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NAD(P)H oxidase-derived reactive oxygen species regulate angiotensin-II induced adventitial fibroblast phenotypic differentiation ☆

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Abstract

Phenotypic differentiation of adventitial fibroblasts into myofibroblasts is an essential feature of vascular remodeling. The present study was undertaken to test the hypothesis that reactive oxygen species (ROS) are involved in rat adventitial fibroblast differentiation to myofibroblast. Activation of α-smooth muscle a'ctin (α-SMA) was used as a marker of myofibroblast. Angiotensin II increased intracellular ROS in adventitial fibroblasts that was completely inhibited by the free radical scavenger NAC, the NAD(P)H oxidase inhibitor DPI, and transfection of antisense gp91phox oligonucleotides. Myofibroblast differentiation was prevented by inhibition of ROS generation with DPI, NAC, and antisense gp91phox as shown by decreased expression of α-SMA. Angiotensin II rapidly induced phosphorylation of p38 MAPK and JNK, both of which were inhibited by DPI, NAC, antisense gp91phox, and the selective AT1 receptor antagonist, losartan. Inhibiting p38MAPK with SB202190 or JNK with SP600125 also reduced angiotensin II-induced α-SMA expression. These findings demonstrate that angiotensin II induces adventitial fibroblast differentiation to myofibroblast via a pathway that involves NADPH oxidase generation of ROS and activation of p38MAPK and JNK pathways.

Keywords: Reactive oxygen species; Adventitial fibroblast; Differentiation

There is emerging evidence that adventitial fibroblasts play a vital role in vascular remodeling [1]. Following injury, adventitial fibroblasts are activated and morphologically differentiation to myofibroblast-like cells, which are characterized by the appearance of the cytoskeletal protein alpha-smooth muscle actin (α-SMA). These myofibroblasts have contractile properties and demonstrate a marked increase in proliferative and synthetic activities. They may also migrate to the intima and participate in neointimal formation [2–4]. Therefore, phenotypic modulation of adventitial fibroblasts is critical to initiate vascular remodeling in response to injury. However, the mechanisms regulating adventitial fibroblast differentiation have not yet been fully defined.

The generation of reactive oxygen species (ROS) has recently been suggested to be involved in inducing growth, apoptosis, migration, and differentiation [5]. Generation of ROS is regulated by cytokines and growth factors, including angiotensin II (AngII), which increase O_2^- and H_2O_2 production in cardiac, vascular smooth muscle, endothelial,

☆ *Abbreviations:* AF, Adventitial fibroblast; p38MAPK, p38 mitogen activated protein kinase; JNK, c-Jun amino terminal kinase; ERK, extracellular signal-regulated kinase; AngII, Angiotensin II; α-SMA, α-smooth muscle actin.

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adventitial, and mesangial cells [6]. In cultured rat aortic vascular smooth muscle cells (VSMC), AngII increases O_2^- production by activating NAD(P)H oxidase. This effect is sustained and contributes to long-term signaling events such as cell growth and hypertrophy [7]. It is well established that angiotensinogen is present in the adventitia and that angiotensin-converting enzyme is activated in the adventitia in response to vascular injury [8]. Moreover, Pagano et al. [9] reported the adventitia as a major source of O_2^- in the rabbit and rat aorta. NADPH oxidase-derived ROS also appear to be involved in adventitial fibroblast proliferation and extracellular matrix deposition [10]. However, little is known about the functional role of ROS in the differentiation of fibroblasts to myofibroblasts.

The purpose of this study was to determine whether AngII induced NAD(P)H oxidase-derived ROS in the vascular adventitia was involved in fibroblast phenotype differentiation into myofibroblast initiated by the activation of mitogen-activated protein kinases (MAPK). Our data demonstrated that AngII could induce adventitial fibroblast phenotype differentiation via ROS generation. We also evaluated the role of JNK and p38MAPK activation in fibroblast differentiation into myofibroblast.

