"Molecular mechanisms in wound healing in the heart: Regulation of the cardiac fibroblast AT1 receptor"
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Project Completion Report

Title of the Project: Molecular mechanisms in wound healing in the heart: Regulation of the cardiac fibroblast AT1 receptor

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Objectives:

Cardiac fibroblasts, the most prevalent cell type in the heart, play a key role in wound healing and in maintaining the structural and functional integrity of the heart. Angiotensin II, acting via its GPCR-linked receptor, AT1, is the most important regulator of cardiac fibroblast function. Activation of AT1 in concert with other components of the renin-angiotensin system in the heart is among the sequelae of myocardial infarction that contribute to wound healing and myocardial remodeling, and AT1 antagonists are well known for their beneficial effects on the heart *post injury.* Investigations carried out by the Principal Investigator in collaboration with the NIH, USA, had confirmed earlier reports that cardiac fibroblasts express the components of the renin-angiotensin system, and had demonstrated that Angiotensin II regulates the expression of AT1 in these cells. Interestingly, apart from Angiotensin II, many factors in the diseased heart are reported to impact AT1 expression in other cell types, a phenomenon termed "heterologous receptor regulation". The molecular mechanisms involved in the regulation of AT1 expression in the heart, however, remain largely unclear although AT1 receptor regulation links a variety of traditional risk factors to cellular events that could potentially culminate in cardiovascular pathology. H_2O_2 is recognized as a potent endogenous mediator and an important exogenous cardiovascular research tool to model oxidant-dependent changes in the cardiovascular system. Against this backdrop, the proposed study tests the hypothesis that oxidative stress may regulate AT1

expression in cardiac fibroblasts via redox-sensitive pathways involving MAPK kinases, NF-kB and AP-1.

The key questions include:

1) Does oxidative stress enhance AT1 receptor expression in cardiac fibroblasts?

2) Do NF-kB and AP-1 play a role in regulating AT1 expression in cardiac fibroblasts exposed to oxidative stress?

3) Do mitogen-activated protein kinases regulate AT1 upstream of NF-kB and AP-1 in cardiac fibroblasts exposed to oxidative stress?

4) Does H2O2 induce oxidative stress and AT1 expression via NADPH oxidase-dependent ROS generation?

Deviation from the proposed objectives: None

Methodology in brief

Isolation of cardiac fibroblasts: Cardiac fibroblasts were isolated by enzymatic digestion of ventricular tissue from young adult male Sprague Dawley rats (2-3 months) followed by pre-plating in Medium 199 with 10% FBS for 150 minutes to separate the fibroblasts. Cells from passage 2 or 3 were used.

Intracellular ROS measurement in cardiac fibroblasts exposed to H_2O_2: Cardiac fibroblasts pre-treated with 10uM dichlorodihydroflourescein diacetate were incubated with H_2O_2 for 10-30 minutes and fluorescence was read at excitation and emission wavelengths of 485 nM and 530nM, respectively.

Western blot analysis for protein expression and MAPK status was performed using standard protocols. β-actin was used as loading control.

Real Time PCR assay: Taqman quantitative real-time polymerase chain reaction (RT-PCR) analysis was carried out using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, CA) under the following thermal cycling conditions: 95°C for 10 min followed by denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 min for each of 40 cycles. 18S rRNA served as endogenous control.

Electrophoretic Mobility Shift Assay: EMSA was performed by the biotin-labelled non-radioactive method using the protocol provided by Thermo Scientific (Light Shift Chemiluminescent Assay). Competition assay was carried out by co-incubating the nuclear extract with NF-κB (wild) oligos and excess unlabelled NF-κB oligonucleotides (200-fold).

Dual luciferase assay: Rat genomic DNA was subjected to PCR to amplify the 500 bp AT1 gene promoter regions with forward primer, 5' GGTGGTGCTAGCCCTTCCTTCCATCTTTCCTTTCC-3' and reverse primer, 5'-GGTGGTCCCGGGGTCCAACCCGCTCCCTCTC-3', and cloned into TA cloning vector, pCRII. AT1 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 hrs of transfection, cardiac fibroblasts were treated with 25 µM of H_2O_2 for 3 hrs and total cell lysate was prepared and luciferase activity was determined by the Dual Luciferase reporter assay and normalized to Renilla.

