inhibition of ERK1/2, p38 MAPK and JNK. Representative profiles from one of three experiments are shown for each of the transcription factors.

using a Dual luciferase kit with *Renilla* as internal control. H_2O_2 induced a significant increase in luciferase activity over the control group, showing H_2O_2-dependent activation of the AT1R promoter (Fig. 2h).

To ascertain the binding of NF-κB and AP-1 to the AT1R promoter in vivo, we performed ChIP assay with the 500 bp DNA fragments, which would report the binding of the NF-κB p65 or c-Fos/c-Jun antibody to the chromatin region corresponding to the AT1R promoter site. Cross-linked chromatin preparations from control and H_2O_2-treated cells were immunoprecipitated individually with anti-NF-κB p65, c-Fos or c-Jun antibody. The anti-NF-κB p65 as well as c-Fos and c-Jun binding site on the immunoprecipitated DNA was determined by PCR using specific primers. Amplification of input chromatin prior to immunoprecipitation served as positive control for chromatin extraction and PCR amplification. Chromatin immunoprecipitation using a non-specific antibody (normal human IgG) served as negative control. Our results showed significantly higher NF-κB p65, c-Fos and c-Jun cross-linking with the AT1R sequence in H_2O_2-treated cells, compared to the control group (Fig. 2i).

3.6. ERK1/2 and p38 MAPK mediate H_2O_2-induced NF-κB activation

To determine whether activation of MAPKs (ERK1/2, p38 MAPK or JNK) is a pre-requisite for NF-κB activation, cells were pre-incubated for 60 min with 10 μM each of ERK1/2 inhibitor (PD98059), p38 MAPK inhibitor (SB203580) or JNK inhibitor (SP600125) and, following treatment with H_2O_2, the nuclear lysates were subjected to EMSA. ERK1/2 and p38 MAPK inhibitors but not the JNK inhibitor (Fig. 3a) exerted significant inhibitory effects on NF-κB activation, suggesting that activation of NF-κB is regulated by ERK1/2 and p38 MAPK but not JNK.

3.7. ERK 1/2, p38 MAPK and JNK mediate H_2O_2-induced AP-1 activation

EMSA showed that inhibition of any of the three MAPKs attenuated AP-1 activation, indicating a role for all three MAPKs in H_2O_2-induced AP-1 activation (Fig. 3b).

3.8. Role of MAPKs in H_2O_2-mediated AT1R up-regulation

Subsequent studies investigated the role of MAPKs in H_2O_2-induced AT1R up-regulation. MAPK inhibitors attenuated H_2O_2-induced increase in AT1R mRNA, suggesting that activation of these three kinases is necessary for the H_2O_2 effect (Fig. 4a–c). Further, chemical inhibitors and siRNA-mediated ERK1/2, p38 MAPK and JNK inhibition attenuated the H_2O_2-induced increase in AT1R protein expression (Fig. 4d–i). siRNA-mediated knockdown of MAPKs is shown in Supplementary Fig. 2a–c.

3.9. H_2O_2 triggers local Ang II production to augment AT1R and collagen expression

Serendipitously, candesartan, the AT1R antagonist, and enalapril, the angiotensin converting enzyme inhibitor that prevents Ang I conversion to Ang II, were found to abolish H_2O_2-induced increase in AT1R mRNA and protein expression in cardiac fibroblasts (Fig. 5a–d), indicating that H_2O_2 triggers local Ang II production that in turn enhances AT1R expression in these cells. Consistent with these observations, H_2O_2 was found to cause a 5-fold increase in Angiotensinogen mRNA expression and a 3-fold increase in secreted Ang II levels (Fig. 5e & f). Further, Ang II per se was found to enhance AT1R mRNA and protein expression, which was abolished by Vas, showing that Ang II enhances AT1R expression in cardiac fibroblasts via NADPH oxidase-dependent ROS (Fig. 5g & h).

Further, H_2O_2 significantly increased collagen mRNA and protein expression in these cells, which was decreased by candesartan (Fig. 6a & b), confirming the involvement of Ang II in the H_2O_2 effect on collagen. Moreover, inhibition of NF-κB, AP-1, NADPH oxidase, ERK1/2, p38 MAPK and JNK attenuated collagen protein expression (Fig. 6ci & cii), indicating that the same pathway mediates H_2O_2-induced increase in AT1R and collagen expression. Together, the data show for the first time the existence of a redox-sensitive renin-angiotensin system in cardiac fibroblasts that is activated under conditions of oxidative stress, leading to enhanced expression of Ang II, AT1R and collagen.

4. Discussion

Cardiac fibroblasts, which constitute about 90% of stroma and two-thirds of the myocardial cell population, are the major effector of wound repair and regenerative healing in the heart [33]. The ability to phenotypically transform into active myofibroblasts upon myocyte loss, the potential to proliferate throughout adult life and persistence in the scar long after the termination of the wound healing process underlie their pivotal role in stromal expansion that causes myocardial stiffness and compromised ventricular compliance [34–36]. Ang II is a critical regulator of cardiac fibroblast function, and its stimulatory effect on collagen and the underlying mechanisms have been of great interest in the context of cardiac fibrogenesis [37,38]. Ang II exerts its manifold actions via the GPCR pathway downstream of AT1R [39]. The present study demonstrated up-regulation of AT1R gene expression in H_2O_2-treated cardiac fibroblasts. A major goal of the present study was to

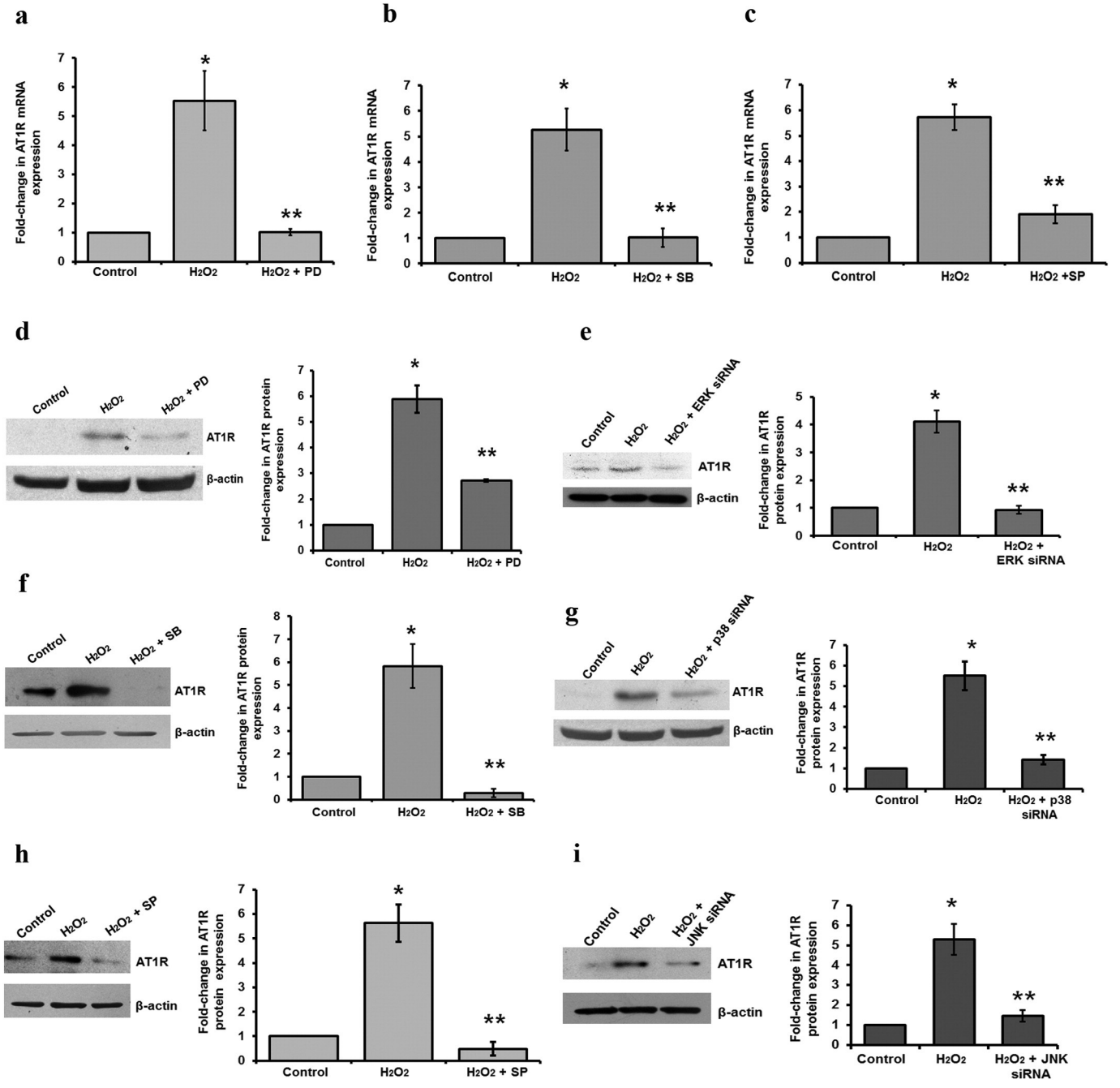

Fig. 4. Regulation of AT1R expression by ERK1/2, p38 MAPK and JNK. Effect of MAPK inhibition by chemical inhibitors and siRNA on AT1R mRNA and protein expression was assessed as described under experimental procedures. (a–c) Inhibition of ERK1/2, p38 MAPK and JNK by PD, SB and SP, respectively, attenuated the H_2O_2-induced increase in AT1R mRNA expression. a, *p < 0.02 vs control, **p < 0.001 vs H_2O_2. b, *p < 0.02 vs control. **p < 0.001 vs H_2O_2. c, *p < 0.03 vs control. **p < 0.01 vs H_2O_2 (d and e) ERK1/2 inhibition by PD or ERK siRNA attenuated the H_2O_2-induced increase in AT1R protein expression. d, *p < 0.03 vs control, **p < 0.001 vs H_2O_2. e, *p < 0.01 vs control, **p < 0.05 vs H_2O_2. (f and g) p38 MAPK inhibition by SB or p38 MAPK siRNA abolished the H_2O_2-dependent increase in AT1R protein expression. f, *p < 0.01 vs control. **p < 0.001 vs H_2O_2. g, *p < 0.01 vs control, **p < 0.01 vs H_2O_2. (h and i) JNK inhibition by SP or JNK siRNA abolished the H_2O_2-dependent increase in AT1R protein expression. h, *p < 0.04 vs control. **p < 0.001 vs H_2O_2. i, *p < 0.01 vs control, **p < 0.01 vs H_2O_2. H_2O_2 was used at a concentration of 25 μM in all the experiments. Values in all the graphs represent mean ± SD of 3 determinations. Representative profiles from one of three experiments are shown. (ANOVA p ≤ 0.05). Validation of siRNA-mediated MAPK inhibition is shown in Supplementary Fig. 2.

define the molecular basis of ROS-dependent increase in AT1R expression in cardiac fibroblasts exposed to oxidative stress that is associated with several disease states of the myocardium [23]. We focused on NF-κB and AP-1 because they are redox-sensitive transcription factors with a significant role in various processes like immune response, inflammation, cell cycle and apoptosis [40]. Importantly, NF-κB is reported to play a central role in many cardiovascular pathologies, including atherosclerosis, myocardial ischemia/reperfusion injury, ischemic preconditioning, cardiac hypertrophy and heart failure [41]. It has also been shown to act in concert with AP-1 to regulate many genes involved in myocardial pathophysiology [32]. However, although AT1R mediates the diverse actions of Ang II in the cardiovascular system, with significant implications in the pathogenesis of several clinical conditions, the possible involvement of these transcription factors in the regulation of AT1R expression in cardiac cells remains largely unclear. In the present study, activation of NF-κB and AP-1, effect of their inhibition on AT1R