Materials and methods

Materials. All the experimental procedures were performed according to the guidelines for the care and use of laboratory animals as established by our institute. AngII and PD 123319 were obtained from Sigma–Aldrich (St. Lous, Missouri, USA). Losartan was kindly provided by Merck (Rahway, New Jersey, USA). *N*-Acetylcysteine (NAC), diphenylene iodonium (DPI), SB202190, SP600125 and 2′, and 7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were purchased from Calbiochem (Darmstadt, Germany). Anti-phospho-ERK, anti-phospho-p38MAPK, and anti-phospho-JNK antibodies were purchased from New England Biolabs (Hitchin, Herts, UK). β-Actin and α-smooth muscle actin were purchased from Sigma–Aldrich (St. Louis, Missouri, USA).

Cell culture. Adventitial fibroblasts were isolated from thoracic aortas of 200 g male Sprague–Dawley rats [11]. Cells were grown in DMEM supplemented with 10% heat-inactivated FCS, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The subconfluent cells were made quiescent by incubation in DMEM supplemented with 0.1% BSA for 48 h before stimulation. Myofibroblast differentiation was induced by exposure to AngII (10^{-7} mol/L) for 24–48 h. Cells used passages 3–7.

Immunofluorescence. Adventitial fibroblasts were growth arrested with medium containing 0.1% BSA for 48 h. Quiescent cells were treated without (control) or with AngII (10^{-7} mol/L) for 24–48 h. After treatment, cells were fixed in methanol, blocked with 1% BSA, and incubated with an anti-α-SM actin antibody (1:200). Immunofluorescent staining of α-SMA was completed by incubating the cells with a rabbit anti-mouse IgG conjugated with TRITC (1:100). Cellular nuclei were stained with DAPI dye (1:1000). Zeiss epifluorescence microscope was used to visualize the stained cells, and photographs were taken by using a Zeiss digital camera and Axiovision 2.05 software [12].

Intracellular redox state. Intracellular ROS production was measured as described by Baas et al. [13]. 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFH-DA) is a nonpolar compound that is converted into a nonfluorescent polar derivative (H_2DCFH) by cellular esterase after incorporation into cells. H_2DCFH is rapidly oxidized to the highly fluorescent DCF in the presence of intracellular hydrogen peroxide and peroxidases. Briefly, serum-starved cells on round coverslips were incubated with H_2DCFH-DA 5×10^{-6} mol/L for 30 min at 37 °C. Sometimes, cells were pre-incubated with NAC (5×10^{-3} mol/L) or DPI (5×10^{-6} mol/L) for 30 min. The cells were stimulated and then immediately observed by a laser scanning confocal microscope. The samples were excited by a 488-nm Ar laser and images were filtered by a longpass 515 nm filter. About 30 cells were randomly selected from three separate experiments and DCF fluorescence intensities of treated cells were compared with those of unstimulated control cells.

Lysate protein analysis. Following treatment, cells were washed twice with ice-cold PBS containing protease inhibitors (chymostatin, antipain, pepstatin, each at 15.7 μg/ml, leupeptin, 57.7 μg/ml; AEBSF, 250 μg/ml), lysed in sample buffer (62.5 mM Tris–Cl, pH 6.8, 2% SDS, and 5 mM DTT) at room temperature, and vortexed. Cell lysates were then boiled for 5 min and cleared by centrifugation (13,000 rpm, 10 min at 4 °C). Protein concentration was determined using Bio-Rad DC protein assay [14].

Western blotting. The soluble lysates (10 μg per lane) were subjected to 10% SDS–PAGE as described. Proteins were then transferred to nitrocellulose membranes and blocked with 5% nonfat milk/TBST for 1 h at room temperature. Membranes were incubated with primary antibodies directed against α-SMA (1:1000), β-actin (1:5000), p-ERK (1:1000), p-JNK (1:1000), and p-p38MAPK (1:500) in 5% milk/TBST at 4 °C overnight. After washing membranes with TBST three times, membranes were incubated with horseradish peroxidase-conjugated antibody for 1 h at room temperature. Western blots were developed using ECL (Roche, Mannheim, Germany) and quantified by scanning densitometry.