Chromatin immunoprecipitation (ChIP) assay: 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 H2O2 for 3 hrs, 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'-GTCCAACCCGCTCCCTCTC-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.

RESULTS

H_2O_2 enhances AT1 expression in cardiac fibroblasts

Based on cell viability analysis using Hoechst 33342 and Annexin V /PI staining, we chose a concentration of 25 µM H_2O_2 for the experiments. To evaluate the effect of H_2O_2 on AT1 gene expression in cardiac fibroblasts, cells were incubated with 25 µM H_2O_2 for different durations and AT1 mRNA and protein levels were determined by Real-time PCR and western blot analyses, respectively. H_2O_2 produced a 6-fold increase in AT1 mRNA levels at 3 hrs of treatment, which dropped to 2-fold at 12 hrs (Fig. 1). Consistent with the increase in transcript

levels, western blot analysis showed a significant increase in AT1 protein expression as well, which was maximum at 6 and 12 hrs of H_2O_2 treatment (Fig. 2).

H_2O_2 increases AT1 expression via NADPH oxidase-dependent reactive oxygen species (ROS) generation

Cardiac fibroblasts were pre-incubated with specific pharmacological inhibitors of NADPH-oxidase, DPI or VAS2870, and the effect of H_2O_2 on AT1 mRNA and protein levels was assessed. NADPH oxidase inhibition completely abolished the effects of H_2O_2 on AT1 mRNA and protein, indicating that NADPH oxidase-dependent ROS generation augments AT1 expression in H_2O_2-treated cells. Consistent with these observations, a significant increase in intracellular ROS levels was observed in H2DCFDA-loaded cardiac fibroblasts exposed to H_2O_2, which was inhibited by DPI.

ROS signaling activates NF-κB and AP-1

Since ROS mediates H_2O_2-induced AT1 expression, the involvement of redox-sensitive transcription factors, NF-κB and AP-1, in the regulation of AT1 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 hrs. H_2O_2-induced activation and nuclear translocation of NF-κB and AP-1 (Fig. 3) 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.

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

To determine whether NF-κB activation and AP-1 activation are coupled in H_2O_2-treated cardiac fibroblasts, nuclear lysates from NF-kB-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.

Transcriptional regulation of AT1 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 AT1 gene using specific pharmacological inhibitors of NF- κB and AP-1. Pre-incubation with BAY-11-7085 for 60 min prior to the addition of 25 μM H_2O_2 prevented H_2O_2-induced AT1 mRNA (Fig. 4) and protein (Fig. 5) up-regulation, suggesting that NF-κB activation is required for AT1 up-regulation by H_2O_2. The

possibility that AP-1 may also be involved in H_2O_2-mediated AT1 expression in cardiac fibroblasts was examined following its inhibition. Pre-incubation of cells for 60 min with SR11302 (10 μM) before H_2O_2 treatment attenuated AT1 mRNA (Fig. 6) and protein expression (Fig. 7).

AT1 gene promoter-binding activity of NF-κB and AP-1

Luciferas assay: Subsequent experiments probed the AT1 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 AT1 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 hrs and luciferase activity was assayed using a Dual luciferase kit (Promega) 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 AT1 promoter (Fig. 8).

ChIP assay: To confirm the binding of NF-κB and AP-1 to the AT1R promoter, 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 H2O2-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 H2O2-treated cells, compared to the control group (Fig. 9*)*.

Do MAPKs mediate H2O2-induced increase in AT1 expression via NF-kB and AP-1 activation?

Prior to probing the involvement of MAPKs in AT1 regulation in response to H2O2, the possible role of MAPKs in H2O2-induced NF-kB and AP-1 activation was first assessed.

ERK1/2 and p38 MAPK mediate H2O2-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 H2O2, the nuclear lysates were subjected to EMSA. ERK1/2 and p38 MAPK inhibitors but not the JNK inhibitor (Fig. 10) 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.