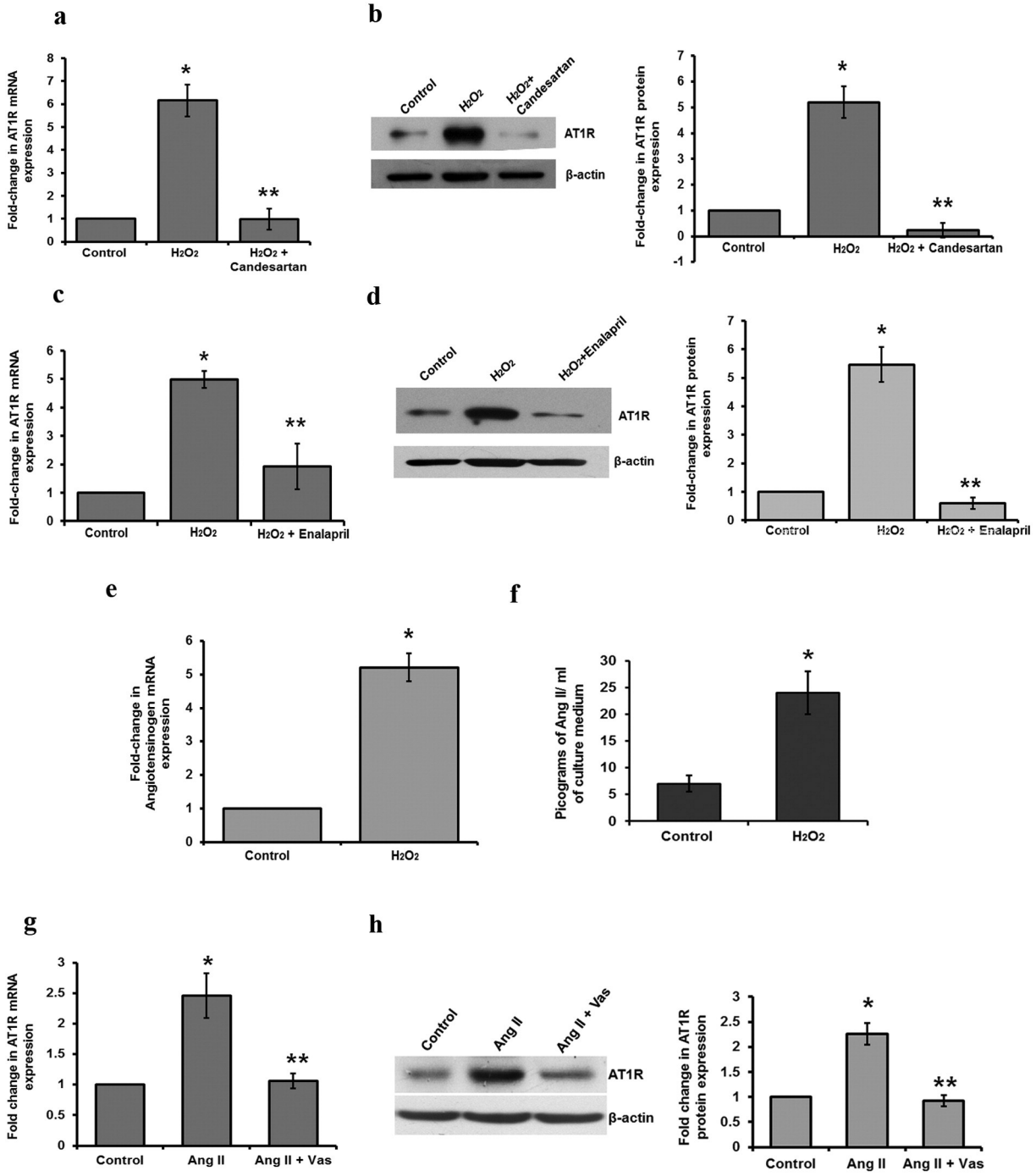

Fig. 5. Oxidative stress induces endogenous Ang II production. (a and b) Pre-treatment with candesartan abolished H_2O_2-induced increase in AT1R expression. a, *p < 0.03 vs control. **p < 0.01 vs H_2O_2. b, *p < 0.05 vs control. **p < 0.001 vs H_2O_2. (c and d) Pre-treatment with enalapril abolished H_2O_2-induced increase in AT1R expression. c, *p < 0.03 vs control. **p < 0.05 vs H_2O_2. d, *p < 0.04 vs control. **p < 0.001 vs H_2O_2. (e) Angiotensinogen mRNA expression was examined in cells treated with H_2O_2 for 3 h. *p < 0.04 vs control. (f) Cardiac fibroblasts were treated with H_2O_2 for 12 h and Ang II in culture medium was measured. *p < 0.05 vs control. (g and h) Ang II (100 nM) increased AT1R mRNA and protein expression in cardiac fibroblasts, which was significantly attenuated upon pre-treatment with Vas (5 μM). g, *p ≤ 0.01 vs control. **p ≤ 0.01 vs H_2O_2. h, *p ≤ 0.001 vs control. **p ≤ 0.001 vs H_2O_2. H_2O_2 was used at a concentration of 25 μM in all the experiments. Values in all the graphs represent mean ± SD of 3 determinations. Representative profiles from one of three experiments are shown. (ANOVA p ≤ 0.05).

mRNA and protein expression and luciferase and chromatin immunoprecipitation assays indicate a role for both of these transcription factors in the regulation of AT1R in cardiac fibroblasts exposed to H_2O_2. It is noteworthy that Zucker and co-workers have investigated AT1R regulation in the cardiovascular regulatory regions of the brain and reported the involvement of NF-κB in AT1R regulation in neurons via intermediates like CREB and ELK-1 rather than through direct binding to the AT1R promoter [42,43]. Further, it was reported that pressure overload by aortic coarctation increased the expression of AT1R promoter luciferase reporter gene injected into the apex and left ventricular free wall of the heart and this response is mediated partly by the functional interaction between AP-1 and GATA-4 transcription factors [44]. To the best of our knowledge, ours is the first demonstration of the direct interaction of NF-κB and the two subunits of AP-1, c-Jun and c-Fos, with the AT1R

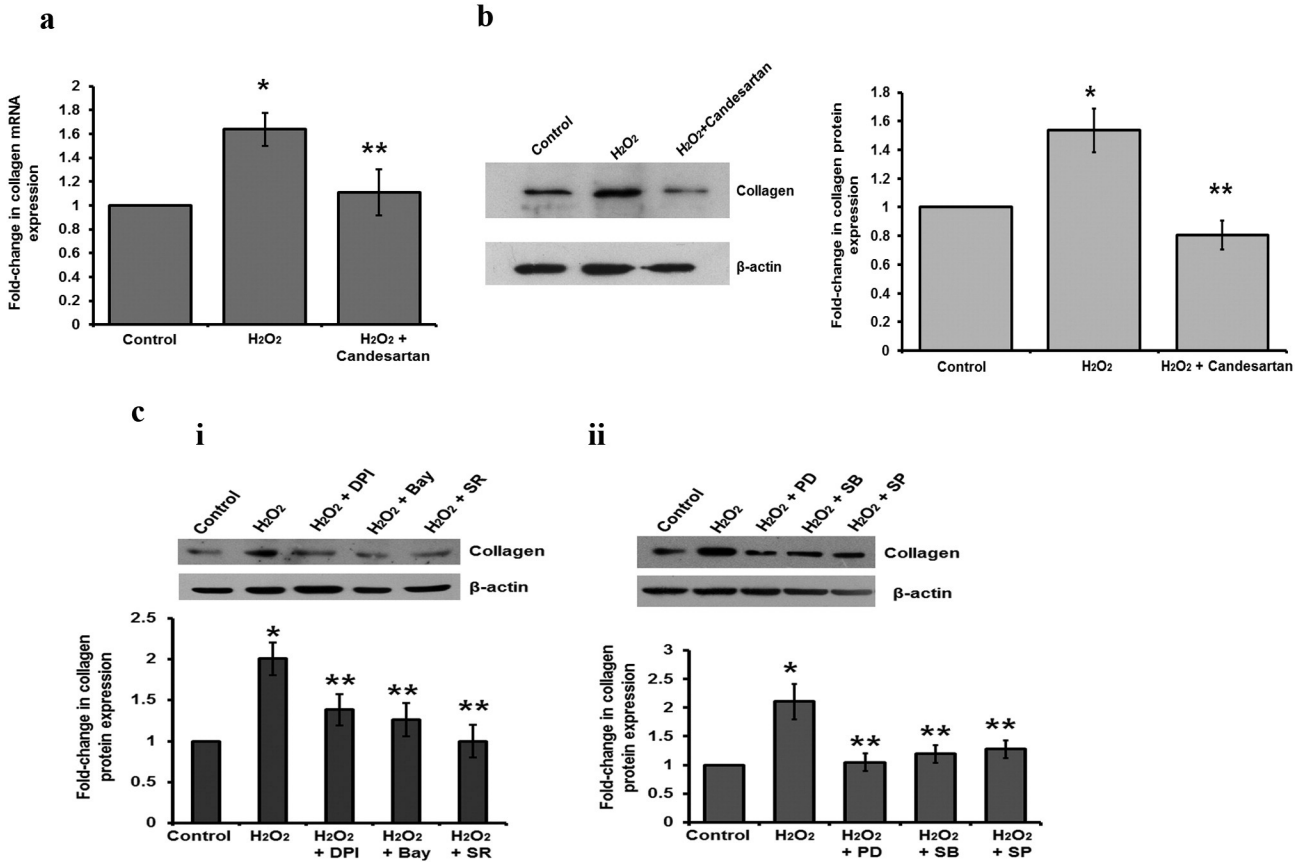

Fig. 6. Ang II mediates oxidative stress-induced collagen production via NADPH oxidase, MAPKs, NF-κB and AP-1. (a and b) Pre-treatment of cells with candesartan reduced H_2O_2-induced increase in collagen expression. a, *p < 0.02 vs control. **p < 0.01 vs H_2O_2. b, *p < 0.04 vs control. **p < 0.001 vs H_2O_2. (c) H_2O_2-induced increase in collagen expression was significantly reduced upon inhibition of: (i) NADPH oxidase, NF-κB and AP-1 by DPI, Bay and SR, respectively; *p < 0.05 vs control. **p < 0.05 vs H_2O_2, and (ii) ERK1/2, p38 MAPK and JNK by PD, SB and SP, respectively. *p < 0.05 vs control. **p < 0.05 vs H_2O_2. (ANOVA p ≤ 0.05).

promoter, which confirms the presence of binding sites for these transcription factors on the AT1R gene promoter. This study also shows for the first time that NF-κB and AP-1 act in tandem to regulate AT1R gene expression in cardiac fibroblasts.