Antisense gp91phox oligonucleotide (ODN) transfection. The gp91phox antisense (AS) ODN had the sequence 5′-AACTGGG CTGTGAATGAGG-3′, targeting base pairs 7–25 downstream of the translation initiation start in the coding sequence of gp91phox mRNA (GenBank library Accession No. NM_000397). The scrambled control ODN had the sequence 5′-CATTGTGGAGTGACAGGAG-3′. FITC was labeled on the 3′ end of the ODNs. ODNs were transfected into cells by using Lipofectamine Plus reagent (Invitrogen). According to the manufacturer's protocol, adventitial fibroblasts were incubated in serum-free medium and then transfected with 2 μmol/L ODN for 24 h.

Statistical analysis. Statistical evaluation of the data was performed by Student's *t* test for paired or unpaired observations and by analysis of variance (ANOVA). Scheffe's test for multiple comparisons was used to identify differences among groups. Values were considered to be significantly different when p was <0.05.

Results

AngII induced phenotypic modulation of fibroblasts

To determine the role of AngII in adventitial fibroblast transition to myofibroblast, we examined the effects of AngII on α-SM actin expression, an indicator of fibroblast differentiation to myofibroblast. As shown in Fig. 1A, in AngII-treated fibroblast, the myofibroblast phenotype was identified by the examination of α-SM actin staining using confocal immunofluorescence microscopy. After AngII treatment, adventitial fibroblasts acquired phenotypic features of myofibroblasts, showing an intense α-SM actin expression (Fig. 1A). AngII-treated cells were uniformly positive for α-SM actin staining and displayed numerous actin microfilaments (stress fibers). In contrast, untreated fibroblasts were very weakly stained for α-SM actin expression. Fig. 1B immunoblotting also shows α-SMA expression was increased after 24 h of AngII (10^{-7} mol/L) treatment and reached a maximum by 48 h.

