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Arachidonic Acid–Dependent Activation of a p22^{phox}-Based NAD(P)H Oxidase Mediates Angiotensin II–Induced Mesangial Cell Protein Synthesis and Fibronectin Expression via Akt/PKB

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ABSTRACT

Angiotensin II (Ang II) induces protein synthesis and hypertrophy through arachidonic acid (AA)- and redox-dependent activation of the serine-threonine kinase Akt/PKB in mesangial cells (MCs). The role of NAD(P)H oxidase component p22^{phox} was explored in this signaling pathway and in Ang II-induced expression of the extracellular matrix protein fibronectin. Ang II causes activation of Akt/PKB and induces fibronectin protein expression, effects abrogated by phospholipase A_2 inhibition and mimicked by AA. Ang II and AA also elicited an increase in fibronectin expression that was reduced with a dominant negative mutant of Akt/PKB. Exposure of the cells to hydrogen peroxide stimulates Akt/PKB activity and fibronectin synthesis. The antioxidant *N*-acetylcysteine abolished Ang II- and AA-induced Akt/PKB activation and fibronectin expression. Western blot analysis revealed high levels of p22^{phox} in MCs. Antisense (AS) but not sense oligonucleotides for p22^{phox} prevented ROS generation in response to Ang II and AA. AS p22^{phox} inhibited Ang II- or AA-induced Akt/PKB as well as protein synthesis and fibronectin expression. These data provide the first evidence, in MCs, of activation by AA of a p22^{phox}-based NAD(P)H oxidase and subsequent generation of ROS. Moreover, this pathway mediates the effect of Ang II on Akt/PKB-induced protein synthesis and fibronectin expression. *Antioxid. Redox Signal.* 8, 1497–1508.

INTRODUCTION

CELLULAR HYPERTROPHY AND EXTRACELLULAR MATRIX accumulation in the glomeruli contributes to the pathogenesis of numerous renal diseases, such as diabetic nephropathy, leading to the development of irreversible renal changes including glomerulosclerosis (4, 13, 31, 37, 41). Data from animal models, as well as cultured renal cells, indicate that the octapeptide hormone angiotensin II (Ang II) contributes to the initiation and the progression of these events via induction of hypertrophy and extracellular matrix expansion in glomerular mesangial cells (MCs) (4, 35, 41).

Ang II-induced oxidative stress has emerged as a critical pathogenic factor in the development of renal and vascular diseases (21, 35). For instance, diabetes is accompanied by an upregulation of the components of the renin–angiotensin system that may contribute to the increased generation of reactive oxygen species (ROS) in the kidney (35). NAD(P)H oxidases of the Nox family are a major source of ROS in many nonphagocytic cells, including renal cells such as MCs (17, 27, 39) . These NAD(P)H oxidases are isoforms of the neutrophil oxidase, in which the catalytic subunits, termed Nox proteins, correspond to homologues of gp91^{phox} (or Nox2), the catalytic moiety found in phagocytes (11, 27, 39). The catalytic center of this oxidase is the membrane-integrated protein gp91^{phox}/Nox2, tightly complexed in the membrane with p22^{phox}, and its activation requires the association with p47^{phox}, p67^{phox}, and the small GTPase Rac, which normally

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resides in the cytoplasm (39). Seven members of the Nox family have been identified in the human genome: Nox1 through Nox5, and the dual oxidases Duox1 and Duox2 (11, 39). We have previously reported that the homologue Nox4 mediates the arachidonic acid (AA)-dependent signaling pathway leading to Ang II-induced protein synthesis in MCs (17, 18), suggesting its potential involvement in kidney hypertrophy under pathologic conditions. However, the molecular composition of the Nox oxidase present in MCs and its biological role(s) are not well understood at present. Since recent studies suggested that Nox4 forms a complex with $p22^{phox}$ to function as a ROS-producing oxidase (2, 29, 39), we investigated the role of $p22^{phox}$ in Ang II-induced MC hypertrophy and expression of the extracellular matrix protein fibronectin.

We show that a $p22^{phox}$-containing NAD(P)H oxidase is present in MCs and provide the first evidence, in MCs, that ROS derived from this oxidase contribute to Ang II-induced protein synthesis and fibronectin expression. We propose that PLA_2-mediated generation of AA is responsible for the activation by Ang II of the $p22^{phox}$-based mesangial Nox. In turn, ROS generated by the oxidase stimulate protein synthesis and fibronectin expression through Akt/PKB activation. The physiological relevance of these findings will be discussed.

MATERIAL AND METHODS

Cell culture and transfections

Rat glomerular MCs were isolated and characterized as described (16). Cells were maintained in RPMI 1640 tissue culture medium supplemented with antibiotic/antifungal solution and 17% fetal bovine serum. MCs were transiently transfected with plasmid DNA [15 μg of vector alone or HA-Akt(K179M)] via electroporation (Gene pulser, Bio-Rad, Hercules, CA), as previously described (16). Antisense oligonucleotides were designed near the ATG start codon of rat $p22^{phox}$ (5′-GATCTGCCCCATGGTGACGACC-3′). Antisense and the corresponding sense oligonucleotides were synthesized as phosphorothiolated oligonucleotides and purified by high performance liquid chromatography (Sigma Genosys, The Woodlands, TX and University of Texas Health Science Center at San Antonio). Antisense and sense oligonucleotides for $p22^{phox}$ were transfected by electroporation as described above.

Immunoprecipitation and Akt/PKB activity assay

MCs were grown in 60- or 100-mm dishes and serum-deprived for 48 h. All incubations were carried out in serum free RPMI 1640 at 37°C for specified duration. The cells were lysed in radioimmune precipitation buffer [20 mmol/L tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.5, 150 m*M* NaCl, 5 m*M* EDTA, 1 m*M* Na_3VO_4, 1 m*M* phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1% NP-40] at 4°C for 30 min. The cell lysates were centrifuged at 10,000 *g* for 30 min at 4°C. Protein was determined in the cleared supernatant using the Bio-Rad method. Immunoprecipitation and Akt/PKB activity assay were performed as described (16). The bands were quantitated by densitometric analysis using Phosphor-Imager analysis (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

MC lysates were prepared as described above for Akt/PKB activity assay. For immunoblotting, proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% low fat milk in Tris-buffered saline, and then incubated with a rabbit polyclonal $p22^{phox}$ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (dilution 1:200), a rabbit polyclonal anti-fibronectin antibody (Sigma) (1:2,500) or a mouse monoclonal anti-β-actin (1:4,000), and a rabbit anti-phospho-Akt (Ser473) antibody or a rabbit polyclonal anti-Akt1/PKBα (Cell Signaling Technology Inc., Danvers, MA) (1:1,000). The appropriate horseradish peroxidase-conjugated secondary antibodies were added and bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using the public domain NIH Image software developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