Redox-sensitive protein kinase-driven pathways are known to regulate NF-κB and AP-1 activation. Sano et al. [45] have reported that extracellular administration of H_2O_2 activates p38 MAPK, ERK1/2 and JNK in cardiac fibroblasts. In the present study, p38 MAPK and ERK1/2 were found to be required for NF-κB activation and consequent up-regulation of AT1R. Interestingly, JNK, ERK1/2 and p38 MAPK were necessary for AP-1 activation since inhibition of any one of them abolished H_2O_2-dependent AP-1 activation. It is, however, pertinent to note that the involvement of these MAPKs in the activation of NF-κB and AP-1 is cell type- and stimulus-specific. For instance, while we had reported recently that ERK1/2-mediated NF-κB activation enhances cIAP-2 expression in H_2O_2-treated cardiac fibroblasts [28], doxorubicin-induced pro-inflammatory cytokine expression involved NF-κB activation by p38 MAPK in H9c2 cardiac cells [46]. Further, it has also been shown that while both H_2O_2 and Ang II activate MAPKs, H_2O_2 but not Ang II-mediated MAPK activation results in NF-κB-dependent effects on IL-6 in cardiac fibroblasts [45]. While p38 MAPK is reported to induce IL-8 gene transcription in human VSMCs via AP-1 activation [47], it has also been reported that ERK1/2 but not p38 MAPK is required for AP-1 activation in cardiomyocytes [48]. Interestingly, in the regulation of AT1R in neuronal cells, JNK is reported to be the sole regulator of AP-1 with no role for ERK1/2 or p38 MAPK [49]. Admittedly, the complexity and heterogeneity inherent in MAPK-mediated transcription factor regulation may potentially determine cellular responses to a plethora of stimuli to achieve specific endpoints, which calls for further investigations on different cell types exposed to diverse stimuli.

Our study also demonstrates repression of AP-1 activation upon NF-κB inhibition, revealing an essential role for NF-κB in regulating AP-1. Although more studies are required to ascertain how NF-κB might regulate AP-1, previous studies [32,42], seem to suggest that NF-κB may interact with CBP and activate Elk-1, which in turn may bind to the c-Fos promoter and influence its transcription. c-Fos may then bind with JNK-activated c-Jun, resulting in AP-1 activation. We propose that NF-κB may regulate AT1R gene expression not only by direct transcriptional regulation but also indirectly via AP-1, thus allowing multiple regulatory steps and maximal target gene expression in response to H_2O_2. In contrast to previous reports, the findings presented here indicate a complex mechanism of AT1R regulation involving the three MAPKs acting in tandem to activate the two transcription factors and enhance AT1R expression in cardiac fibroblasts.

Apart from delineating the molecular basis of oxidative stress-induced increase in AT1R gene expression in a cardiac cell, an important offshoot of the study is the serendipitous and novel finding that H_2O_2 triggers Ang II production in cardiac fibroblasts. This study also shows that H_2O_2 triggers NADPH oxidase-dependent ROS production in cardiac fibroblasts. Notably, many previous studies have shown that Ang II induces NADPH oxidase-dependent ROS production [45,50]. Consistent with earlier reports (7, 50), we also observed that exogenously added Ang II exerts stimulatory effect on AT1R expression via NADPH oxidase-dependent ROS production. Together, these findings point to the possible existence of a positive feedback loop wherein ROS and Ang II-AT1R may potentially trigger a cascade of reciprocal regulation

that may serve to sustain Ang II signalling via NADPH oxidase-dependent ROS generation after its initial trigger by exogenous H_2O_2. In turn, such a cascade of reciprocal regulation by ROS and Ang II-AT1R would perturb the myocardial redox milieu and promote AT1R up-regulation in these cells.

Ang II per se is an important factor in cardiovascular responses to external stress [51]. Association of Ang II with AT1R activates a series of signalling cascades, involving NADPH oxidase-dependent ROS [45], MAPK [45] and a variety of transcription factors such as NF-κB [52,53], AP-1 [54] and KLF5 [55] that in turn impact target gene expression. In cardiac fibroblasts exposed to H_2O_2, the likely sequence of events that our findings support involves H_2O_2-mediated Ang II production and Ang II-induced AT1R activation that in turn leads to activation of the three MAPKs and the two transcription factors (NF-κB/AP-1), culminating in enhanced AT1R gene expression (Fig. 7). However, since the signalling cascade triggered by Ang II in this instance is in cells exposed to H_2O_2, further studies are needed to clarify if all of the effects of H_2O_2 are attributable to Ang II with no contribution from H_2O_2 per se. In this regard, it is noteworthy that Sano et al. [45] had reported that Ang II generates H_2O_2 in cardiac fibroblasts but, whereas H_2O_2 promotes NF-κB activation, Ang II does not.

It is well known that oxidative stress and increased RAS activity co-exist in conditions such as obesity, diabetes and hypertension [24] but, to the best of our knowledge, oxidative stress-dependent increase in Ang II production has not been reported previously. The significance of our findings lies in the demonstration that oxidative stress up-regulates not only AT1R but also Ang II, which can act via AT1R to impact multiple aspects of myocardial pathophysiology. In this study, H_2O_2 enhanced collagen expression via Ang II production, which represents a shift to a phenotype characterized by augmented matrix production, indicating that oxidative stress may act via Ang II to impact cardiac fibroblast activity. Our observations are in line with the possibility that RAS inhibition may confer collateral benefits in a setting of oxidative stress.

To conclude, oxidative stress and Ang II are both reported to be independently involved in the pathogenesis of many cardiovascular diseases [56,57]. By extrapolation, it is tempting to propose that oxidative stress in the heart may, on the one hand, compromise myocyte viability and, on the other, contribute to the consequent reparative response through enhanced expression of RAS components and collagen in cardiac fibroblasts. By linking oxidative stress to augmented expression of Ang II, AT1R and collagen in cardiac fibroblasts, this study expands our understanding of the implications of oxidative stress in cardiac pathobiology and provides a new paradigm to understand the pathogenesis of cardiovascular conditions associated with oxidative stress.

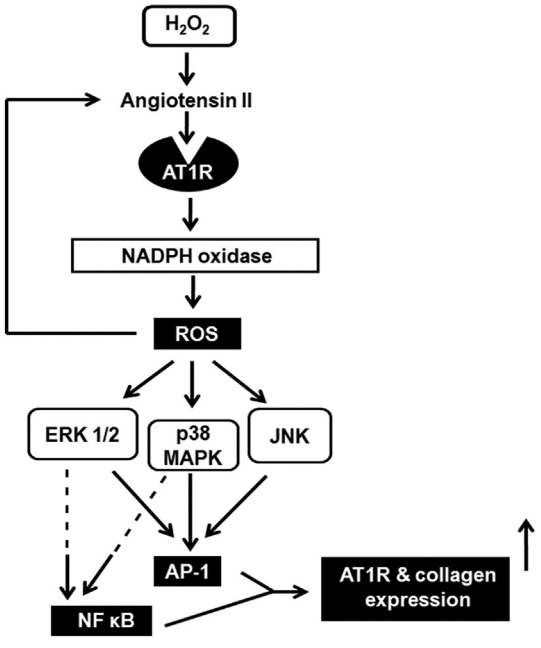

Fig. 7. Molecular mechanisms underlying H_2O_2-induced increase in AT1R gene expression in cardiac fibroblasts. H_2O_2 activates local RAS in cardiac fibroblasts to enhance AT1R gene expression by a complex mechanism involving NF-κB and AP-1 that are activated by the co-ordinated action of ERK1/2, p38 MAPK and JNK. The existence of a positive feedback loop between ROS and Ang II may serve to sustain Ang II signalling via NADPH oxidase-dependent ROS generation after its initial trigger by exogenous H_2O_2.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2016.05.010.

Conflict of interest

The authors declare that they have no conflict of interest.

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Molecular basis and functional significance of Angiotensin II-induced increase in Discoidin Domain Receptor 2 gene expression in cardiac fibroblasts

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ABSTRACT

Delineation of mechanisms underlying the regulation of fibrosis-related genes in the heart is an important clinical goal as cardiac fibrosis is a major cause of myocardial dysfunction. This study probed the regulation of Discoidin Domain Receptor 2 (DDR2) gene expression and the regulatory links between Angiotensin II, DDR2 and collagen in Angiotensin II-stimulated cardiac fibroblasts. Real-time PCR and western blot analyses showed that Angiotensin II enhances DDR2 mRNA and protein expression in rat cardiac fibroblasts via NADPH oxidase-dependent reactive oxygen species induction. NF-κB activation, demonstrated by gel shift assay, abolition of DDR2 expression upon NF-κB inhibition, and luciferase and chromatin immunoprecipitation assays confirmed transcriptional control of DDR2 by NF-κB in Angiotensin II-treated cells. Inhibitors of Phospholipase C and Protein kinase C prevented Angiotensin II-dependent p38 MAPK phosphorylation that in turn blocked NF-κB activation. Angiotensin II also enhanced collagen gene expression. Importantly, the stimulatory effects of Angiotensin II on DDR2 and collagen were inter-dependent as siRNA-mediated silencing of one abolished the other. Angiotensin II promoted ERK1/2 phosphorylation whose inhibition attenuated Angiotensin II-stimulation of collagen but not DDR2. Furthermore, DDR2 knockdown prevented Angiotensin II-induced ERK1/2 phosphorylation, indicating that DDR2-dependent ERK1/2 activation enhances collagen expression in cells exposed to Angiotensin II. DDR2 knockdown was also associated with compromised wound healing response to Angiotensin II. To conclude, Angiotensin II promotes NF-κB activation that up-regulates DDR2 transcription. A reciprocal regulatory relationship between DDR2 and collagen, involving cross-talk between the GPCR and RTK pathways, is central to Angiotensin II-induced increase in collagen expression in cardiac fibroblasts.

1. Introduction

Delineation of molecular mechanisms relevant to tissue repair and fibrosis is a clinically important goal since parenchymal tissue destruction and fibrosis are a major cause of organ dysfunction. In the heart, fibroblast proliferation associated with augmented matrix production is a critical event in wound healing following parenchymal cell loss [1]. However, excessive hyperplasia of fibroblasts, in conjunction with unchecked matrix deposition, results in myocardial fibrosis and compromised ventricular function. Cardiac fibroblast response to injury is a complex process involving many factors and the intricate interplay between them that controls matrix homeostasis. In this regard, a major focus of research over the years in several laboratories has been the regulation of collagen turnover by Angiotensin II (Ang II) in cardiac fibroblasts, which are the only source of type I and type III collagens in the heart [2–3]. These investigations have convincingly demonstrated that Ang II, whose intracardiac generation is reported to be enhanced following myocardial injury [4], is a potent pro-fibrogenic factor in the myocardium with marked stimulatory effects on collagen expression in cardiac fibroblasts [4–5].

In the overall context of matrix biology, there has also been a surge of interest in collagen receptors that mediate fibroblast responses during tissue remodeling [6]. Discoidin domain receptor 2 (DDR2) is a collagen-specific receptor noted for its association with fibrotic diseases besides regulation of a wide array of fundamental cellular processes [7–9], including extracellular matrix remodeling and cell proliferation, differentiation, migration and adhesion [7,10]. It is a unique tyrosine kinase receptor (RTK) expressed predominantly in mesenchymal cells, with a marked regulatory role in epithelial-mesenchymal transition [11–13]. Over-expression of DDR2 is associated with pathological scarring in non-cardiac tissues and fibrotic diseases of the lung, kidney and liver [6,14–15] besides atherosclerosis, osteoarthritis and several tumors [16–19]. Sporadic reports suggest that the relationship between DDR2 and collagen may be of immense relevance in a setting of tissue injury [20–21]. In fact, collagen-DDR2 interaction as a key determinant

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of matrix remodeling *post injury* is an emerging paradigm. In the heart, DDR2 is expressed predominantly in fibroblasts that are of mesenchymal origin [22] and is likely to play a key role in tissue repair and fibrogenesis. Surprisingly, however, regulation of DDR2 gene expression and its possible involvement in collagen production have not been investigated yet in relation to the heart.

