Vascular Smooth Muscle Cell NAD(P)H Oxidase Activity During the Development of Hypertension: Effect of Angiotensin II and Role of Insulinlike Growth Factor-1 Receptor Transactivation

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Objective: We investigated whether angiotensin II (Ang II)-induced reactive oxygen species (ROS) generation is altered in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHR) during the phases of prehypertension, developing hypertension, and established hypertension and assessed the putative role of insulinlike growth factor-1 receptor (IGF-1R) in Ang II-mediated actions.

Methods: The VSMCs from SHR and Wistar-Kyoto rats (WKY) aged 4 (prehypertensive), 9 (developing hypertension), and 16 (established hypertension) weeks were studied. The ROS production and NAD(P)H oxidase activation were determined by fluorescence and chemiluminescence, respectively. The role of IGF-1R was assessed with the selective inhibitor AG1024. The ROS bioavailability was manipulated with Tiron (10^{-5} mol/L) and diphenylene iodonium (DPI) (10^{-6} mol/L).

Results: Angiotensin II dose dependently increased ROS production in WKY and SHR at all ages. The Ang II-induced responses were greater in SHR versus WKY at 9 and 16 weeks ($P < .05$). The Ang II-stimulated ROS increase was greater in 9- and 16-week-old SHR versus 4-week SHR ($P < .05$). These effects were reduced by AG 1024. Basal NAD(P)H oxidase activity was higher in VSMCs from 9-week-old SHR versus 4-week-old rats ($P < .05$). Angiotensin II induced a significant increase in oxidase activity in VSMCs from 9- and 16-week-old SHR ($P < .001$), without influencing responses in cells from 4-week-old SHR. Pretreatment of 9- and 16-week-old SHR cells with AG1024 reduced Ang II-mediated NAD(P)H oxidase activation ($P < .05$).

Conclusions: Basal and Ang II-induced NAD(P)H-driven ROS generation are enhanced in VSMCs from SHR during development of hypertension, but not in cells from prehypertensive rats. Transactivation of IGF-1R by Ang II may be important in vascular oxidative excess in the development of hypertension in SHR. Am J Hypertens 2005;18:81–87 © 2005 American Journal of Hypertension, Ltd.

Key Words: Spontaneously hypertensive rats, intracellular signaling, growth, hypertrophy.

Reactive oxygen species (ROS), including superoxide ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2) are signaling molecules that regulate vascular smooth muscle cell (VSMC) function.[1,2] In vascular cells, the major source of $\cdot O_2^-$ is a neutrophil-like, membrane-associated NAD(P)H oxidase, which when activated, catalyzes the 1-electron reduction of oxygen using NAD(P)H as the electron donor, to produce $\cdot O_2^-$.[3,4] Of the many factors regulating NAD(P)H oxidase in the vasculature, angiotensin II (Ang II) appears to be one of the most important.[5–7] NAD(P)H-driven generation of $\cdot O_2^-$ plays an important role in Ang II-mediated VSMC growth, contraction/relaxation, and inflammation. These pro-

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cesses are mediated through redox-dependent signaling events, including activation of MAP kinases, particularly p38MAP kinase, ERK5 and JNK, phosphorylation of tyrosine kinases, mobilization of intracellular Ca^{2+}, and activation of transcription factors.[8,9] In addition, growth factor receptors such as platelet-derived growth factor (PDGFR), epidermal growth factor receptor (EGFR), and insulinlike growth factor-1 receptor (IGF-1R) are activated by H_2O_2 and $\cdot O_2^-$.[1,2,10,11] Activated EGFR, in turn, stimulates generation of intracellular ROS,[12,13] suggesting that ROS are both upstream and downstream of receptor tyrosine kinases.

In conditions associated with vascular damage, such as in hypertension, ischemia–reperfusion injury, atherosclerosis, and diabetes, Ang II-induced stimulation of NAD(P)H oxidase is increased and generation of ROS is enhanced.[6,14,15] Underlying mechanisms have not yet been fully identified, but increased activation of c-Src, protein kinase C, and phospholipase D (PLD) may be important.[7,16–18] It is also possible that receptor tyrosine kinases may play a role,[19] especially because AT_1 receptor-mediated transactivation of EGFR is increased in VSMCs from spontaneously hypertensive rats (SHR).[20] We recently demonstrated that in VSMCs from adult normotensive Wistar Kyoto rats (WKY) Ang II stimulates NAD(P)H-inducible ROS production through transactivation of EGFR and IGF-R1.[21] Whether these processes also play a role in Ang II-mediated vascular oxidative excess in hypertension remain unclear.

Although it is now well established that bioavailability of ROS is increased in various models of experimental hypertension,[6,22,23] it is still uncertain whether vascular oxidative stress is a primary event or a consequence of development of hypertension. Zalba et al[24] demonstrated that vascular p22phox mRNA level and NAD(P)H-driven generation of $\cdot O_2^-$ are increased in 30-week-old SHR but not in 16-week-old SHR compared to age-matched WKY. Hamilton et al[25] reported that $\cdot O_2^-$ increases with aging, possibly due to enhanced activation of NAD(P)H oxidase.