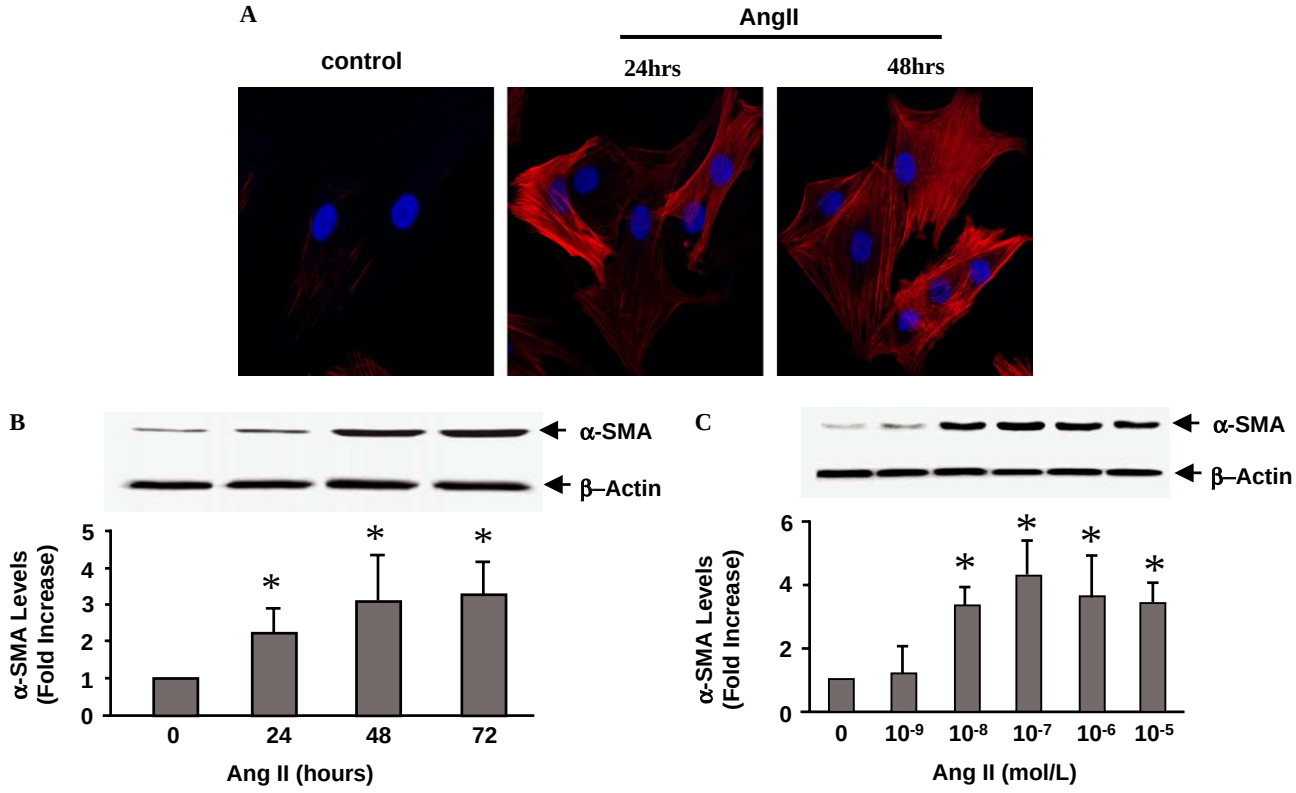

Fig. 1. Time-course and dose-dependent of Angiotensin II on α-SMA expression in adventitial fibroblasts. (A) AngII induces redistribution of α-SMA along stress fibers in fibroblasts. Cells were exposed to AngII (10^{-7} mol/L) for 24 and 48 h, and fixed with cold methanol. Indirect immunofluorescent staining was performed with an anti-α-SMA antibody. Nuclei were stained with DAPI dye. Magnification: 40×. (B) Rat adventitial fibroblasts were treated with AngII (10^{-7} mol/L) for the times indicated. Top panel shows representative immunoblots of AngII induced the expression of α-SM actin. β-Actin was assayed to verify equal loading of cell lysates. Bottom panel shows the quantification of the bands by densitometry. Results are shown as fold increases of control from three independent experiments compared with unstimulated control cells. Data are means ± SD. *$p < 0.05$ vs. control. (C) The cells were stimulated with different concentrations of AngII for 48 h. Top panel shows representative immunoblots of AngII induced the expression of α-SM actin. β-Actin was assayed to verify equal loading of cell lysates. Bottom panels show the quantification of the bands by densitometry. Results are shown as fold increases of control from three independent experiments compared with unstimulated control cells. *$p < 0.05$ vs. control.

The effect of AngII on α-SMA expression was also dose-dependent (Fig. 1C). Maximal stimulation was reached at 10^{-7} mol/L.

AngII-induced ROS formation via NADPH oxidase stimulation

It is well known that AngII stimulates ROS production in various cell types [10]. To determine whether AngII induces intracellular ROS in fibroblasts, we measured ROS levels using the ROS-sensitive fluoroprobe, DCF-DA, by confocal analysis. AngII (10^{-7} mol/L) increased DCF fluorescence in a time-dependent manner (Fig. 2A). ROS generation increased significantly at 10 min and then further increased until a plateau was reached at 30 min. Furthermore, the free radical scavenger NAC (5×10^{-3} mol/L) or NADPH oxidase inhibitor DPI (5×10^{-6} mol/L) markedly blunted AngII-induced ROS formation (Fig. 2B). To determine the specific role of gp91phox in AngII-induced ROS production, we examined the effect of blocking gp91phox expression by antisense gp91phox oligonucleotides on AngII-induced ROS production. Transfection of antisense gp91phox reduced pg91phox expression by 80% (Fig. 2C). We found that transfection of antisense gp91phox oligonucleotides significantly reduced AngII-induced ROS induction compared with control or transfection with mismatched oligonucleotides (Fig. 2B).

Role of ROS and AT1R in AngII-induced adventitial fibroblast differentiation

We next determined the effects of losartan, NAC, and DPI on Ang II-induced fibroblast phenotype differentiation by measuring α-SMA induction. As shown in Fig. 3A losartan, DPI, and NPAC significantly inhibited AngII-stimulated induction of α-SMA, suggesting that AngII-induced adventitial fibroblast differentiation is mediated by ROS generation via the AT1R. We further determine the role of gp91phox in AngII-induced fibroblast phenotype differentiation. As shown in Fig. 3B, AngII-induced α-SMA expression was significantly blocked by antisense