Against this backdrop, the objective of this study was to examine the regulation of DDR2 expression in cardiac fibroblasts by Ang II and probe its functional link with collagen. We present evidence, for the first time, that Ang II acts via protein kinase C to trigger p38 mitogen-activated protein kinase (p38 MAPK)-mediated activation of Nuclear Factor-κB (NF-κB) that in turn associates with the DDR2 promoter to enhance DDR2 transcription in cardiac fibroblasts. Importantly, using an siRNA-based gene silencing approach, we have demonstrated that DDR2 and collagen type 1 are locked in a cycle of mutual regulation in Ang II-stimulated cardiac fibroblasts, which can potentially impact tissue response to injury.

2. Materials and methods

2.1. Materials

Angiotensin II, Tri reagent, Bay 11–7085, PD 98059, Chelerythrine, U73122, VAS2870, Diphenyleneiodonium chloride, NAC and M199 were obtained from Sigma-Aldrich, USA. Dual Luciferase assay kit, Random primers, Reverse transcriptase, RNAase inhibitor, dNTPs and SB203580 were obtained from Promega. Low cell# ChIP kit protein A × 48 was from Diagenode. NE-PER Nuclear and Cytoplasmic Extraction Reagents and Chemiluminescent nucleic acid detection module were from Thermoscientific. DDR2 siRNA and Col1a1 siRNA were from Ambion. Lipofectamine was from Invitrogen. Opti-MEM and fetal bovine serum were from GIBCO. All cell culture ware was purchased from BD Falcon, USA. Primary antibodies against DDR2, p38 MAPK and ERK1/2 MAPK were obtained from Cell Signaling Technology, USA. Primary antibody for Collagen type I was from Santa Cruz Biotechnology, USA, and that for p65 NF-κB was from Abcam. All antibodies were used after dilution (1:1000), except anti-p65 NF-κB (1:30). This study was approved by the Institutional Animal Ethics Committee (SCT/IAEC122/AUGUST/2014/85).

2.2. Isolation of fibroblasts

Cardiac fibroblasts were isolated from young adult male Sprague–Dawley rats (2–3 months) as described earlier [23]. Sub-confluent cultures of cardiac fibroblasts from passage 2 or 3 were used for the experiments.

2.3. Real-time polymerase chain reaction analysis

Sub-confluent cultures of cardiac fibroblasts were subjected to the indicated treatments and total RNA was isolated using TRI Reagent, according to the manufacturer's instructions. Following DNase I treatment, 2 μg of total RNA was reverse transcribed to cDNA with random primers and M-MLV reverse transcriptase. TaqMan quantitative Real-time polymerase chain reaction (RT-PCR) analysis was carried out using the ABI prism 7500 Sequence Detection System (Applied Biosystems, CA) with specific FAM-labeled probes. PCR reactions were performed over 40 cycles, as per the manufacturer's instructions. DDR2 expression was normalized to β-actin.

2.4. Western blot analysis

Sub-confluent cultures of cardiac fibroblasts in serum-free M199 were treated with Ang II and relative DDR2 protein abundance was determined by western blot analysis following standard protocols, using β-actin as loading control. Enhanced chemiluminescence reagent was used to detect the proteins, and protein expression was quantified by densitometric scanning (Bio-Rad Laboratories).

2.5. Electrophoretic mobility shift assay

DNA-binding activity of NF-κB was assessed by electrophoretic mobility shift assay (EMSA) using the LightShift Chemiluminescent EMSA Kit. Sub-confluent cardiac fibroblast cultures were serum-starved for 24 h followed by incubation in M199 with or without Ang II, and nuclear extracts were prepared using the NE-PER nuclear extraction kit. Protein concentration of the nuclear extracts was determined using the BCA protein assay method. Primers for NF-κB were biotinylated using the Thermo Scientific 3′-end biotin labeling kit. The nuclear extracts were incubated with the biotinylated probes and components of the Light Shift Chemiluminescent kit at 37 °C for 60 min and electrophoresed on 6% non-denaturing gel. After transfer to nylon membrane, DNA was UV cross-linked at a wavelength of 254 nm for 10 min. After blocking, streptavidin-conjugated HRP was applied and bands were visualized by enhanced chemiluminescence.

2.6. Luciferase assay

Rat genomic DNA was subjected to PCR to amplify the DDR2 gene promoter region with forward primer, 5'GGTGGTAAGCTTTCTAGTCG AGGACCCAAACAG 3′, and reverse primer, 5'GGTGGTACGCGTTGCAGG CCACCAATAATGC 3′, and subsequently cloned into TA cloning vector, pCRII (Invitrogen). DDR2 promoter luciferase (pDDR2-Luc) plasmid was constructed by directionally cloning HindIII and MluI-digested 1.5 Kb promoter fragment from pCRII-DDR2 into pGL3 basic vector (Promega). The orientation and fidelity of the sequence was confirmed using restriction enzyme digestion and gene sequencing. Cardiac fibroblasts were plated on a 24-well plate and co-transfected with the constructed luciferase plasmid, pGL3-DDR2, in the presence of Renilla control vector (Promega, Madison, WI, USA). Total cell lysate was prepared 48 h after transfection and Luciferase activity was determined using the Luciferase reporter assay system (Promega) and normalized to Renilla.

2.7. Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using the low cell ChIP kit (Diagenode), according to the manufacturer's protocol. After treatment of cardiac fibroblasts with Ang II, immunoprecipitation was carried out overnight at 4 °C using 10 μl of anti-p65 NF-κB antibody (Abcam). Immune complexes were pulled down with Protein A-coated magnetic beads. DNA cross-links of the immune complexes were reverted by proteinase K digestion followed by heating. DNA isolated from an aliquot of the total sheared chromatin was used as loading control for PCR (input control). The retrieved DNA was analyzed by PCR amplification using the following DDR2 promoter-specific primers containing binding site for NF-κB: 5′-AGCGAATCAACATGGCAGATAC-3′ (Forward), 5′-ACTCCTAACTCCTCACATATAAGC 3′ (Reverse). The PCR products were subjected to electrophoresis on 1% agarose gel. Chromatin immunoprecipitation using a non-specific antibody (normal human IgG) served as negative control.

2.8. RNA interference

RNA interference protocol was as reported by us earlier [24]. Briefly, cells were seeded on 12-well plates at 8×10^4 cells/well. After 24 h, the cells were incubated in Opti-MEM with Ambion pre-designed Silencer-Select siRNA [5 pmol DDR2, 5 pmol collagen α1 type1 or scrambled siRNA, control] and Lipofectamine (2 μl) for 19 h. Following an additional incubation in M199 with 10% FBS for 12 h, the cells were treated with Ang II for the indicated duration. Cell lysate was prepared in SDS lysis buffer, denatured and used for western blot analysis.

2.9. Scratch wound assay

Cells were seeded on 35 mm culture dishes and grown to 90%–100% confluence. DDR2 knockdown by RNA interference was performed as described under Section 2.8. Following serum deprivation, a single scratch gap was created using a 200 μl pipet tip. After wash with 10% phosphate-buffered saline, the cells were treated with Ang II in serum-free M199 for the indicated durations. About 3–4 fields were examined per dish and images of the wound closure pattern in the Ang II-treated groups with and without DDR2 and the control group were acquired using a Nikon inverted phase contrast microscope.

2.10. Statistical analysis

Data were expressed as Mean ± S.D. Statistical analysis was performed using Student's *t* test. $p \leq 0.05$ was considered significant. Data were also analyzed by one-way ANOVA and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Ang II stimulates DDR2 expression in cardiac fibroblasts

The effect of different concentrations of Ang II on DDR2 protein and mRNA expression in cardiac fibroblasts was determined by western blot and real time PCR analyses, respectively. A concentration of 1 μM was found to have the most marked effect on DDR2 expression (Fig. 1A) and was used in all experiments. A significant increase in DDR2 protein expression was observed at 6 and 12 h but the Ang II effect was not sustained at 24 h (Fig. 1B). mRNA levels were elevated significantly at 6 and 12 h of Ang II treatment but significantly below control levels upon prolonged exposure (Fig. 1B). Since Ang II exerts some of its effects through NADPH oxidase (NOX)-driven ROS generation [25], we checked the effect of NOX inhibition on Ang II-induced DDR2 expression. NOX inhibitors, DPI and VAS2870 (Fig. 1C), were found to abolish the Ang II effects on DDR2 expression, indicating a role for NOX-dependent ROS in Ang II-induced stimulation of DDR2 mRNA expression. N-acetyl cysteine (NAC) also reduced Ang II-induced increase in