ERK 1/2, p38 MAPK and JNK mediate H2O2-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 H2O2-induced AP-1 activation (Fig. 10).

H2O2 triggers local Ang II production to augment AT1R and collagen expression

Serendipitously, candesartan, the AT1R antagonist was found to abolish H2O2-induced increase in AT1R mRNA and protein expression in cardiac fibroblasts (Fig. 11), indicating that H2O2 triggers local Ang II production that in turn enhances AT1R expression in these cells. Consistent with these observations, H2O2 was found to cause a 5-fold increase in Angiotensinogen mRNA expression and a 3-fold increase in secreted Ang II levels (Fig. 11). 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. 11). Further, H2O2 significantly increased collagen mRNA and protein expression in these cells, which was decreased by candesartan (Fig. 12), confirming the involvement of Ang II in mediating the H2O2 effect on collagen. Moreover, inhibition of NF-κB, AP-1, NADPH oxidase, ERK1/2, p38 MAPK and JNK attenuated collagen protein expression (Fig. 12), indicating that the same pathway mediates H2O2-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.

Summary: The AT1 receptor mediates the manifold actions of Angiotensin II in the cardiovascular system. This study probed the effect of oxidative stress on AT1 gene expression in cardiac fibroblasts. Real-time PCR and western blot analysis showed that H_2O_2 enhances AT1 mRNA and protein expression via NADPH oxidase-dependent reactive oxygen species induction. Activation of NF-κB and AP-1, demonstrated by mobility shift assay, abolition of AT1 expression by their inhibitors, Bay-11-7085 and SR11302, respectively, and promoter assays indicated transcriptional control of AT1 by NF-κB and AP-1 in H_2O_2-treated cells. Further, while ERK 1/2 and p38 MAPK JNK mediate H2O2-induced NF-kB activation, ERK 1/2, p38 MAPK and JNK are obligatory requirements for H2O2-induced AP-1 activation. Serendipitously, it was found that H2O2 triggers local Ang II production that in turn enhances AT1R expression in these cells. H2O2 also significantly increased collagen mRNA and protein expression in these cells via Ang II production and by a mechanisms involving NF-κB, AP-1, NADPH oxidase, ERK1/2, p38 MAPK and JNK. 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. By causally linking oxidative stress to Angiotensin II, AT1R and

collagen up-regulation in cardiac fibroblasts, this study offers an extremely important and novel perspective on the pathogenesis of cardiovascular diseases associated with oxidative stress. Further, the observation is in line with the possibility that drugs that block Angiotensin II action may confer collateral benefits in a setting of oxidative stress.

Total Sanctioned Amount (for 3 years): Rs. 42,38,539/-

Publication: We have been able to publish the findings in the very prestigious Journal of Molecular and Cellular Cardiology.

1. Anupama V, Mereena George, SB Dhanesh, Aneesh C, Jackson James K, Shivakumar K. Molecular mechanisms in H2O2-induced increase in AT1 receptor gene expression in cardiac fibroblasts. J Mol Cell Cardiol 97: 295-306; 2016. (Impact Factor: 5.7)