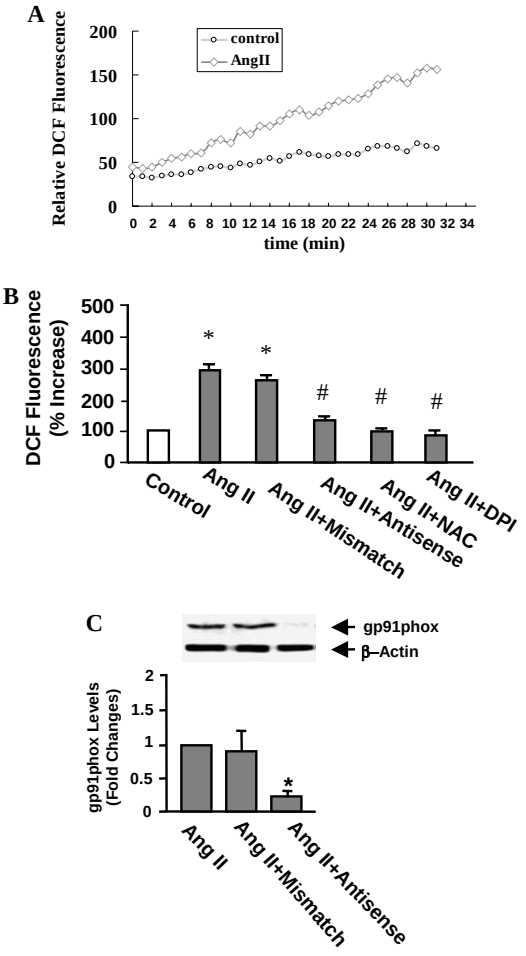

Fig. 2. Analysis of ROS generation by AngII in adventitial fibroblasts. (A) Effects of AngII on intracellular redox state in vascular adventitial fibroblasts. Cells were incubated for 30 min at 37 °C with 5×10^{-6} mol/L DCFH-DA in HBSS–Hepes and stimulated with 10^{-7} mol/L AngII, and fluorescence intensity was measured with a confocal laser scanning microscope. Cell fields consisted of 10–20 cells and were scanned over 30-min periods, and the light emitted at 513 nm was recorded. Relative fluorescence intensity of each cell was calculated relative to untreated control cells. For each treatment group, 5 fields were scanned. Results are means ± SD ($n = 3$). (B) Wildtype, mismatch- and antisense gp91phox-transfected cells were incubated with 5×10^{-6} mol/L H_2DCF-DA in HBSS–Hepes for 30 min at 37 °C and stimulated with 10^{-7} mol/L AngII. Furthermore, the wildtype cells were preincubated with the free radical scavenger NAC (5×10^{-3} mol/L) or NADPH oxidase inhibitor DPI (5×10^{-6} mol/L) for 30 min before AngII: relative fluorescence intensity of each cell was calculated relative to untreated control cells. For each treatment group, 5 fields were scanned. Results are means ± SD ($n = 3$). $^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. AngII. (C) The transfection efficiency of the antisense gp91phox ODNs. Western blot shows expression of gp91phox protein in wildtype AF, AF transfected with mismatch and AF transfected with antisense gp91phox. Lysates were subjected to SDS–PAGE, followed by immunoblotting with anti-gp91phox antibody. β-Actin was measured to demonstrate equal loading of cell lysates. Results are shown as fold increases of control from three independent experiments compared with wildtype cells. $^{*}p < 0.05$ vs. wildtype.

gp91phox oligonucleotides, whereas mismatch-transfected cells remained unchanged, consistent with the effect of antisense gp91phox on AngII-induced ROS production (Fig. 2C).