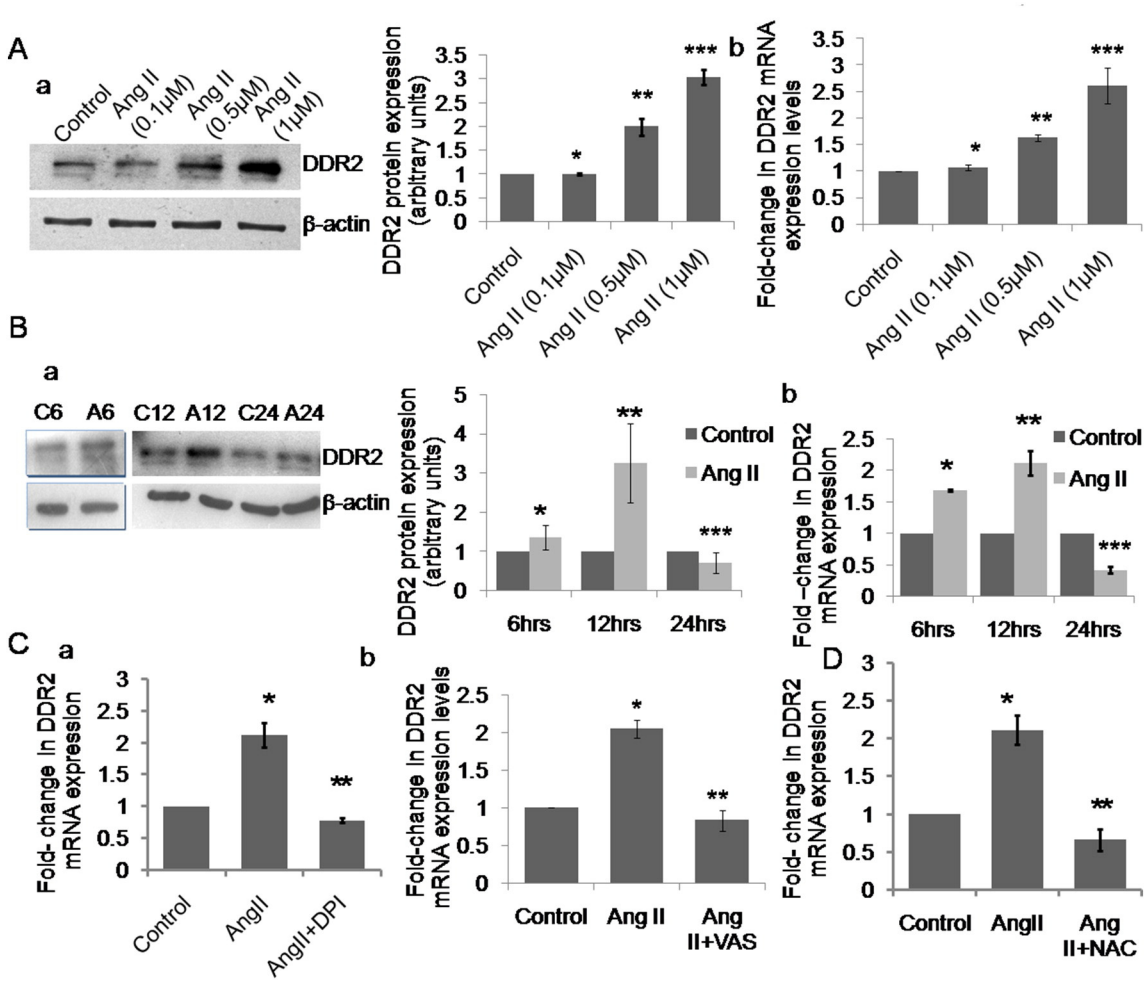

Fig. 1. Ang II increases DDR2 expression in cardiac fibroblasts. A, sub-confluent quiescent cultures of cardiac fibroblasts in M199 were stimulated with Ang II at the indicated concentrations. (a) Protein was isolated and subjected to western blot analysis, with β-actin as loading control. A representative blot from one of three independent experiments is shown. Values in the graph represent Mean ± SD of three determinations; *not significant vs. control, **$p < 0.05$ vs. control, ***$p < 0.05$ vs. control. (b) DDR2 mRNA levels were determined by Real-time PCR analysis. *Not significant vs. control, **$p < 0.05$ vs. Ang II. ***$p < 0.001$ vs. control ($n = 3$). B, sub-confluent quiescent cultures of cardiac fibroblasts in M199 were stimulated with Ang II (1 μM) and (a) protein was isolated at 6 (C6/A6), 12 (C12/A12) and 24 (C24/A24) h (replenishing Ang II at 12 h) and subjected to western blot analysis, with β-actin as loading control. A representative blot from one of three independent experiments is shown. Values in the graph represent Mean ± SD of three determinations; *$p < 0.05$ vs. control, **$p < 0.05$ vs. control, ***$p < 0.05$ vs. control. The 6 h protein blot from a separate run has been juxtaposed. (b) DDR2 mRNA expression in cardiac fibroblasts treated with Ang II for 6, 12 & 24 h was examined by Real-time PCR analysis, *$p < 0.0001$ vs. control, **$p < 0.01$ vs. control, ***$p < 0.01$ vs. control ($n = 3$). C, sub-confluent quiescent cultures of cardiac fibroblasts in M199 were treated with Ang II in the presence of DPI (a) or VAS2870 (b) for 12 h and DDR2 mRNA levels were determined by Real-time PCR analysis. (a) *$p < 0.001$ vs. control, **$p < 0.0005$ vs. Ang II. ($n = 3$). (b) *$p < 0.05$ vs. control, **$p < 0.0005$ vs. Ang II. ($n = 3$). D, sub-confluent quiescent cultures of cardiac fibroblasts in M199 were treated with Ang II in the presence of N-acetyl cysteine (NAC) for 12 h and DDR2 mRNA levels were determined by Real-time PCR analysis. *$p < 0.001$ vs. control, **$p < 0.05$ vs. Ang II. ($n = 3$) (ANOVA $p < 0.05$).

DDR2 mRNA expression (Fig. 1D), indicating ROS-dependence of the Ang II effect.

3.2. NF-κB up-regulates DDR2 expression in Ang II-treated cells

Since ROS mediates Ang II-induced DDR2 expression, the involvement of redox-sensitive NF-κB in the regulation of DDR2 expression was probed. Activation status of NF-κB in response to Ang II was first assessed by EMSA using nuclear extracts from cardiac fibroblasts following 30 min or 3 h of Ang II treatment. As shown in Fig. 2A, Ang II induced nuclear translocation of NF-κB, which was significantly reduced by an excess (200-fold) of wild type NF-κB oligos and its inhibitor, Bay 11-7085, showing that the binding was specific. Real time PCR analysis revealed that the Ang II-induced increase in DDR2 mRNA levels is abolished upon NF-kB inhibition, which demonstrated that NF-κB transcriptionally up-regulates DDR2 in response to Ang II (Fig. 2B). Experiments were therefore initiated to probe the DDR2 gene promoter-binding activity of NF-κB in Ang II-stimulated cardiac fibroblasts.

Sub-confluent cardiac fibroblasts were transiently co-transfected with 0.8 μg of DDR2 promoter-luciferase DNA construct (pGL3-DDR2) carrying the NF-κB binding site and 50 μg of Renilla plasmid. Following transfection, the cells were treated with Ang II for 3 h and luciferase activity was determined using a luminometer with Renilla as internal control. Ang II induced a significant increase in luciferase activity over the control group, showing Ang II-dependent activation of the DDR2 promoter (Fig. 2C).

To confirm NF-κB binding to the DDR2 promoter in Ang II-treated cardiac fibroblasts in vivo, we performed chromatin immunoprecipitation assay, which would report the binding of the p65 subunit of NF-κB to the chromatin region corresponding to the DDR2 promoter site. Cross-linked chromatin preparation from Ang II-treated cells was immunoprecipitated with anti-NF-κB antibody. The NF-κB binding site on the immunoprecipitated DNA was determined by PCR using specific primers. Amplification of input chromatin prior to immunoprecipitation served as positive control for chromatin extraction and PCR amplification. Chromatin immunoprecipitation using a non-specific antibody (normal human IgG) served as negative control. Our results, after normalization to input DNA, confirmed enhanced NF-κB binding to the DDR2 sequence in Ang II-stimulated cells (Fig. 2D).

3.3. Phospholipase C (PLC) and Protein Kinase C (PKC) mediate Ang II-induced increase in DDR2 expression in cardiac fibroblasts

To identify the signaling pathways that mediate the Ang II-induced increase in DDR2 expression in cardiac fibroblasts, the cells were treated with Ang II in the presence of specific inhibitors of these pathways. Chelerythrine and U73122, inhibitors of PKC and PLC respectively, reduced the Ang II-induced increase in DDR2 mRNA and protein expression significantly (Fig. 3, A and B). Moreover, chelerythrine also abolished Ang II-induced activation of NF-κB (Fig. 3C), which indicated that PKC is required for NF-κB activation in response to Ang II.

3.4. p38 MAPK mediates NF-κB activation in Ang II-treated cardiac fibroblasts

Since p38 MAPK is reported to be involved in stretch-induced DDR2 expression in VSMCs [26], we checked the possible involvement of p38 MAPK in Ang II-induced increase in DDR2 expression using its specific

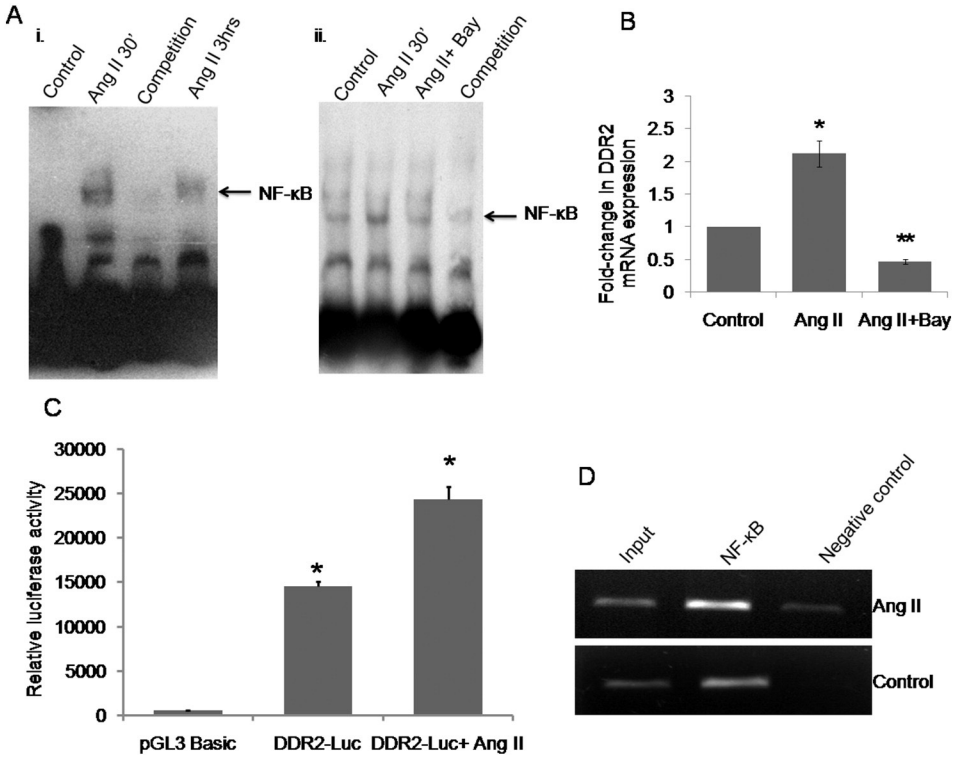

Fig. 2. NF-κB is a transcription factor for DDR2 in Ang II-treated cardiac fibroblasts. A, sub-confluent cardiac fibroblasts in M199 were serum deprived for 24 h and then exposed to Ang II for the indicated duration in serum-free M199. Nuclear extracts prepared using NE-PER extraction kit were incubated with biotinylated primers for NF-κB and the light shift Chemiluminescent EMSA kit components. DNA binding activity of NF-κB was observed in response to Ang II, which was significantly reduced in the presence of an excess of wild type oligos due to competition (i) and in the presence of its inhibitor, Bay 11-7085 (ii). A representative profile from one of three experiments is shown. B, expression levels of DDR2 mRNA, assessed by Real-time PCR analysis, were significantly lower in NF-κB-inhibited cells (Ang II + Bay 11-7085) exposed to Ang II for 12 h. *$p < 0.001$ vs. control, **$p < 0.005$ vs. Ang II. ($n = 3$). C, control and Ang II-treated cells were co-transfected with pGL3-DDR2 in the presence of Renilla-expressing vector. Luciferase activity was assayed 48 h after transfection and normalized to Renilla. Activities relative to the vector-alone control are shown. *$p < 0.05$ vs. relevant control. ($n = 3$). D, binding of NF-κB to the DDR2 promoter was confirmed by ChIP. ChIP was performed with an antibody specific for p65 as well as a non-specific anti-mouse IgG antibody (negative control). PCR was carried out using primers specific for the NF-κB binding site in the DDR2 promoter. Prior to immunoprecipitation, an aliquot of each sample was stored as an 'input' fraction (PCR control). (ANOVA $p \leq 0.05$).