Assets acquired wholly out of government grants

a. Real Time PCR machine: ABI
b. Equipment for western blot analysis: Biorad

Figure 1: AT1 mRNA expression upon exposure of cardiac fibroblasts to H2O2

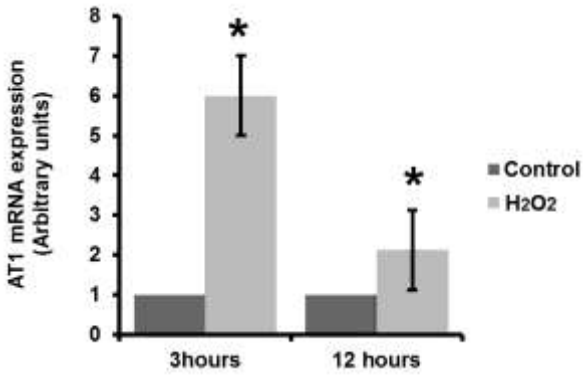


Figure 2: AT1 protein expression upon exposure of cardiac fibroblasts to H2O2

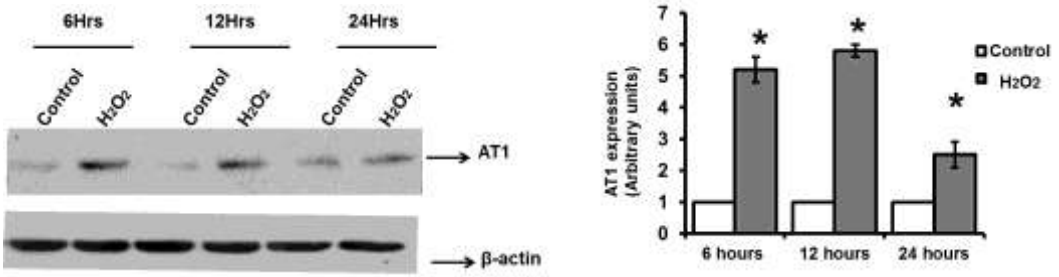


Figure 3: Nuclear translocation of NF-κB and AP-1 in cardiac fibroblasts exposed to H_2O_2