NADPH oxidase assay

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method (17, 20). MCs were washed five times in ice-cold phosphate-buffered saline and were scraped from the plate in the same solution followed by centrifugation at 800 *g*, 4°C, for 10 min. The cell pellets were resuspended in lysis buffer containing 20 m*M* KH_2PO_4, pH 7.0, 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin. Cell suspensions were homogenized with 100 strokes in a Dounce homogenizer on ice, and aliquots of the homogenates were used immediately. To start the assay, 100 μl of homogenate was added into 900 μl of 50 m*M* phosphate buffer, pH 7.0, containing 1 m*M* EGTA, 150 m*M* sucrose, 5 μ*M* lucigenin as the electron acceptor, and 100 μ*M* NADPH as an electron donor in the presence or absence of 30 μ*M* AA. Photon emission in terms of relative light units was measured either every minute for 12 min or every 30 sec for 10 min in a luminometer. There was no measurable activity in the absence of NADPH. A buffer blank (less than 5% of the cell signal) was subtracted from each reading before calculation of the data. Superoxide production was expressed as relative chemiluminescence (light) units/mg protein. Protein content was measured using the Bio-Rad protein assay reagent.

Detection of intracellular ROS

2′,7′-Dichlorodihydrofluorescein fluorescence. The peroxide-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescin diacetate (Invitrogen/Molecular Probes, Carlsbad, CA) was used to assess the generation of intracellular ROS, as described previously (16). This compound is converted by intracellular esterases to 2′,7′-dichlorodihydrofluorescin, which is then oxidized by hydrogen peroxide to the highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). Differential interference contrast images were obtained simultaneously using an inverted microscope with ×40 Aplanfluo objective (Olympus

America Inc. Center Valley, PA) and an Olympus fluoview confocal laser scanning attachment. The DCF fluorescence was measured with an excitation wavelength of 488 nm light and its emission was detected using a 510–550 nm bandpass filter.

Alternatively, cells were grown in 12- or 24-well plates and serum-deprived for 48 h. Immediately before the experiments, cells were washed with Hank's balanced salt solution (HBSS) and loaded with 50 μ*M* 2′,7′-dichlorodihydrofluorescin diacetate dissolved in HBSS for 30 min at 37°C. They were then incubated with the selected agonist or vehicle for various time-periods. Subsequently, DCF fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, and measured with a multiwell fluorescence plate reader (Wallac 1420 Victor^2, Perkin Elmer, Boston, MA), as described (26).

Dihydroethidium fluorescence. Superoxide anion concentrations within MCs were monitored by measuring the changes in fluorescence resulting from the oxidation of dihydroethidium (DHE) (Molecular Probes). DHE can enter the cell and be oxidized by superoxide to yield ethidium (Eth), which binds to DNA to produce bright red fluorescence. The increase in Eth-DNA fluorescence is suggestive of superoxide production within cells. MCs were stimulated, washed with HBSS, and loaded with 50 μ*M* DHE for 10–30 min. Fluorescence was monitored by laser confocal fluorescence microscopy.

Protein synthesis

[^{3}H]Leucine incorporation into trichloroacetic acid-insoluble material was used to assess protein synthesis, as described (16).

Statistical analysis

Results are expressed as mean ± S.E. Statistical significance was assessed by Student's unpaired *t* test. Significance was determined as probability (p) <0.05.

RESULTS

Ang II induces fibronectin expression via an AA-dependent mechanism in MCs

To assess the effect of Ang II on matrix accumulation, MCs were treated with 1 μ*M* Ang II, and fibronectin protein expression was measured. Lysates of MCs incubated with Ang II for different periods of time were immunoblotted with antibody against fibronectin. Ang II increased fibronectin expression within 12 h and the expression was sustained until 48 h (Fig. 1A). AA mimicked the effect of Ang II on fibronectin expression. Indeed, the direct addition of AA (30 μ*M*) to MCs induced the expression of fibronectin protein in a time-dependent manner with a time course that correlated well with the kinetic of fibronectin expression after exposure to Ang II (Fig. 1A). One of the mechanism by which Ang II elicits an increase in AA production is hydrolysis of phospholipids by PLA_2. We examined the effect of PLA_2 inhibitors, mepacrine and aristolochic acid. Preincubation of MCs with mepacrine or aristolochic acid abolished induction of fibronectin synthesis by Ang II (Fig. 2B). Collectively, these data indicate that the effect of Ang II on fibronectin synthesis is mediated by AA via activation of PLA_2.

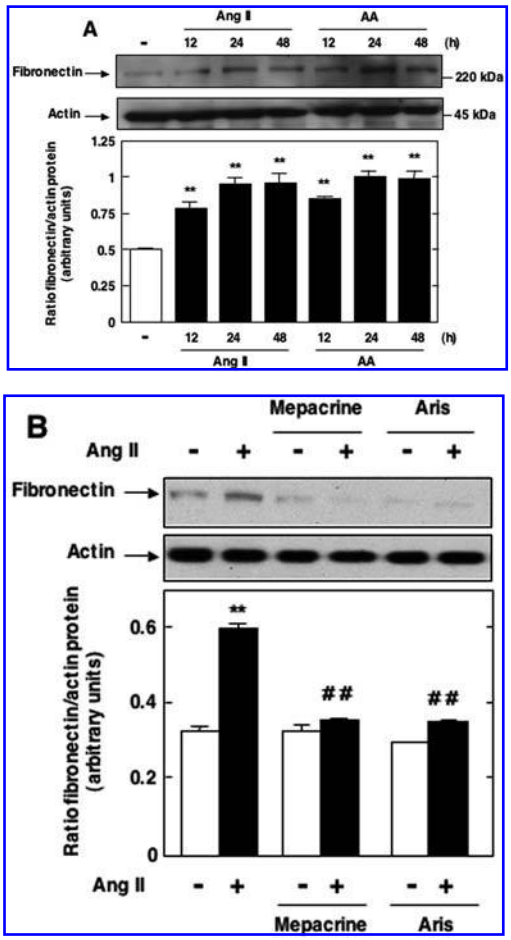


FIG. 1. AA mediates Ang II-induced fibronectin expression in MCs. (**A**) Time course of fibronectin expression by Ang II and AA. Serum-deprived MCs were treated with 1 μ*M* Ang II or 30 μ*M* for the indicated time periods. Fibronectin protein expression was determined by direct immunoblotting of cell lysates. Actin was included as a control for loading and the specificity of change in protein expression. Representative results of Western blot analysis were obtained from three independent experiments. (**B**) Effect of PLA_2 inhibitors on Ang II-induced fibronectin expression. Serum-deprived MCs were preincubated with mepacrine (500 μ*M*, 5 min) or aristolochic acid (50 μ*M*, 30 min), followed by 1 μ*M* Ang II for 24 h. Data are expressed as in (**A**). In (**A**) and (**B**), each histogram at the *bottom panel* represents the ratio of the intensity of fibronectin bands quantified by densitometry factored by the densitometric measurement of actin band. Values are the means ± S.E from three independent experiments.**$p < 0.01$ versus control and ##$p < 0.01$ versus Ang II.