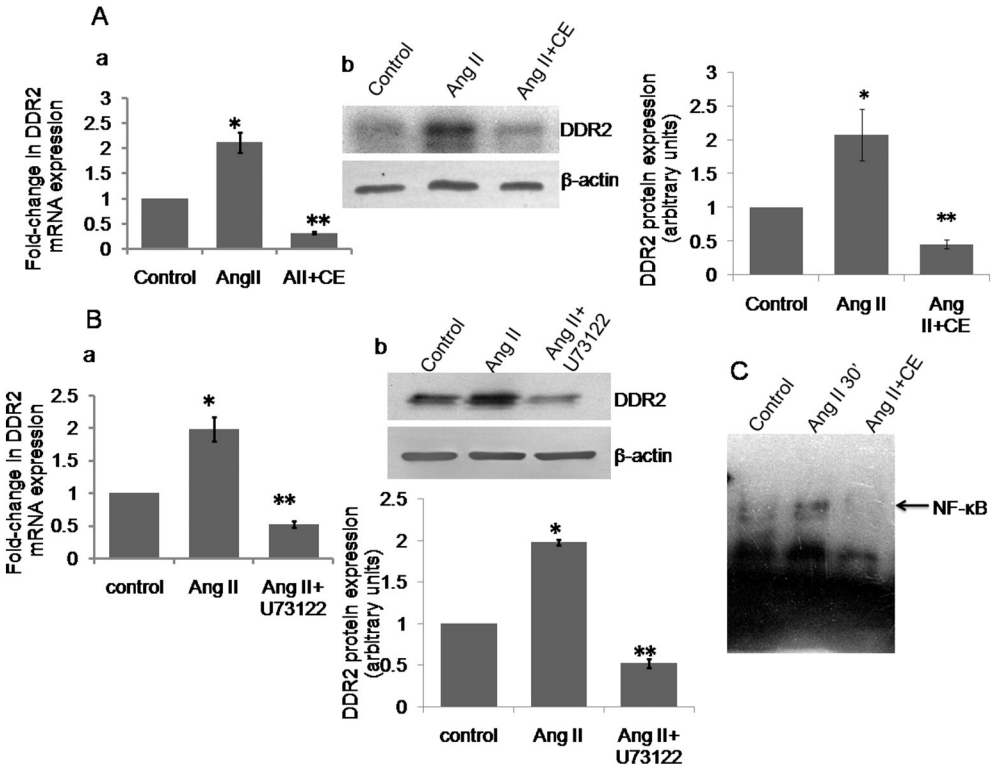

Fig. 3. Phospholipase C and Protein Kinase C mediate Ang II-induced increase in DDR2 expression in cardiac fibroblasts. A, PKC inhibition by chelerythrine abolished Ang II-induced increase in DDR2 mRNA (a) and protein expression (b) in cardiac fibroblasts. a, *$p < 0.001$ vs. control, **$p < 0.005$ vs. Ang II. ($n = 3$). b, Representative profile from one of three experiments is shown. *$p < 0.05$ vs. control, **$p < 0.05$ vs. Ang II. B, Ang II-induced increase in DDR2 mRNA and protein expression is abolished upon PLC inhibition by U73122. a, *$p < 0.001$ vs. control, **$p < 0.001$ vs. Ang II. ($n = 3$). b, representative profile from one of three experiments is shown, *$p < 0.001$ vs. control, **$p < 0.001$ vs. Ang II. C, electrophoretic mobility shift assay showed that the nuclear translocation of NF-κB observed in response to Ang II is abolished upon PKC inhibition by chelerythrine, showing that NF-κB activation in Ang II-treated cells is PKC-dependent. A representative profile from one of three experiments is shown. (ANOVA $p \leq 0.05$).

inhibitor, SB203580. Western blot analysis showed that Ang II promotes p38 MAPK activation in these cells (Fig. 4A). Further, the Ang II-induced increase in DDR2 mRNA and protein expression was significantly reduced upon treatment with the p38 MAPK inhibitor (Fig. 4B), showing that p38 MAPK is required for DDR2 expression.

Importantly, EMSA showed that inhibition of p38 MAPK abolished Ang II-induced NF-κB activation (Fig. 4C), indicating that p38 MAPK activates NF-κB and increases DDR2 expression. Further, inhibition of PLC or PKC abolished Ang II-induced p38 MAPK activation (Fig. 4A), suggesting that p38 MAPK acts downstream of PLC and PKC to activate NF-κB.

3.5. DDR2 and collagen type I are locked in a cycle of mutual regulation in Ang II-treated cardiac fibroblasts

Consistent with previous reports [27], Ang II was found to significantly increase collagen mRNA (Fig. 5A) and protein (Fig. 5B) expression in cardiac fibroblasts. Next, we checked the possibility that DDR2 and collagen type 1 may regulate each other in Ang II-stimulated cardiac fibroblasts. To test this, cells were transfected with DDR2 and collagen siRNA as described under Materials and methods. After confirming knockdown, expression levels of DDR2 and collagen proteins were determined following treatment with Ang II (Fig. 6, A–D). Interestingly, RNA interference-based inhibition of DDR2 attenuated the stimulatory effect of Ang II on collagen expression (Fig. 6B). Moreover, siRNA-mediated type 1 collagen knockdown significantly reduced the Ang II-induced increase in DDR2 (Fig. 6D). Together, the data point to reciprocal regulation of DDR2 and collagen type I in Ang II-treated cardiac fibroblasts.

3.6. DDR2 regulates collagen gene expression via ERK1/2 MAPK activation

We performed preliminary experiments to determine the mechanism by which DDR2 may regulate collagen type 1 expression in Ang II-treated cells. Since ERK1/2 was found to be a necessary mediator of Ang II-dependent increase in collagen (Fig. 7A, a and b) but not DDR2 expression (Fig. 7B), we tested the possibility that ERK1/2 acts downstream of DDR2 to regulate collagen. DDR2 knockdown abolished the Ang II-induced activation of ERK1/2 in cardiac fibroblasts (Fig. 7C), supporting the postulation that DDR2 may be involved in collagen regulation, at least in part, through activation of ERK1/2.

3.7. Wound healing ability of cardiac fibroblasts is impaired upon DDR2 knockdown

Since DDR2 is reported to play a role in wound healing [6], we checked whether DDR2 knockdown would modify the wound healing response of the cells to Ang II. We performed the scratch wound assay that revealed compromised wound closure in DDR2-silenced cells exposed to Ang II (Fig. 8), indicating that DDR2 is essential for the wound healing ability of cardiac fibroblasts.

4. Discussion

The quantitative relationship between parenchyma and stroma is critically important for the structural and functional integrity of the heart [28]. Pathological myocardial growth due to myocyte hypertrophy or fibroblast hyperplasia perturbs the ratio of functional parenchymal cells to connective tissue and contributes to the onset and progression

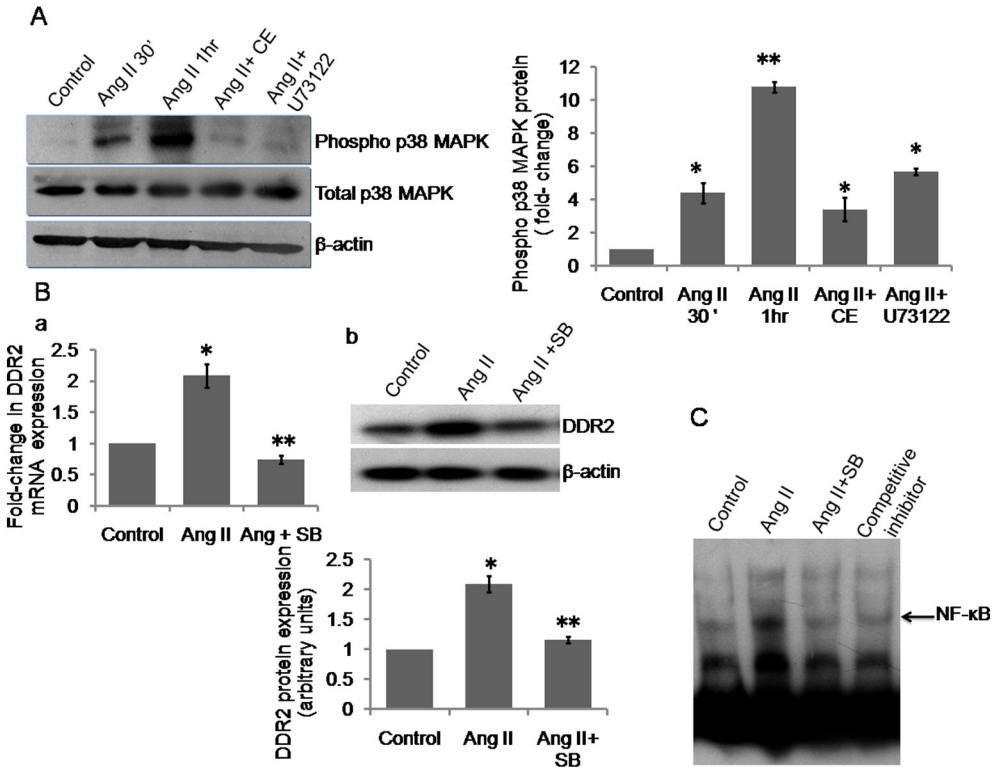

Fig. 4. p38 MAPK mediates NF-κB activation in Ang II-treated cardiac fibroblasts. A, sub-confluent cultures of cardiac fibroblasts in M199 were serum deprived for 24 h and then treated with Ang II for the indicated durations. Western blot analysis was performed using monoclonal anti-phospho p38 MAPK antibody, and phospho p38 MAPK levels were normalized to total p38 MAPK levels. Representative profile from one of three experiments is shown. Inhibition of PKC or PLC abolished Ang II-induced (1 h) p38 MAPK activation, showing p38 MAPK acts downstream of PKC and PLC to activate NF-κB. *$p < 0.05$ vs. relevant control, **$p < 0.005$ vs. control. B, p38 MAPK inhibition by SB203580 attenuated the Ang II-induced increase in DDR2 mRNA (a) and protein (b) expression in cardiac fibroblasts, showing that p38 MAPK is required for DDR2 expression. a, *$p < 0.0001$ vs. control, **$p < 0.0005$ vs. Ang II. ($n = 3$). b, representative profile from one of three experiments is shown. *$p < 0.0005$ vs. control, **$p < 0.0005$ vs. Ang II. C, electrophoretic mobility shift assay showed that inhibition of p38 MAPK abolished Ang II-induced NF-κB activation, indicating its requirement for NF-κB activation. Excess of wild type oligos significantly reduced NF-κB binding by competition. A representative profile from one of three experiments is shown. (ANOVA $p \leq 0.05$).

of heart failure. Notably, in response to myocyte loss, quiescent cardiac fibroblasts get transformed into myofibroblasts that proliferate and deposit collagenous extracellular matrix to facilitate wound healing. In contrast to fibroblasts from non-cardiac tissues, cardiac fibroblasts are resistant to apoptosis and persist in the infarct scar even after the healing response is completed [29], leading eventually to myocardial fibrosis that increases ventricular stiffness and impairs pump function. In this regard, recent studies from our laboratory have demonstrated that NF-κB and ERK1/2 are major determinants of apoptosis resistance [30] and cell cycle progression [24] in cardiac fibroblasts. Because of the widely appreciated role of DDR2 in matrix turnover and fibrosis in non-cardiac tissues, the present study was undertaken to examine the regulation of DDR2 gene expression by Ang II in cardiac fibroblasts, probing its transcriptional activation by NF-κB and its functional link with collagen via ERK1/2. The focus on DDR2 rather than DDR1 is on account of the fact that DDR2 is found predominantly in cells of mesenchymal origin in contrast to DDR1, which is expressed primarily in epithelial tissues, particularly in the epithelium of skin, kidney, gut and brain [31].

The diverse effects of Ang II are mediated by a variety of signaling cascades in a cell type- and tissue-specific manner [32]. A major mechanism by which Ang II exerts its actions via the AT1 receptor involves the classic G protein-mediated pathways. However, Ang II also cross-talks with several tyrosine kinases, including RTKs and non-receptor tyrosine kinases. Ang II stimulation of AT1 is also reported to activate serine/threonine kinases like PKC and MAPKs like ERK1/2, p38 MAPK and c-Jun. N-terminal kinase that have significant roles in cellular growth. Further, many actions of Ang II involve generation of NAD(P)H oxidase-dependent ROS that in turn can activate ROS-sensitive transcription factors such as NF-κB and AP-1 [33]. It is also recognized that stimulation of NF-κB determines some of the effects of Ang II, particularly its pro-inflammatory effect [34].