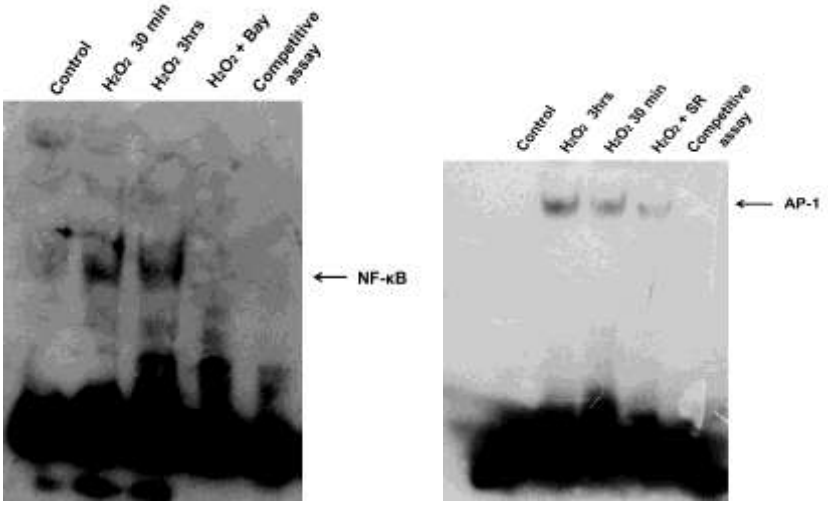


Figure 4: NF-κB activation is required for AT1 mRNA up-regulation by H_2O_2

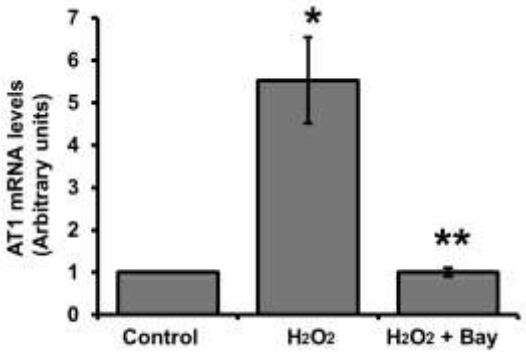


Figure 5: NF-κB activation is required for AT1 protein up-regulation by H_2O_2

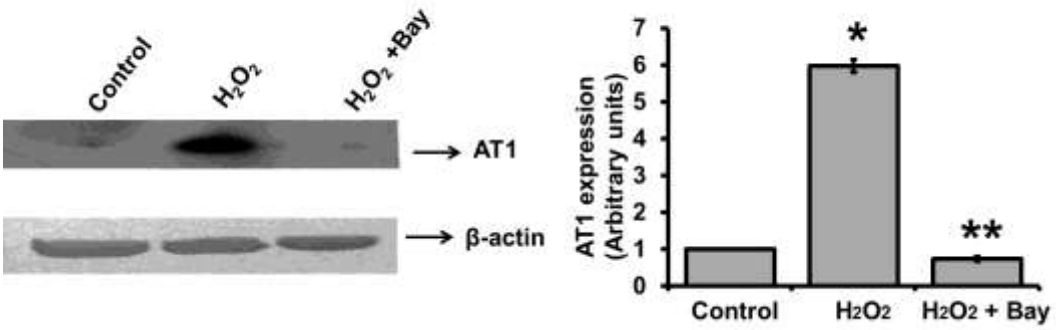


Figure 6: AP1 activation is required for AT1 mRNA up-regulation by H_2O_2

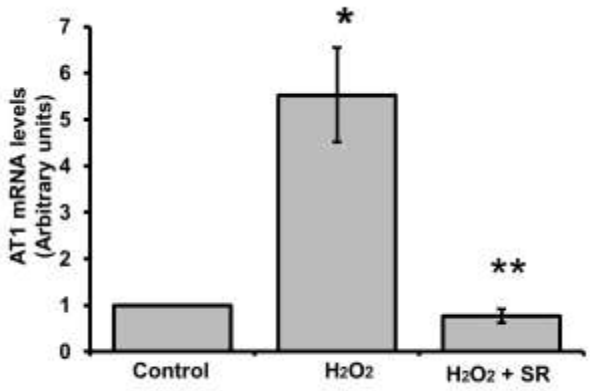


Figure 7: AP1 activation is required for AT1 protein up-regulation by H_2O_2.

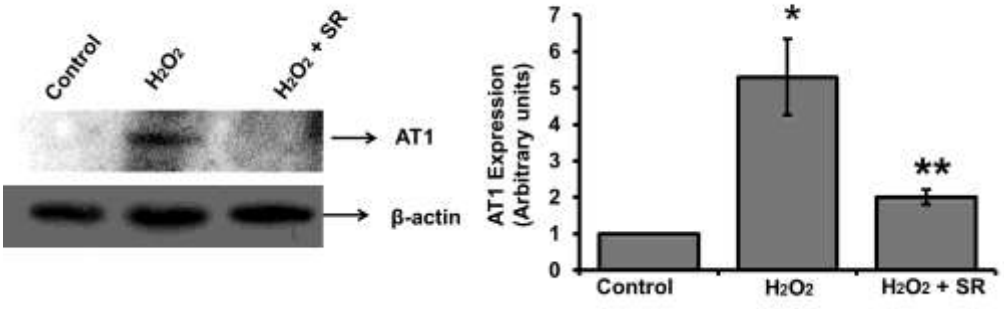


Figure 8: Enhanced AT1 promoter activity in H_2O_2-treated cells

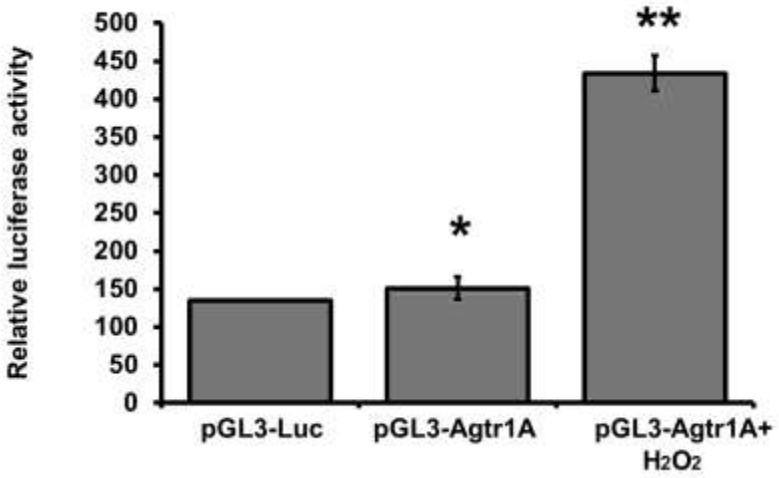


Figure 9: Chromatin immunoprecipitation assay confirmed that NF-kB and AP-1 bind to the AT1 promoter to turn on its transcription