AA-dependent Akt/PKB activation mediates Ang II-induced fibronectin expression

We have recently shown that treatment of MCs with Ang II or AA increases Akt/PKB activity (measured by *in vitro* kinase assay), protein synthesis, and cell hypertrophy (16). To

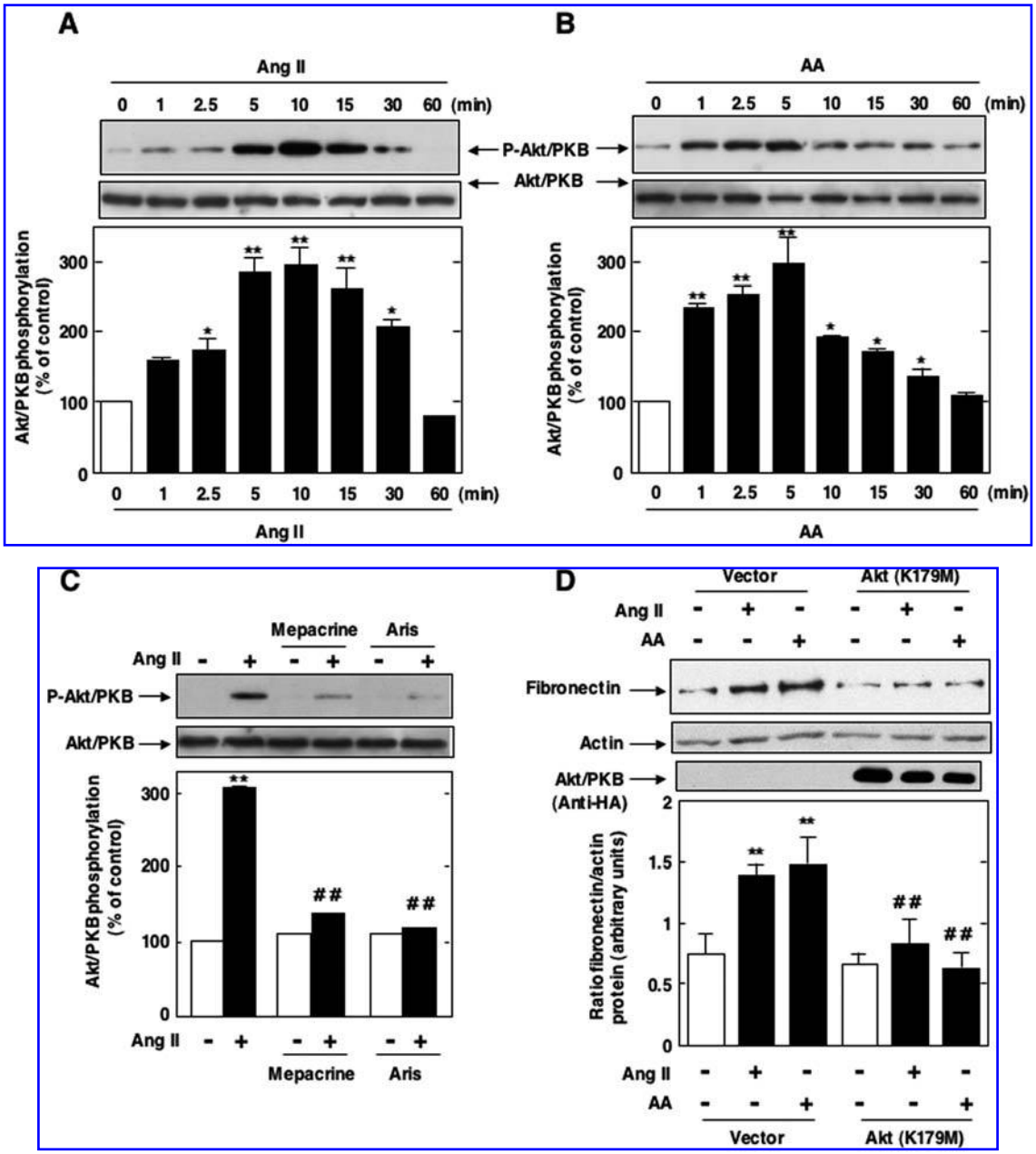


FIG. 2. AA-dependent Akt/PKB activation mediates Ang II-induced fibronectin expression. Akt/PKB activation by Ang II (**A**) or AA (**B**) was assessed using anti-phospho-specific Akt/PKB antibodies (Serine 473). Serum-deprived MCs were treated with 1 μ*M* Ang II and 30 μ*M* AA. (**C**) Effect of PLA_2 inhibitors on Ang II-induced Akt/PKB activation. Serum-deprived MCs were preincubated with mepacrine (500 μ*M*, 5 min) or aristolochic acid (50 μ*M*, 30 min), followed by 1 μ*M* Ang II for 10 min. In (**A**), (**B**) and (**C**), the *middle panels* show the immunoblot analysis of cell lysates with Akt/PKB antibody. Representative results of Western blot analysis were obtained from three independent experiments. Each histogram at the *bottom panel* represents the ratio of the intensity of phosphorylated Akt/PKB bands quantified by densitometry factored by the densitometric measurement of actin band. The data are expressed as percent of control where the ratio in the control was defined as 100%. Values are the means ± S.E from three independent experiments.*$p < 0.05$; **$p < 0.01$ versus control and ##$p < 0.01$ versus Ang II. (**D**) MCs were transfected with HA-tagged inactive Akt/PKB mutant [HA-Akt(179M)] or vector as control and treated with 1 μ*M* Ang II or 30 μ*M* AA for 24 h. Fibronectin protein expression was determined by direct immunoblotting of cell lysates. Actin was included as a control for loading and the specificity of change in protein expression. The *third panel* from the top shows immunoblot of cells transfected with vector or the dominant negative form of Akt/PKB using anti-HA antibody. Representative results of Western blot analysis were obtained from three independent experiments. Data are expressed as in Fig. 1. Values are the means ± S.E from three independent experiments.**$p < 0.01$ versus control and ##$p < 0.01$ versus Ang II.

further confirm Akt/PKB activation, Akt/PKB activity was measured using an antibody that recognizes its phosphorylation on serine 473. Ang II (1 μ*M*) induced a rapid and time-dependent phosphorylation of Akt/PKB (Fig. 2A). Treatment of MCs with AA (30 μ*M*) also resulted in a time-dependent increased phosphorylation of Akt/PKB (Fig. 2B). The time course of Akt/PKB phosphorylation demonstrated that the effect of AA on Akt/PKB phosphorylation was more rapid (maximum at 2.5–5 min) than the effect of Ang II (maximum at 10 min), consistent with the contention that AA mediates Akt/PKB phosphorylation induced by Ang II. This is further confirmed by the finding that preincubation of MCs with the same PLA_2 inhibitors as described above dramatically reduced Akt/PKB phosphorylation induced by Ang II (Fig. 2C). We next investigated the involvement of Akt/PKB in Ang II and AA-induced fibronectin protein synthesis by transiently transfecting the cells with HA-tagged kinase-inactive Akt/PKB with a point mutation in the ATP-binding site [HA-Akt(K179M)]. As shown in Fig. 2D, expression of a dominant-negative form of Akt/PKB dramatically reduced stimulation of fibronectin synthesis by Ang II and AA. Immunoblotting of the cell lysates using anti-HA antibody confirmed the expression of the mutant protein (Fig. 2D, bottom panel). These data demonstrate that Akt/PKB contributes to Ang II- and AA-induced fibronectin synthesis.