A major objective of this study was to probe the molecular basis of Ang II-induced increase in DDR2 expression. Several transcription factors/complexes have been implicated in the regulation of DDR2 expression in different systems. During osteogenic differentiation, the ATF4-C/EBPb transcription factor complex is found to induce DDR2 upregulation [35]. In rat vascular smooth muscle cells, hypoxia or hyperbaric oxygen is reported to enhance Myc-Max DNA binding to the DDR2 promoter region to increase its transcription [36–37]. In nasopharyngeal carcinomas, direct activation of the DDR2 promoter by the EBV-Z transactivator protein has been reported [38]. In looking for the transcriptional regulation of DDR2 in Ang II-treated cardiac fibroblasts, we focused on NF-κB because it is a target of Ang II and a redox-sensitive transcription factor with a major role in multiple functions like immune response, inflammation, embryonic development, cell cycle and apoptosis [34,39–42]. NF-κB is involved in Ang II-mediated inflammatory responses [43] and its role in tissue fibrosis is underscored by the report that hepatic activation of NF-κB induces liver fibrosis [44]. In the cardiovascular system, a role for NF-κB has been suggested in many pathologies, including atherosclerosis, myocardial ischemia/reperfusion injury, ischemic pre-conditioning, cardiac hypertrophy and heart failure [45]. However, involvement of NF-κB in fibrosis-related genes in the heart remains unexplored. In the present study, activation of NF-κB, the effect of NF-κB inhibition on DDR2 expression and the Luciferase and chromatin immunoprecipitation assays provide evidence for the first time that Ang II-dependent increase in DDR2 expression in cardiac fibroblasts is mediated by NF-κB.

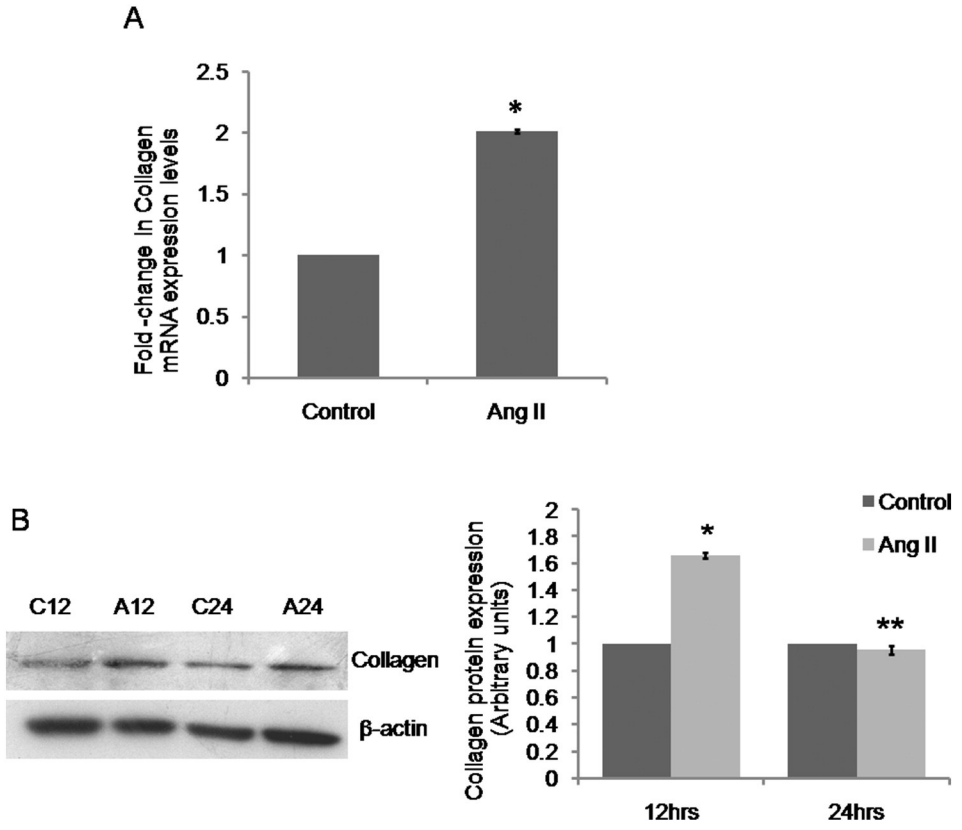

Fig. 5. Ang II increased collagen expression in cardiac fibroblasts. A, Real-time PCR analysis showing increase in collagen mRNA expression in cardiac fibroblasts treated with Ang II for 6 h. *$p < 0.001$ vs. control. ($n = 3$). B, cardiac fibroblasts in M199 were serum deprived for 24 h and then treated with Ang II for 12 (C12/A12) and 24 (C24/A24) h. Western blot analysis was performed using anti-collagen type I antibody and collagen levels were normalized to β-actin. Ang II significantly increased collagen protein expression in these cells. Representative profile from one of three experiments is shown. *$p < 0.05$ vs. control, **$p < 0.005$ vs. control. (ANOVA $p \leq 0.05$).

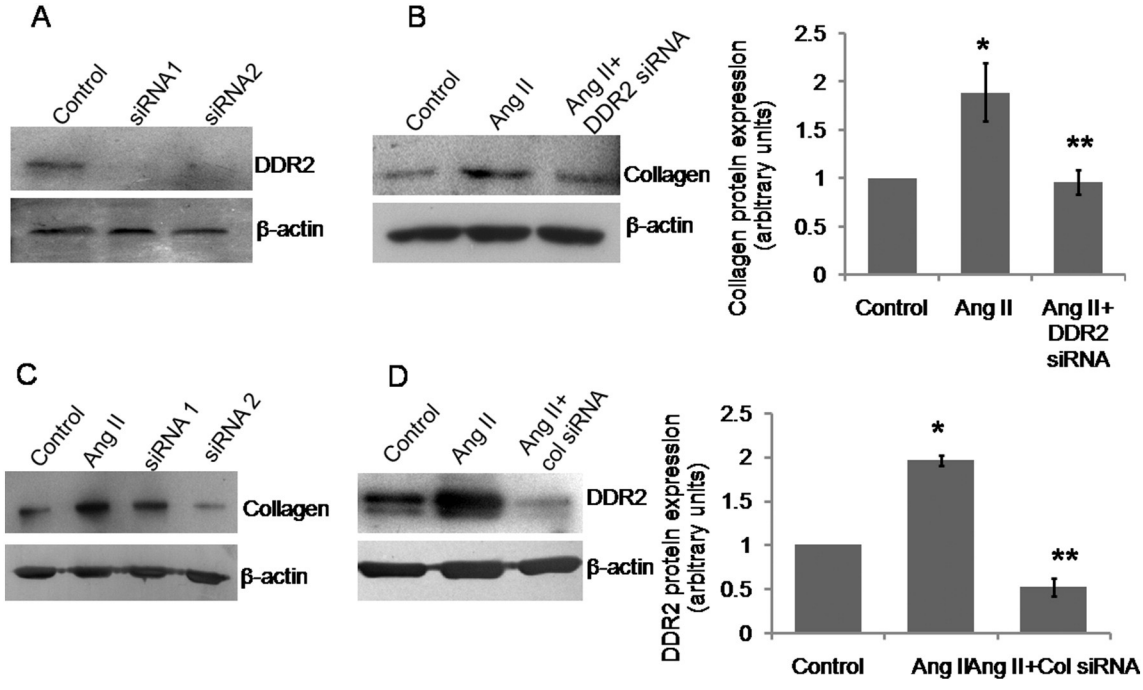

Fig. 6. Reciprocal regulation of DDR2 and collagen type I in Ang II-treated cardiac fibroblasts. A, cardiac fibroblasts were transiently transfected with siRNA1 or siRNA2 to silence DDR2. siRNA1 was found to be more effective and was used in subsequent experiments. B, Following exposure of the transfected cells to Ang II for 12 h, protein expression was examined by western blot analysis and collagen levels were normalized to β-actin. DDR2 knockdown attenuated the stimulatory effect of Ang II on collagen expression. Representative profile from one of three experiments is shown. *$p < 0.005$ vs. control, **$p < 0.05$ vs. Ang II. C, Cardiac fibroblasts were transiently transfected with siRNA1 or siRNA2 to silence collagen type1. siRNA2 was found to be effective and was used in subsequent experiments. D, collagen knockdown significantly reduced the Ang II-induced increase in DDR2 expression. Representative profile from one of three experiments is shown. *$p < 0.005$ vs. control, **$p < 0.0005$ vs. Ang II. (ANOVA $p \leq 0.05$).

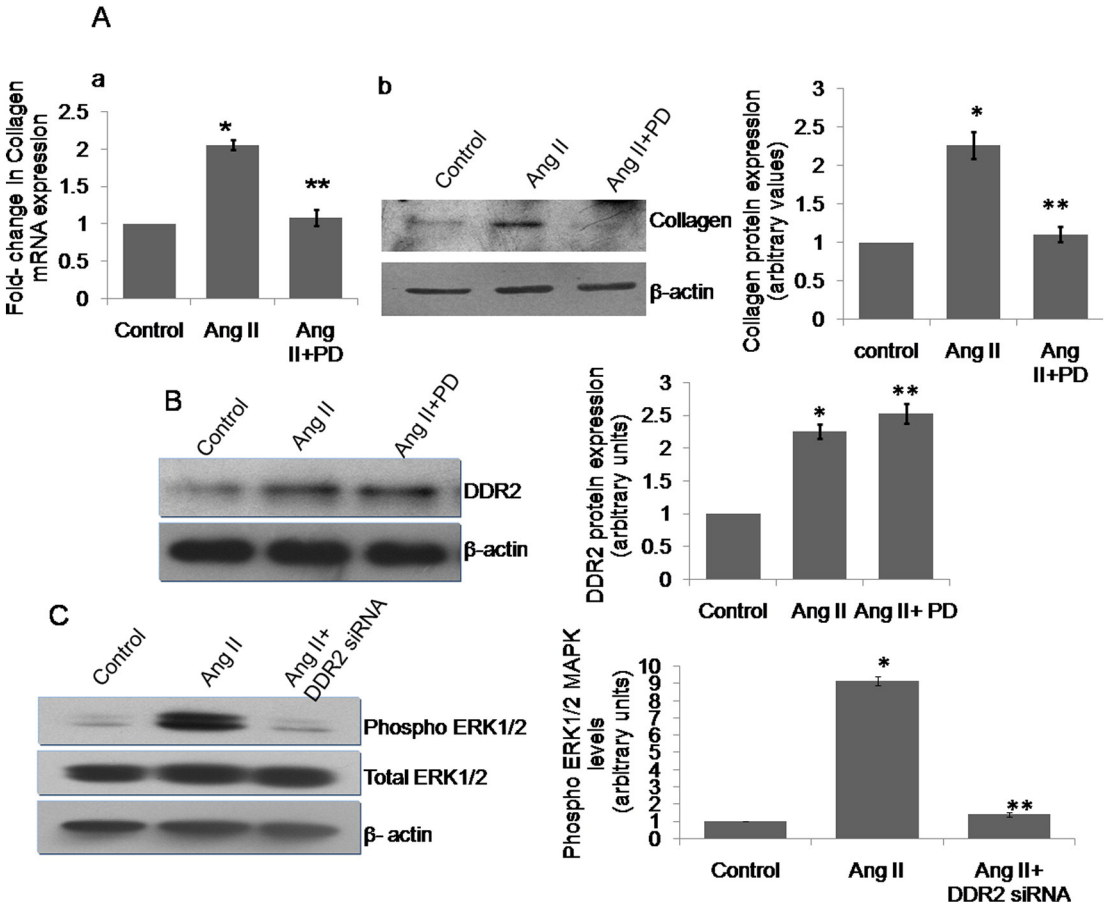

Fig. 7. DDR2 regulates collagen gene expression via ERK1/2 activation. A, a. ERK1/2 inhibition by PD98050 abolished Ang II-dependent collagen mRNA expression in cardiac fibroblasts, determined by Real-time PCR analysis. *$p < 0.005$ vs. control, **$p < 0.005$ vs. Ang II. ($n = 3$). b, ERK1/2 inhibition abolished Ang II-dependent increase in collagen protein expression, determined by western blot analysis using β-actin as loading control. Representative profile from one of three experiments is shown. *$p < 0.005$ vs. control, **$p < 0.005$ vs. Ang II. B, ERK1/2 inhibition does not affect Ang II-dependent DDR2 expression, determined by western blot analysis using β-actin as loading control. Representative profile from one of three experiments is shown. *$p < 0.05$ vs. control, **not significant vs. Ang II. C, DDR2 knockdown abolished the Ang II-induced activation of ERK1/2, determined by western blot analysis using monoclonal anti-ERK1/2 antibody. Phospho ERK1/2 levels were normalized to total ERK1/2 levels. Representative profile from one of three experiments is shown. *$p < 0.001$ vs. control, **$p < 0.005$ vs. Ang II. (ANOVA $p \leq 0.05$).