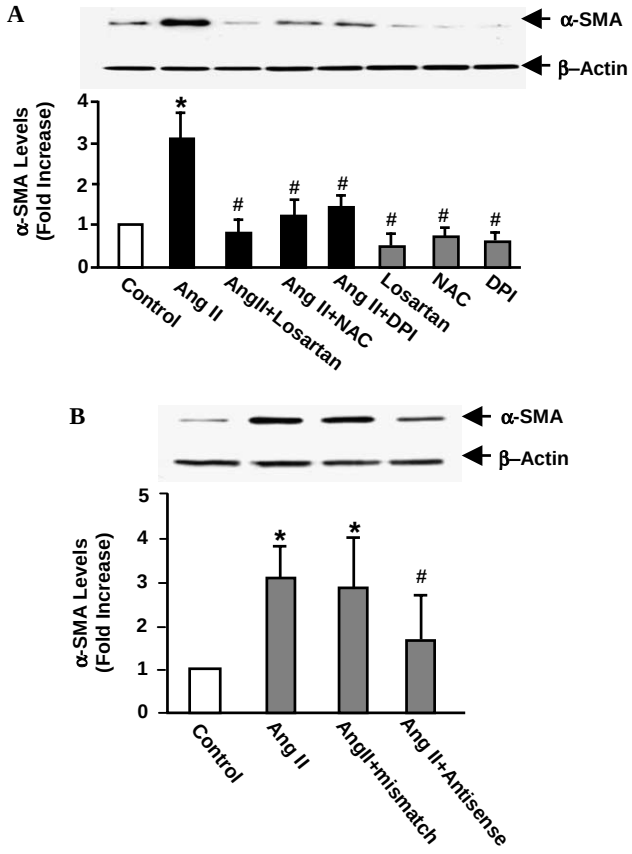

Fig. 3. Effects of Losartan, NAC, DPI, and antisense gp91phox on AngII-induced α-SMA expression. (A) Effects of losartan, NAC, and DPI on AngII-induced α-SMA expression. After treatment with losartan (10^{-5} mol/L), NAC (5×10^{-3} mol/L) or DPI (5×10^{-6} mol/L) for 30 min, the cells were stimulated by AngII (10^{-7} mol/L) for 48 h. Western blot analysis of cell lysates was performed by using an anti-α-SMA antibody. β-Actin was measured to assure equal loading of the cell lysates. Densitometry quantification from three separate experiments is shown in the lower panel. Means ± SD $^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. AngII. (B) Effects of antisense gp91phox on AngII-induced α-SM actin expression. Mismatch and antisense gp91phox-transfected adventitial fibroblasts were assessed for α-SM-actin after AngII treatment for 48 h. β-Actin was measured to assure equal loading of the cell lysates. The intensity of each band on the blot was quantified by densitometric scanning, and the activities are shown as fold increases of the average from three independent experiments compared with unstimulated controls. $^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. AngII.

AngII-mediated activation of p38MAPK and JNK is redox-sensitive and through AT1 R

To determine the signaling molecules involving in AngII-stimulated ROS production and α-SMA induction, we focused on JNK and p38. We found that AngII rapidly induced phosphorylation of p38 and JNK, reaching a maximum at 10–15 min, and declined to baseline after 120 min (data not shown). AngII-induced p38 and JNK activation was mediated by AT1R and dependent upon ROS production because losartan, NAC, and DPI significantly inhibited AngII-induced phosphorylation of p38MAPK and JNK (Figs. 4A and B). Similarly, antisense gp91phox but not mismatched oligonucleotides attenuated AngII-mediated p38