Role of ROS in Akt/PKB-mediated induction of fibronectin synthesis by Ang II and AA

The previous observation that ROS mediate Ang II- and AA-induced Akt/PKB activation in MCs led us to investigate a potential role for ROS on fibronectin synthesis. First, we studied the effect of hydrogen peroxide (H_2O_2) on Akt/PKB phosphorylation and fibronectin protein expression. As shown in Fig. 3A and B, 200 μ*M* H_2O_2 induced a robust phosphorylation of Akt/PKB and increase in fibronectin protein synthesis. This is consistent with the time courses of Akt/PKB activation or fibronectin expression by Ang II or AA described previously. These results confirm the previous observations showing that Akt/PKB phosphorylation and fibronectin synthesis are redox-sensitive in MCs (16, 24). The data also indicate that ROS may play a role in the effects of Ang II and AA on Akt/PKB activation and fibronectin synthesis. To test this hypothesis, we examined the effect of *N*-acetylcysteine (NAC), an antioxidant, on Ang II- and AA-induced Akt/PKB phosphorylation and increase in fibronectin synthesis. As shown in Figs. 3C and 4D, NAC blocked Akt/PKB phosphorylation and fibronectin synthesis stimulated by Ang II or AA. These findings are consistent with a critical role for ROS in Akt/PKB-dependent Ang II- and AA-induced fibronectin synthesis.

Role of p22^{phox}-containing NAD(P)H oxidase in Ang II and AA-induced ROS generation in MCs

We have described previously that the release of ROS elicited by Ang II and AA is mediated by Nox4 NAD(P)H oxidase in MCs (17, 18). It has been recently proposed that interaction between Nox4 and p22^{phox} is required to form an ROS-generating NAD(P)H oxidase(2, 29, 39). Therefore, we investigated whether a p22^{phox}-based oxidase may account for the ROS generation in response to Ang II and AA using an antisense oligonucleotide approach. Western blot analysis revealed that p22^{phox} protein is expressed in rat MCs and that transfection of the cells with phosphorothioate-modified antisense (AS) oligonucleotides but not sense (S) oligonucleotides for p22^{phox} markedly decreased p22^{phox} protein expression (Fig. 4A). The production of intracellular ROS by MCs in response to Ang II and AA was demonstrated with a fluorescence-based assay using peroxide-sensitive fluorophore 2′,7′-dichlorodihydrofluorescin diacetate (DCF). Quantification of DCF fluorescence was performed with a multiwell fluorescence plate reader (Fig. 4B) and laser-scanning confocal microscopy (Fig. 4C). Ang II- and AA-stimulated ROS generation was significantly reduced in MCs transfected with AS p22^{phox} (Fig. 4B and C). Conversely, fluorescence was not affected by transfection of MCs with S p22^{phox}. Alternatively, we evaluated the effect of AS p22^{phox} on ROS generation using the superoxide-sensitive fluorophore dihydroethidium (DHE). A strong increase in fluorescence in MCs stimulated with Ang II or AA compared with controls indicated increased ROS levels. Transfection of AS p22^{phox}, but not S p22^{phox}, prevented the increase in ROS generation in response to Ang II or AA (Fig. 4D). In addition, we also assessed the effect of AS p22^{phox} transfection on AA-induced NADPH-dependent superoxide producing activity in MCs. Using lucigenin-enhanced chemiluminescence, we found that AS p22^{phox}, but not S p22^{phox}, inhibited the increase in NADPH oxidase activity caused by addition of AA to MC homogenates (Fig. 4E). Together, these results indicate that p22^{phox} is required for increase of ROS production by Ang II and AA in MCs.

p22^{phox} mediates Ang II-induced AA/redox-dependent Akt/PKB activation and fibronectin synthesis in MCs

We examined the effect of p22^{phox} antisense oligonucleotides on activation of the AA-mediated redox-dependent activation of Akt/PKB by Ang II. Transfection of MCs with AS p22^{phox}, but not S p22^{phox}, prevented the increase in Akt/PKB kinase activity and phosphorylation in response to Ang II or AA (Fig. 5A). These data support a role for p22^{phox} in the redox signaling cascade triggered by Ang II and mediated by AA to activate Akt/PKB.

To assess the role of p22^{phox} in fibronectin synthesis, we tested the effect of AS p22^{phox} on Ang II-and AA-stimulated fibronectin protein expression by Western blot analysis. As shown in Fig. 5B, AS p22^{phox} but not S p22^{phox} significantly reduced stimulation of fibronectin synthesis induced by Ang II and AA.

p22^{phox} mediates Ang II-induced AA/redox-dependent protein synthesis and MC hypertrophy

We have previously shown that Nox4-derived ROS mediates Akt/PKB activation and MC hypertrophy in response to Ang II and AA (17). Since Nox4 and p22^{phox} both act as upstream regulators of Akt/PKB activation, this suggests that p22^{phox} is part of the signaling cascade engaged by Ang II to