Following identification of NF-κB as a transcription factor for DDR2, we probed the mechanisms involved in NF-κB activation in response to Ang II. Activation of PKC is considered to be a pre-requisite for Ang II-induced NF-κB activation [46] and there is considerable interest in the mechanisms that link Ang II-dependent PKC activation with NF-κB activation. Although PKC is reported to directly activate NF-κB by promoting IκB phosphorylation [47], PKC activation of NF-κB may also involve intermediates [48]. We demonstrate here that Ang II induces activation of p38 MAPK, which is prevented upon inhibition of PKC, showing that PKC is required for p38 MAPK activation. Further, p38 MAPK inhibition was found to prevent NF-κB activation, indicating that PKC does not directly activate NF-κB in Ang II-treated cardiac fibroblasts. Together, our data show that p38 MAPK acts downstream of PLC and PKC to activate NF-κB. It is pertinent to point out in this context that p38 MAPK has, on the one hand, been reported to be involved in NF-κB activation [46] and, on the other, in stretch-induced DDR2 expression in VSMCs [26,37]. The findings (Fig. 1, C and D) also suggest a role for Nox-dependent ROS in mediating the effects of Ang II on DDR2. It is well known that ROS promotes activation of PLC and PKC [49–50]. Thus, it appears that several signaling molecules may act coordinately to promote NF-κB activation in cardiac fibroblasts exposed to Ang II. Because a number of signaling pathways can potentially be involved in mediating the effects of Ang II, more studies are necessary to elucidate the exact sequence of events that follow Ang II-stimulation of the AT1 receptor, culminating in the transcriptional up-regulation of DDR2. Nevertheless, it is reasonable to propose that NOX-dependent ROS may stimulate PLC and PKC to phosphorylate p38 MAPK that in turn activates NF-κB to enhance DDR2 transcription in Ang II-treated cardiac fibroblasts.

We also explored the potential implications of Ang II-induced increase in DDR2 expression. Ang II-dependent enhancement of collagen expression in cardiac fibroblasts is recognized to be a major contributor to cardiac fibrosis. In fact, drugs that target Ang II actions are found to be very effective in limiting tissue fibrosis and adverse myocardial remodeling [51–52]. These facts provide compelling rationale to explore hitherto unknown mechanisms that determine Ang II effects on collagen turnover in cardiac fibroblasts. Although the link between the collagens and their receptors is well known, the possibility that RTK signaling mediated by DDR2 may exert a regulatory influence on collagen gene expression remains unexplored. In this context, it is pertinent to note that DDR2 knockdown in normal skin fibroblasts down-regulates type 1 collagen, indicating that DDR2 is involved in collagen expression [20]. It has been proposed that this may act as a regulatory mechanism against excess collagen production since decreased DDR2 expression would stimulate miR-196a expression that in turn would inhibit collagen expression. Conversely, miR-29b-mediated inhibition of type I collagen synthesis is reported to cause down-regulation of DDR2 in hepatic stellate cells, indicating that type 1 collagen affects DDR2 expression as well [21]. In the present study, we explored the possibility that DDR2 may exert regulatory influence on collagen type 1 in

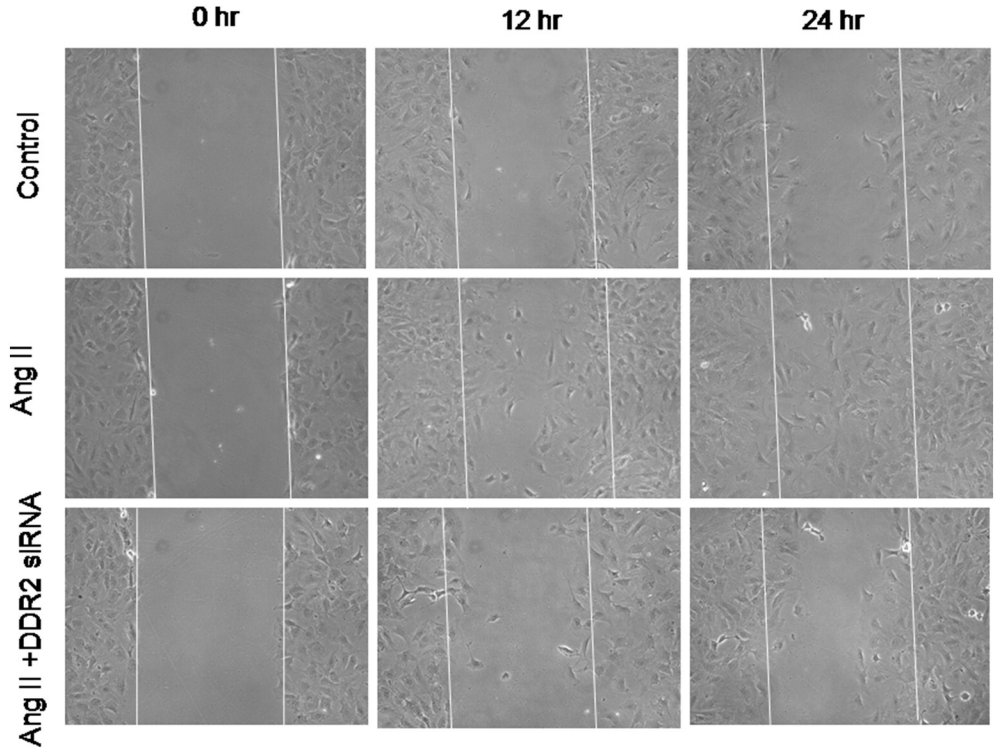

Fig. 8. Wound healing ability of cardiac fibroblasts is impaired upon DDR2 knockdown. Scratch wound assay was performed as described under Materials and methods. About 3–4 fields were examined per dish and images were captured using a Nikon inverted phase contrast microscope. The wound healing ability of the cells in response to Ang II was found to be reduced upon DDR2 knockdown by RNA interference. Representative photomicrograph from one of four different experiments is shown.

Ang II-treated cardiac fibroblasts. We focused on collagen type I rather than type III since, in the cardiac connective tissue, it is far more abundant (about 80%) than collagen type III (about 20%) and, additionally, DDR2 is activated mainly by type I collagen and to a lesser extent by type III [53]. An important outcome of our study is the demonstration of the relationship between DDR2 and collagen type I in cardiac fibroblasts exposed to Ang II. We present evidence based on RNA silencing approach that Ang II-induced increase in collagen expression in these cells has an obligate requirement for DDR2. Moreover, knockdown of collagen, which is the only ligand for DDR2 [54], was also found to prevent Ang II-induced increase in DDR2 expression, which underscores the obligatory role of collagen in DDR2 expression. In the context of the mutual dependence of DDR2 and collagen, it is important to note that the stimulatory effect of Ang II on DDR2 was not prolonged but, interestingly, paralleled the pattern obtained for collagen. Though it is not clear why the Ang II effect on DDR2 was not sustained beyond 12 h (Fig. 1B), it may be related to DDR2 turnover that could serve to avoid over-activation of downstream signaling and its sequelae, including excessive collagen production. Future studies should examine whether dysregulation of the DDR2-collagen link underlies excessive collagen production associated with different pathological states.

The study also provides some insights into a novel mechanism by which DDR2 may regulate collagen type 1 expression in Ang II-treated cardiac fibroblasts. Consistent with earlier reports [55], we found that inhibition of ERK1/2 blocks collagen expression. In contrast, ERK1/2 inhibition had no effect on Ang II-induced increase in DDR2 expression, suggesting that ERK1/2 acts downstream of DDR2 to regulate collagen expression in response to Ang II. Importantly, Ang II-induced activation of ERK1/2 was abolished in DDR2-silenced cells, implying a role for DDR2 in ERK1/2 activation in response to Ang II through a cross-talk between the GPCR and RTK pathways. Similar mechanisms involving the transactivation of RTK signaling by the GPCR pathway have been reported [56–58]. In fact, activation of ERK1/2 downstream of receptor tyrosine kinases such as PDGF-R and EGF-R is well known [59–60]. While DDR2-dependent activation of ERK1/2 may contribute to the increase in collagen expression in response to Ang II, this study did not probe the mechanism underlying the reciprocal regulation of DDR2 by collagen type 1 in Ang II-treated cells but the association of collagen with its receptors may trigger downstream events that culminate in enhanced DDR2 expression.

Apart from demonstrating the obligatory role of DDR2 in the regulation of collagen gene expression in response to Ang II, the study also underscores the importance of DDR2 in cardiac fibroblast function by providing evidence of compromised wound closure in DDR2-silenced cells. It is pertinent to point out that impairment of wound healing in the absence of DDR2 is generally attributed primarily to loss of MMP-2 [53]. Further, since type I collagen is found to be necessary for migration of vascular smooth muscle cells, it has been proposed that impaired wound healing may also be due to reduction in type I collagen [53,61], which is in line with the link between DDR2 and collagen type 1 observed by us. It is also possible that other downstream signals essential for the healing response are lacking in the absence of DDR2.

To conclude, DDR2 is expressed exclusively in mesenchymal cells that mediate tissue response to injury. Its sole ligand is fibrillar collagen, the major element in wound healing and tissue fibrosis. Necessarily, studies on the regulation of DDR2, and its structural and functional relationship with collagen, can generate useful insights into molecular mechanisms that are relevant to tissue remodeling following injury. This study provides evidence for the first time that the DDR2 gene is under the direct transcriptional control of NF-κB. Remarkably, the demonstration of an obligate role for DDR2 in Ang II-induced increase in collagen expression uncovers a hitherto unknown mechanism of regulation of matrix production in cardiac fibroblasts and suggests that DDR2 may be an important factor in wound healing and cardiac fibrogenesis. The data also show that DDR2 and collagen are locked in a cycle of mutual regulation that may turn on a vicious cycle of collagen

production in response to Ang II. The specific localization of DDR2 on fibroblasts and its regulatory role in collagen gene expression identify it as a potential drug target in the control of cardiac fibrogenesis.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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