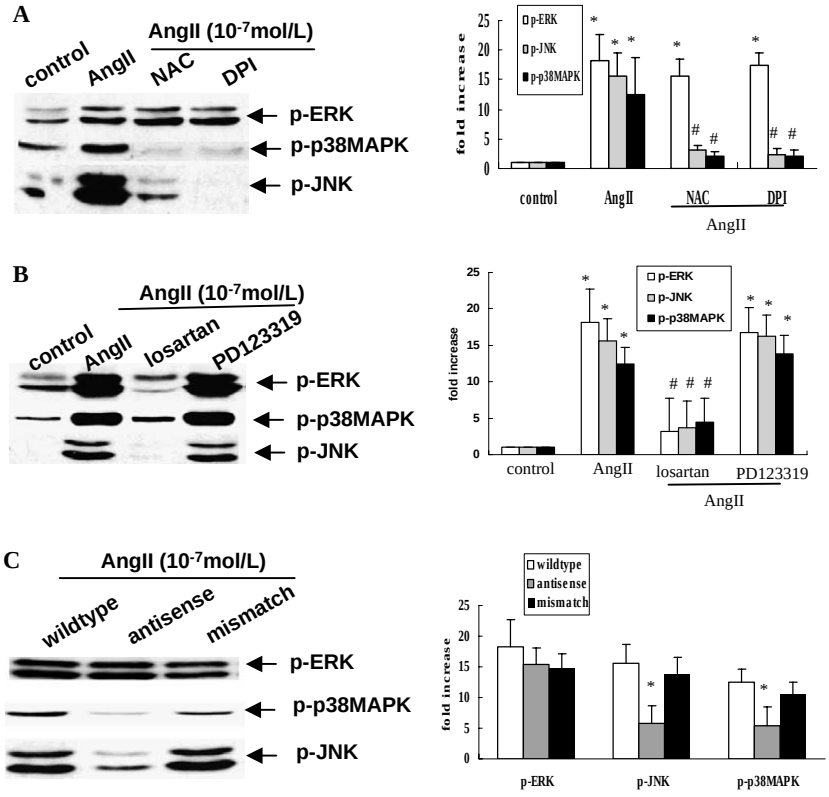

Fig. 4. (A) Effects of NAC and DPI on AngII-induced ERK, JNK, and p38MAPK activation. After preincubated with NAC (5×10^{-3} mol/L) or DPI (5×10^{-6} mol/L) for 30 min, the cells were stimulated by AngII (10^{-7} mol/L) for 10 min. Bottom panels show the quantification of the bands by densitometry. Activities are shown as fold increases of control from three independent experiments compared with unstimulated control cells. Data are means ± SD. *$p < 0.05$ vs. control. #$p < 0.05$ vs. AngII. (B) AngII activates ERK 1/2, p38MAPK, and JNK through the AT1 receptor. AngII receptor antagonists losartan (10^{-5} mol/L, AT1 receptor specific) and PD 123319 (10^{-5} mol/L, AT2 receptor specific) were added to the culture medium for 30 min and exposed to 10^{-7} mol/L AngII for an additional 10 min. Activities are shown as fold increases of control from three independent experiments compared with unstimulated control cells. Data are means ± SD. *$p < 0.05$ vs. control. #$p < 0.05$ vs AngII. (C) Activity of ERK1/2, p38 MAPK, and JNK after stimulation with AngII in wildtype, sense- and antisense gp91phox-transfected AF. Top panels show representative kinase Western blots, β-actin was measured to control for equal loading of cell protein. Densitometric quantification from three separate experiments is shown in the lower panels. Results are expressed as fold increase over control (untreated cells). Data are means ± SD out of three experiments. *$p < 0.05$ vs. wildtype.

and JNK activation (Fig. 4C). In contrast, NAC and DPI had no effect on AngII-mediated ERK1/2 activation. These findings suggest that p38 and JNK activation is dependent on AngII-induced ROS generation, whereas, activation of ERK1/2 by AngII is not mediated through ROS.

Role of JNK, p38, and MAPK in AngII-induced adventitial fibroblast differentiation

To validate the roles of p38 and JNK activation in mediating Ang II-induced α-SMA expression, we examined the effects of pharmacological p38 and JNK inhibition on α-SMA expression. We found that inhibiting p38 activity with SB202190 or JNK activity with SP600125 blocked AngII-induced increases in α-SMA expression (Fig. 5).

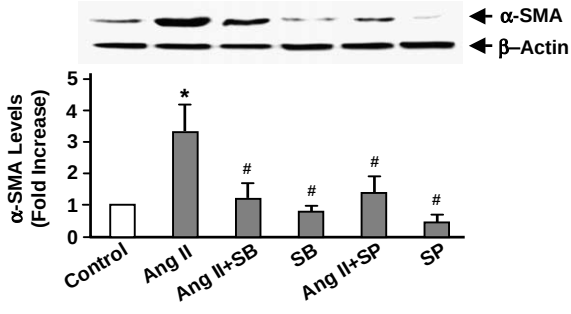

Fig. 5. Effects of SB202190 and SP6000125 on AngII-stimulated α-SM actin expression in adventitial fibroblasts. Quiescent cells were exposed to SB202190 (10^{-5} mol/L) or SP 6000125 (10^{-5} mol/L) for 30 min and then stimulated with AngII 10^{-7} mol/L for 48 h. Western blot analysis of cell lysates was performed with an anti-α-SMA antibody. Densitometric quantification of the bands from three separate experiments is shown in the lower panel, data are means ± SD. *$p < 0.05$ vs. control. #$p < 0.05$ vs. AngII.