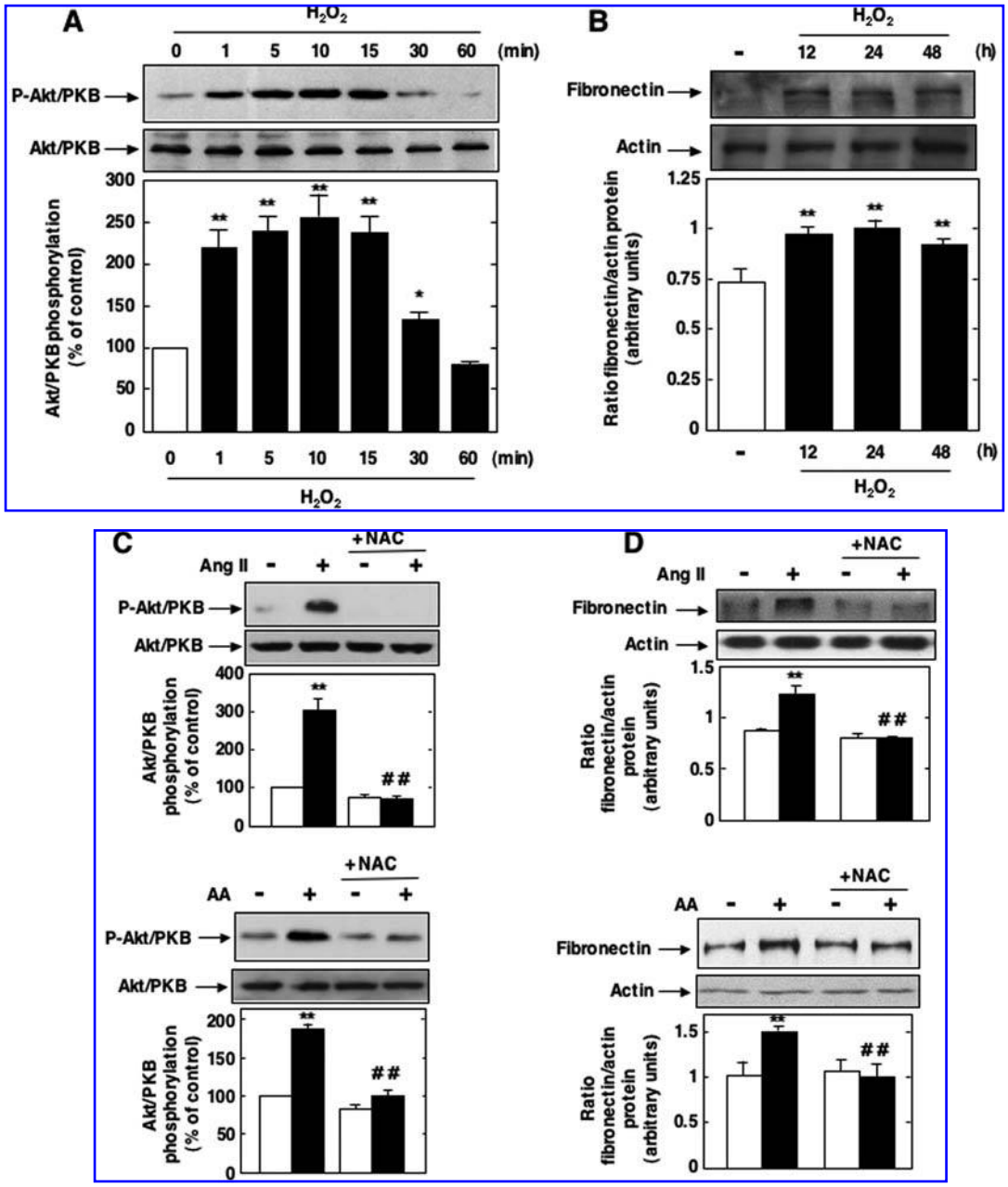


FIG. 3. ROS mediates Akt/PKB-dependent induction of fibronectin synthesis by Ang II and AA. Time courses of Akt/PKB activation (**A**) and fibronectin protein expression (**B**) by H_2O_2. Serum-deprived MCs were treated with 200 μ*M* H_2O_2 for the time periods indicated. Fibronectin protein expression was evaluated as in Fig. 1, and Akt/PKB activation was assessed as in Fig. 2. Representative results of Western blot analysis were obtained from three independent experiments. (**C**) and (**D**) Role of reactive oxygen species in Akt/PKB activation and fibronectin expression by Ang II and AA. Serum-deprived MCs were preincubated with or without NAC (20 m*M*) for 30 min before treatment with 1 μ*M* Ang II (*top panels*) or 30 μ*M* AA (*lower panels*) for 5 min (**C**) and 24 h (**D**). Fibronectin protein expression was evaluated as in Fig.1, and Akt/PKB activation was assessed as in Fig. 2. Representative results of Western blot analysis were obtained from three independent experiments. Data are expressed as in Figs. 1 and 2. Values are the means ± S.E from three independent experiments.*$p < 0.05$; **$p < 0.01$ versus control and ##$p < 0.01$ versus Ang II.

regulate hypertrophy. To test this hypothesis, we evaluated the effect of AS p22^{phox} on Ang II-and AA-stimulated [^{3}H]leucine incorporation. As shown in Fig. 6A and B, transient transfection of the cells with AS p22^{phox} nearly abolished Ang II- and AA-induced [^{3}H]leucine incorporation, whereas S p22^{phox} had no effect. These results suggest that p22^{phox} plays a critical role in Ang II- and AA-induced hypertrophy. Moreover, these data support the concept that AA acts as a mediator of

the signaling pathway activated by Ang II leading to MC hypertrophy and fibronectin synthesis via a p22^{phox}-dependent pathway that results in production of ROS.

DISCUSSION

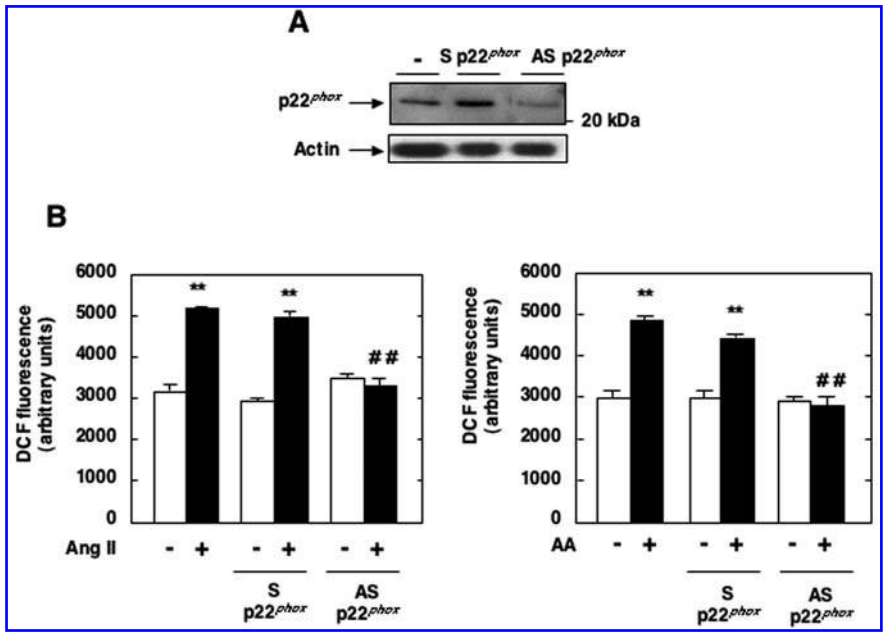
In this study, we provide the evidence that in MCs, Ang II stimulates synthesis of the extracellular matrix protein fibronectin through activation of Akt/PKB and a p22^{phox}-containing NAD(P)H oxidase. We demonstrate that activation of the p22^{phox}-containing oxidase is mediated by a PLA_2-coupled generation of AA and that the ROS act as downstream signal transducers to activate Akt/PKB. Furthermore, p22^{phox} regulates Ang II-stimulated protein synthesis and hypertrophy in MCs.