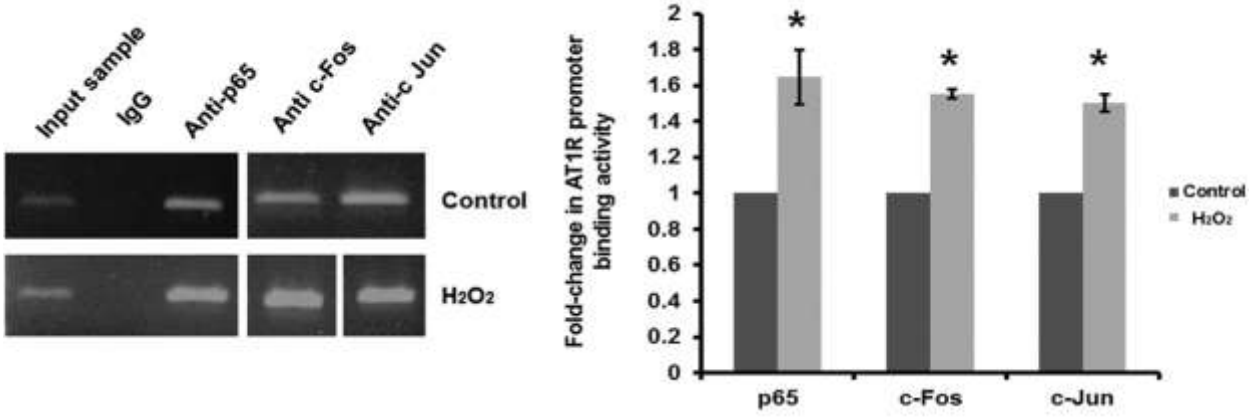
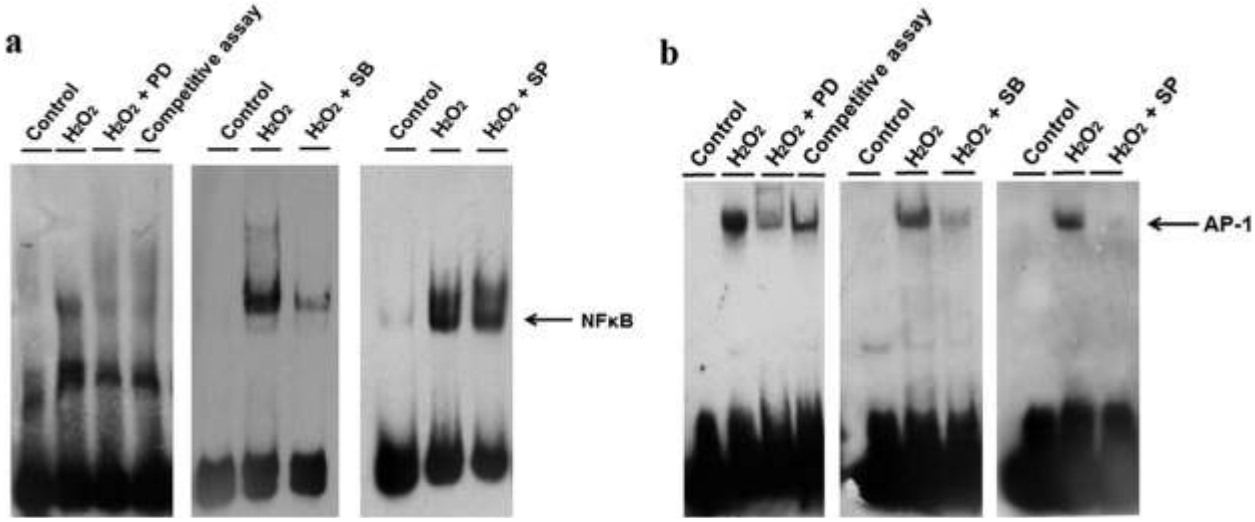


Figure 10: Regulation of NF-κB and AP-1 activation by MAPKs. Activation of NF-κB and AP-1 in response to H2O2 (25 μM) was examined by EMSA. (a) NF-κB activation was significantly reduced upon ERK1/2 and p38 MAPK inhibition using PD and SB, respectively, but was unaffected upon JNK inhibition using SP (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.