Discussion

In the study, we examined the hypothesis that ROS was required for phenotypic transition of fibroblasts into myofibroblasts. The results demonstrated that AngII mediated ROS generation via NADPH oxidase stimulation, which could induce adventitial fibroblast transition to

myofibroblasts through phosphorylation of p38MAPK and JNK pathways.

The contribution of the adventitia to vascular function has largely been considered as support tissue in the past since the discovery of nitric oxide in adventitia [15]. Recently, increasing attention has been given to the role of the vascular adventitia in a variety of vascular diseases, including hypertension and atherosclerosis [16,17]. Several reports have demonstrated that NAD(P)H oxidase is present in the adventitia and is highly regulated [18,19]. Activation of the adventitial fibroblasts with increased production of NAD(P)H oxidase-derived ROS could be a paracrine effect, stimulating cell proliferation and migration [20]. Based on data of Miller et al. [21] gp91phox contributed to the majority of NADPH oxidase activity in fibroblasts. Consistent with this observation, we found that Ang II-induced ROS generation can be almost completely inhibited by antisense gp91phox. In addition, we clearly document a novel role for gp91phox-derived ROS in mediating fibroblast phenotypic differentiation to myofibroblast, as shown by the finding that decreased gp91phox prevented AngII-induced α-SMA expression.

Phenotypic differentiation of fibroblasts into myofibroblasts has been speculated to be a critical step in the pathophysiology of many vascular diseases. To characterize the cell differentiation, we have determined their phenotype by assessing cytoskeletal protein markers. Activation of α-SMA gene during this differentiation is an essential feature and we and others have demonstrated that α-SMA expression in fibroblasts can be induced in response to many factors including TGF-β1, thrombin, and β-FGF [12,22]. However, the role of AngII in α-SMA activation in vascular fibroblasts has never been shown. Several reports indicated that AngII stimulated expression of TGF-β1 in cardiac fibroblasts and myofibroblasts contributing to pathologic myocardial fibrosis [23,24]. Therefore, we studied the ability of AngII to stimulate phenotypic differentiation from vascular adventitial fibroblasts into myofibroblasts. AngII-induced α-SMA expression occurs in 24 h. The effect of AngII on α-SMA expression was also dose-dependent.

Rather this response may be mediated through MAPK kinase signaling pathway activated by AngII in fibroblasts. The generation of ROS in response to various external stimuli has been related to the activation of mitogen-activated protein kinases (MAPK) and transcription factors [25]. MAPK-dependent signaling pathway stimulation induces in a cell specific manner growth and apoptosis, differentiation, and phenotypic transformation [26]. Members of the MAPK family include the extracellular-signal-regulated protein kinases (ERK1/2), stress-activated protein kinases c-Jun-amino terminal kinase (JNK) and p38MAPK [27]. We found that AngII activation of p38MAPK and JNK was redox-sensitive in vascular fibroblasts, while ERK1/2 was not, similar to the findings of Griendling and Fukai in VSMC [28,29]. Of importance, we used different antagonistic strategies to elucidate the function of the JNK and p38MAPK in regulating AngII-induced α-SMA expression. We proved that p38 inhibitor SB202190 and JNK inhibitor SP600125 attenuated the AngII-induced increase in α-SMA expression in fibroblasts. Furthermore, DPI, NAC, and antisense gp91phox also suppressed α-SMA expression in fibroblasts. These data provide strong support to the idea that AngII-mediated NADPH oxidase is capable of inducing the phenotypic differentiation of vascular fibroblasts into myofibroblasts.

The present study was the first to test whether NAD(P)H oxidase-derived reactive oxygen species regulate Angiotensin-II-induced adventitial fibroblast phenotypic transition. Our findings suggest that increased ROS levels induced by AngII lead initially to adventitial fibroblasts activation followed by a phenotypic transformation to myofibroblasts. The dependence of these changes on ROS formation is supported by the observation that inhibition of ROS generation suppressed these responses to AngII. At this juncture it is tempting to speculate that in vivo rise in AngII levels may play a key role in mediating vascular remodeling and possibly hypertension should be the case, our studies provide valuable insight into why AT1 receptor blockers are effective antihypertensive agents.

Acknowledgments

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