Cell hypertrophy and accumulation of extracellular matrix protein represent two major processes by which Ang II exerts its deleterious action in the vasculature and the kidney, thereby contributing to the pathogenesis of atherosclerosis and renal fibrosis (4, 34, 40). Ang II stimulates the release of AA upon PLA_2 activation in a variety of cell types, including MCs (9, 16, 33, 38). AA is a critical mediator of Ang II-induced vascular smooth muscle cell and MC hypertrophy (16–18, 20, 43). The data presented in this study demonstrate that AA mimics the stimulatory effect of Ang II on fibronectin protein synthesis, suggesting that fibronectin upregulation by Ang II is also mediated via AA release. Of note is that the concentrations of AA used in this study are within the range of concentrations that can be achieved in cultured cells or *in vivo* in cells and tissues (3). Use of PLA_2 inhibitors mepacrine and aristolochic acid provides further evidence that AA acts as a second messenger in mediating the stimulatory action of Ang II on fibronectin protein expression. We also demonstrate that AA utilizes the serine-threonine protein kinase Akt/PKB as downstream signal transducer to induce fibronectin synthesis. Although several studies suggested that Akt/PKB is involved in extracellular matrix accumulation (5, 12, 15, 36), the present study, to our knowledge, is the first report that AA-dependent Akt/PKB activation mediates Ang II-induced fibronectin expression.

In most mammalians cells, AA is oxidized through cyclooxygenases, lipoxygenases, and/or cytochrome P450 pathways to yield eicosanoids that mediate most of its biological effects. For instance, metabolites of 12/15-lipoxygenases contribute to Ang II-induced cell growth and matrix protein synthesis in vascular and renal cells (34, 43). Nevertheless, a direct role of AA has been implicated in certain cellular responses via activation of protein kinases and/or phosphatases (1, 6, 23, 30). Importantly, we have previously shown that AA activates Akt/PKB and MC hypertrophy without the requirement of eicosanoid biosynthesis (16). These observations strongly suggest that these metabolites of AA do not mediate its effect on Akt/PKB-induced fibronectin protein expression in MCs. However, further studies are required to confirm this assumption.

There is now considerable evidence that ROS can function as classic second messenger molecules (8). It has been reported that extracellular matrix protein accumulation can be stimulated by oxidative stress in MCs. Here, we provide new insights concerning the molecular mechanism involved in these events and show that redox-dependent activation of Akt/PKB contributes to fibronectin expression. Moreover, the effect of Ang II and AA on Akt/PKB and fibronectin expression is inhibited by the antioxidant NAC, indicating that ROS act as potential signaling molecules responsible for Akt/PKB-mediated induction of fibronectin expression by Ang II and AA. Whereas multiple pathways may result in ROS generation, recent studies indicate that a multicomponent phagocyte-like NAD(P)H oxidase is a major source of Ang II-induced ROS in many nonphagocytic cells, including renal cells such as tubular epithelial cells and MCs (10, 17, 18, 21, 27). NAD(P)H oxidase was originally found in neutrophils and is composed of two plasma membrane-associated proteins, gp91^{phox} (the catalytic subunit) and p22^{phox}, which comprise flavocytochrome b_{558}, and the cytosolic factors, p47^{phox} and p67^{phox}. The small GTPase Rac participates in the assembly of the active complex (39). Electrons from NAD(P)H are transferred through the enzyme to molecular oxygen to generate superoxide and subsequently other ROS such as H_2O_2. Gp91^{phox} is only one member of a family of homologous proteins termed Nox (11, 27, 38). Although the NAD(P)H oxidase component p22^{phox} is known to be expressed in MCs (25), its potential role in Ang II-induced oxidative stress and signaling has not been explored. Our data demonstrate that p22^{phox} is clearly required for Ang II- and AA-induced ROS generation and Akt/PKB activation, indicating that a p22^{phox}-based oxidase is coupled to Ang II redox signaling in MCs. Furthermore, p22^{phox} mediates the stimulation of fibronectin expression and MC hypertrophy by Ang II and AA. This report provides the first evidence that p22^{phox} is a critical component of ROS-generating mesangial NAD(P)H oxidase and suggest a central role for this oxidase system in Ang II-induced hypertrophy and fibrosis in the kidney. The observations described here are consistent with reports showing a role for p22^{phox} in the effects of Ang II and AA on cell growth (22, 40, 43) as well as with data demonstrating that p22^{phox}-containing NAD(P)H oxidase contributes to ROS generation in response to thrombin or urotensin II in vascular cells (7, 19). Importantly, our findings regarding the role of p22^{phox} in fibronectin protein accumulation are in agreement with the recent report from Xia *et al.* (42) showing that p22^{phox} is implicated in collagen type IV expression and support the contention that p22^{phox}-based enzyme NAD(P)H oxidases modulates MC extracellular matrix synthesis.