Figures 11. Oxidative stress induces endogenous Ang II production that in turn enhances AT1 mRNA and protein expression by a mechanism involving NADPH oxidase-dependent ROS.

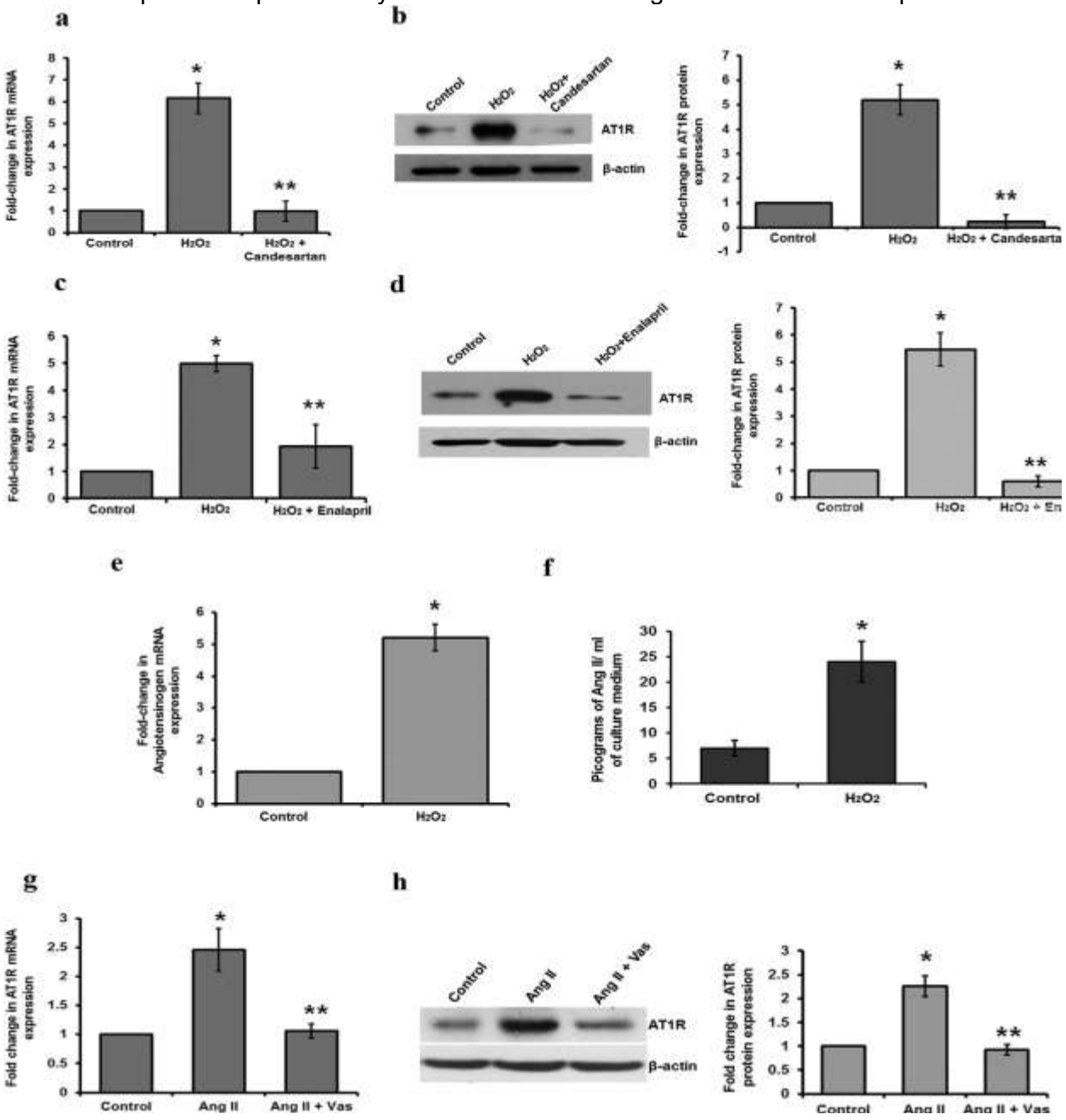
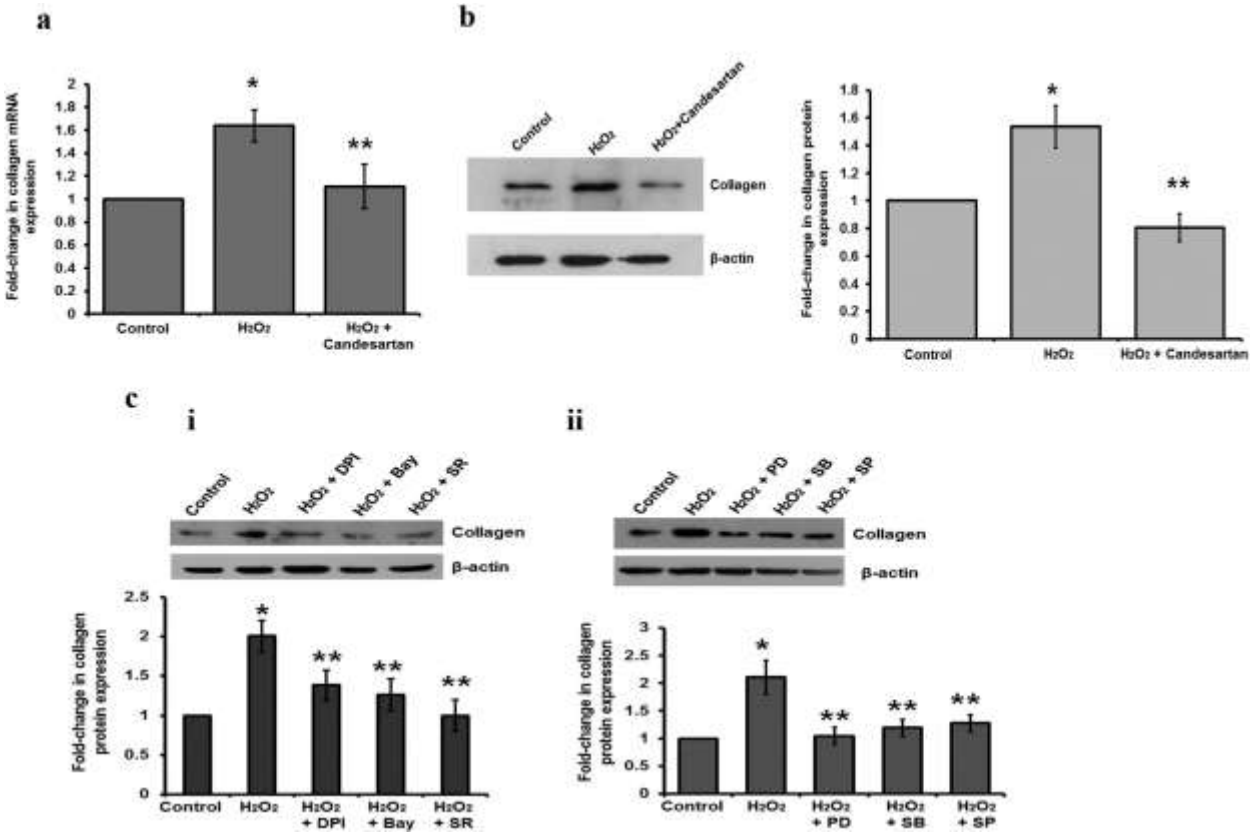


Figure 12. Oxidative stress enhances collagen gene expression via endogenous Ang II production by a mechanism involving NF-κB, AP-1, NADPH oxidase, ERK1/2, p38 MAPK and JNK.



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