We have previously reported that Nox4-derived ROS mediate AA-dependent Akt/PKB and protein synthesis stimulation by Ang II in MCs (17, 18). Hence, p22^{phox} and Nox4 appear to both contribute to Ang II signaling and MC hypertrophy. Interestingly, recent studies show that direct interaction of Nox4 protein with p22^{phox} is required for the formation of a functionally active NAD(P)H oxidase in various cell systems (2, 29, 39). In addition, p22^{phox} expression seems to facilitate the Nox4-dependent ROS production (2, 29, 39). Therefore, it is tempting to speculate that in MCs, p22^{phox} and Nox4 form also a complex that account for Ang II- and AA-induced NAD(P)H-dependent ROS generation and the subsequent hypertrophic and fibrotic responses. It is appealing to consider that p22^{phox}-containing oxidase may be a pivotal signal transducer commonly shared by both hypertrophic and fibrotic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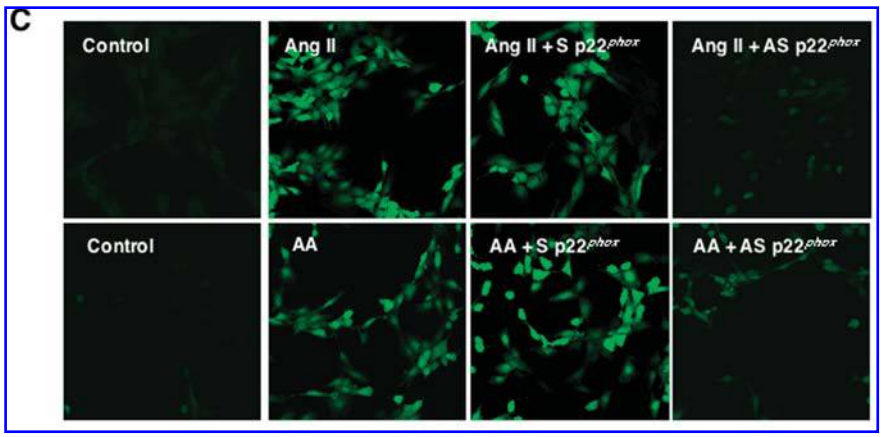
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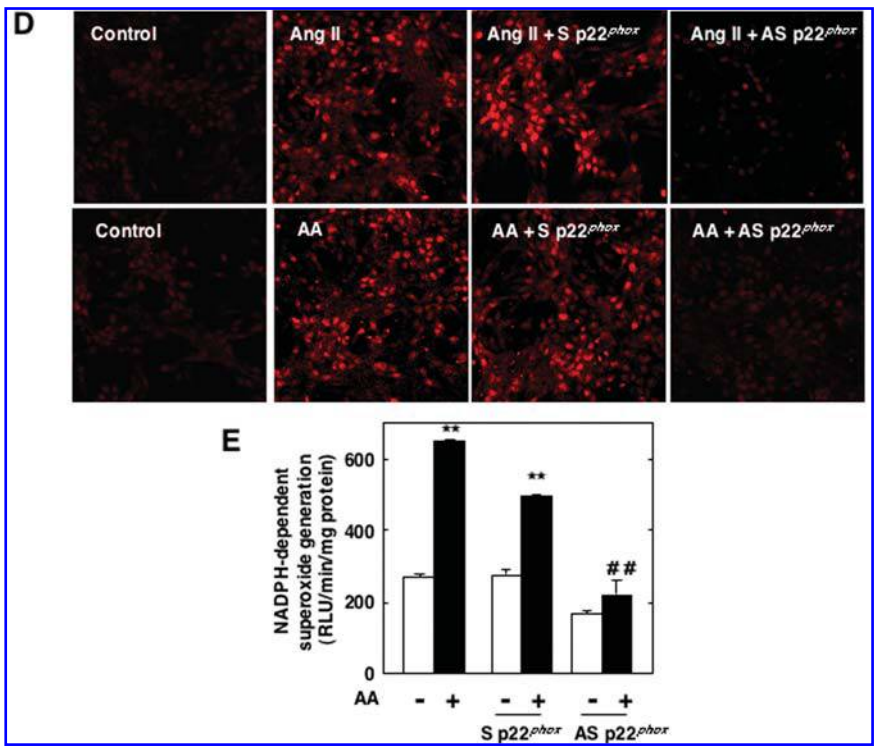
AA
AA + S p22phox
AA + AS p22phox


D
Control
Ang II
Ang II + S p22phox
Ang II + AS p22phox
Control
AA
AA + S p22phox
AA + AS p22phox
E
NADPH-dependent superoxide generation (RLU/min/mg protein)
600
400
200
0
**
**
##
AA - + - + - +
S p22phox
AS p22phox

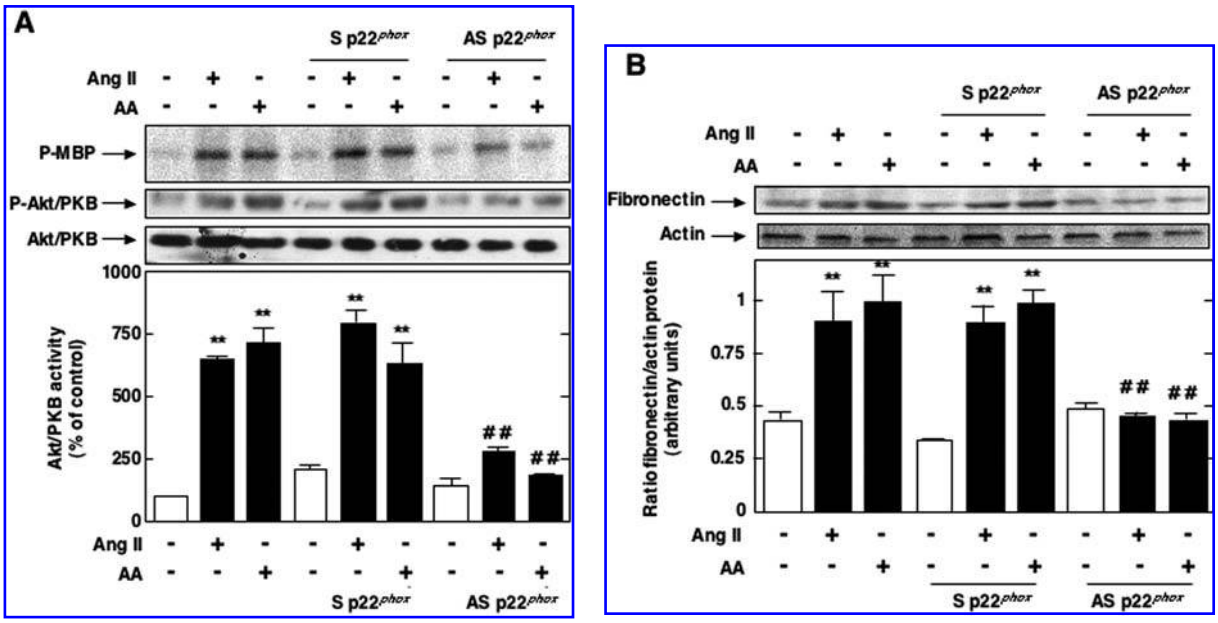


FIG. 5. p22^{phox} mediates Ang II- and AA-induced Akt/PKB activation and fibronectin expression in MCs. (**A**) Serum-deprived MCs were transfected with S p22^{phox} (1 μ*M*) or AS p22^{phox} (1 μ*M*) and treated with 1 μ*M* Ang II or 30 μ*M* AA for 5 min. Akt/PKB activation was assessed either by immunecomplex kinase assay in Akt/PKB immunoprecipitates with myelin basic protein as substrate (*top panel*) or using anti-phospho-specific Akt/PKB antibodies as described in Fig. 2 (*middle panel*). The *bottom panel* shows the immunoblot analysis of cell lysates with Akt/PKB antibody. Representative autoradiogram and immunoblot were obtained from three independent experiments. Each histogram of the *bottom panel* represents the ratio of the radioactivity incorporated into the phosphorylated myelin basic protein quantified by Phosphor-Imager analysis, factored by the densitometric measurement of Akt/PKB band. The data are expressed as percent of control where the ratio in the untreated cells was defined as 100%. Values are the means ± S.E from three independent experiments. $^{**}p < 0.01$ versus control and $\#\#p < 0.01$ versus Ang II. (**B**) Serum-deprived MCs were transfected with S p22^{phox} (1 μ*M*) or AS p22^{phox} (1 μ*M*) and treated with 1 μ*M* Ang II or 30 μ*M* AA for 24 h, and fibronectin protein expression was determined by direct immunoblotting of cell lysates. Representative results of Western blot analysis were obtained from three independent experiments. Data are expressed as in Fig. 1. Values are the means ± S.E from three independent experiments. $^{**}p < 0.01$ versus control and $\#\#p < 0.01$ versus Ang II.

pathways triggered by Ang II in MCs. This observation is highly relevant for the design of therapeutic strategies able to prevent the initiation or progression of the numerous renal diseases where hypertrophic and fibrotic events are involved. For instance, renal hypertrophy and extracellular matrix accumulation are early and late features of diabetic nephropathy (35, 37, 41). Moreover, we have recently described that activation of NAD(P)H oxidase Nox4 plays a critical role in diabetes-induced oxidative stress, kidney hypertrophy, and fibronectin expression (15). However, it is important to note that p22^{phox} interacts with other Nox isoform such as gp91^{phox} or Nox1 (2, 38). It has been suggested that these two Nox isoforms are expressed in MCs (28, 32). Therefore, their involvement in Ang II effects cannot be excluded. Importantly, diabetes is a disease characterized by activation of the renal renin–angiotensin system, where it is well established that

FIG. 4. Role of p22^{phox}-containing NAD(P)H oxidase mediates Ang II and AA-induced ROS generation in MCs. (**A**) MCs were not (-) or transfected by electroporation with S p22^{phox} (1 μ*M*) or AS p22^{phox} (1 μ*M*), and p22^{phox} protein expression was determined by direct immunoblotting with rabbit polyclonal p22^{phox} antibodies. (**B**) Untransfected and S p22^{phox}- or AS p22^{phox}-transfected MCs were serum-starved and then treated with 1 μ*M* Ang II or 30 μ*M* for 5 min. DCF fluorescence, reflecting the relative levels of intracellular ROS, was measured with a multiwell fluorescence plate reader as described under "Materials and methods". Values are the means ± S.E. of three independent experiments. $^{**}p < 0.001$ versus control; $\#\#p < 0.01$, compared with treatment with Ang II or AA in untransfected cells. (**C**) and (**D**) Representative photomicrographs of DCF fluorescence (**C**) and DHE staining (**D**) imaged with a confocal laser scanning fluorescence microscope in untransfected, S p22^{phox}-transfected or AS p22^{phox}-transfected MCs after exposure to 1 μ*M* Ang II or 30 μ*M* AA for 10 min. (**E**) NADPH oxidase activity was measured by incubating homogenates from MCs untransfected, S p22^{phox}-transfected or AS p22^{phox}-transfected with 100 μ*M* NADPH and 5 μ*M* lucigenin alone (*open bars*) or lucigenin in the presence of 30 μ*M* AA (*filled bars*). Superoxide generation was determined by photoemission every minute for 12 min and expressed as relative light units (RLU)/mg protein. The initial rate of enzyme activity was calculated over the first 30 to 120 sec of exposure to NADPH. NADPH-driven superoxide production was expressed as RLU/min/mg protein. Values are the mean ± S.E. of three independent experiments. $^{**}p < 0.01$ versus control and $\#\#p < 0.01$ versus treatment with AA in untransfected cells.

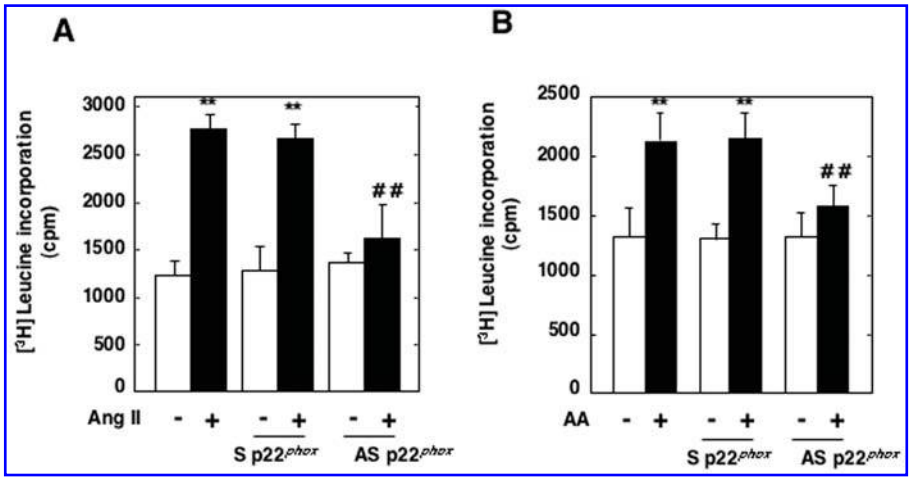


FIG. 6. p22^{phox} mediates Ang II- and AA-induced protein synthesis in MCs. Serum-deprived MCs were transfected with S p22^{phox} (1 μ*M*) or AS p22^{phox} (1 μ*M*) and treated with (*filled bars*) or without (*open bars*) 1 μ*M* Ang II (**A**) or 30 μ*M* AA (**B**) for 48 h. Protein synthesis was measured by [^{3}H]leucine incorporation into TCA precipitable material. Values are the means ± S.E. of three independent experiments. $^{**}p < 0.01$ compared with control. $^{\#\#}p < 0.01$, compared with treatment with Ang II or AA.

Ang II exerts potent effects on several renal cell types to stimulate hypertrophy and synthesis of extracellular matrix proteins (35, 41). Therefore, it is possible that diabetes and high glucose cause the release of Ang II that in turn stimulates hypertrophy and fibronectin expression via activation of the p22^{phox}-containing NAD(P)H oxidase. This hypothesis is supported by recent studies in MCs showing that high glucose-induced ROS generation by p22^{phox} is required for collagen IV accumulation (42) and that Nox4 mediates the upregulation of fibronectin expression by glucose (15). Interestingly, transforming growth factor-β, the major mediator of Ang II fibrotic effects in diabetes, also causes fibronectin and collagen IV expression via Akt/PKB activation in MCs (12, 36). Furthermore, transforming growth factor-β also upregulates extracellular matrix expression via NAD(P)H oxidase-derived ROS in MCs (14, 28). In conclusion, specific inhibition of the redox pathway involving AA- and p22^{phox}-dependent Akt/PKB activation leading to MC hypertrophy and fibronectin expression may selectively target several important biological responses to prevent or reverse pathophysiologic manifestations of renal diseases including diabetic nephropathy.

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ABBREVIATIONS

AA, arachidonic acid; Akt/PKB, Akt/protein kinase B; Ang II, angiotensin II; AS, phosphorothioated antisense oligonucleotides; DCF, 2′,7′-dichlorodihydrofluorescein; DHE, dihydroethidium; Eth, ethidium; H_2O_2, hydrogen peroxide; MCs, mesangial cells; NAC, *N*-acetylcysteine; Nox4, NAD(P)H oxidase 4; PLA_2, phospholipase A_2; ROS, reactive oxygen species; S, phosphorothioated sense oligonucleotides.

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