In the present study we questioned whether augmented Ang II-mediated generation of vascular ROS is a primary or secondary event in the development of hypertension in SHR, by studying VSMCs from SHR during the phases of prehypertension, developing hypertension, and established hypertension. In addition, we assessed whether receptor tyrosine kinases, specifically IGF-1R, play a role in Ang II-induced production of ROS in VSMCs from rats at different stages in the development of hypertension.

Methods

Animals and Experimental Design

The study was approved by the Animal Ethics Committee of the Clinical Research Institute of Montreal (IRCM) and carried out according to the recommendations of the Canadian council for Animal Care. Male SHR (Taconic Farms Inc, Germantown, NY) of 4, 9, and 16 weeks (n = 10/group), corresponding to the prehypertensive, developing, and established phases of hypertension, were studied. Rats were housed under standardized conditions of controlled temperature (22°C) and humidity (60%) and a 12-h light/dark cycle. Animals were fed regular commercial pelleted rat chow and given tap water ad libitum. Systolic blood pressure (BP) was monitored indirectly in conscious prewarmed slightly restrained rats by the tail–cuff method and recorded on a Grass model 7 polygraph (Grass Medical Instruments, Quincy, MA), 1 to 2 days before experimentation.

Cell Culture

The VSMCs derived from the mesenteric vascular bed were isolated and characterized as previously described in detail.[26,27] Mesenteric arteries were cleaned of adipose and connective tissue, VSMCs were dissociated by digestion of vascular arcades with 2 mg/mL collagenase and 0.15 mg/mL elastase, the tissue was filtered, and the cell suspension centrifuged and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing heat-inactivated calf serum, L-glutamine, HEPES, penicillin, and streptomycin. Cells were cultured in DMEM containing 10% fetal calf serum (FCS). Early passage mesenteric VSMCs (passages 3 to 6) were studied. For NAD(P)H oxidase assay, cells were seeded into 100-mm dishes, fed every other day, and used at confluence. Cells were rendered quiescent by serum deprivation for 30 h before experimentation as previously described.[21]

Measurement of ROS in Intact Cells

The Ang II-induced generation of ROS was measured with the fluorescent dye 5-(and 6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) (Molecular Probes, Inc., Eugene, OR), which is a chloromethyl derivative of dichlorodihyrofluorescein diacetate (DCFDA) that exhibits much better retention in living cells than DCFDA. Cells were washed in modified Hank's buffered saline containing (in mmol/L) 137 NaCl, 4.2 $NaHCO_3$, 3 $NaHPO_4$, 5.4 KCl, 0.4 KH_2PO_4, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 0.8 $MgSO_4$, 10 glucose, and 5 HEPES (pH 7.40) and loaded with CM-H_2DCFDA (5 μmol/L), which was dissolved in dimethyl sulfoxide and incubated for 40 to 50 min at room temperature. CM-H_2DCFDA fluorescence was measured by fluorescence digital imaging using an Axiovert 135 inverted microscope (×40 oil immersion objective) and Attofluor Digital Fluorescence System (Zeiss, Oberkochen, Germany) with an excitatory wavelength of 495 nm. Video images of fluorescence at 520 nm emission were obtained using an intensified CCD camera system (Zeiss) with the output digitized to a resolution of 512 × 480 pixels. Although CM-H_2DCFDA reacts with intracellular H_2O_2 as well as with other peroxides, the fluorescence signal elicited by Ang II appears to be derived primarily from H_2O_2, as we and other investigators previously reported.[7,28]

Generation of ROS was measured in unstimulated cells and in cells exposed to increasing concentrations of Ang II

(10^{-11} to 10^{-5} mol/L) in the absence and presence of diphenylene iodinium (DPI) (10^{-6} mol/L), which inhibits flavin-containing enzymes, including NAD(P)H oxidase. To evaluate the role of IGF-1R, cells were pre-exposed to AG 1024 (5×10^{-6} mol/L), a selective IGF-1R inhibitor.[29] Cells were pretreated with inhibitors for 15 to 20 min before Ang II addition.

Measurement of NAD(P)H Oxidase Activity

Quiescent VSMCs were stimulated with Ang II for 15 min. In some experiments, cells were pre-exposed for 30 min to AG 1024. Cell fractionation was performed according to the method described by Griendling et al.[30] Cells were washed twice in ice-cold phosphate-buffered saline (PBS), scraped, transferred to centrifuge tubes, and the plate was washed with an additional volume of PBS. Cells were then centrifuged at 750 *g*, at 4°C for 10 min. The supernatant was discarded, and the pellet was resuspended in 0.5 mL of lysis buffer (20 mmol/L monobasic potassium phosphate, 1 mmol/L EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.75 μg/mL pepstatin, and 0.5 mmol/L phenylmethylsulfonyl fluoride), sonicated, and ultracentrifuged at 29,000 *g* for 20 min at 4°C. The supernatant (cytosolic fraction) was removed, and the pellet (membrane fraction) was resuspended in the original volume of lysis buffer and dounced 20 to 30 times, and stored on ice until use. Protein content was measured in aliquots using the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hercules, CA).

The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in the membrane fraction,[30] which is the major source of the activated oxidase. NAD(P)H (10^{-4} mol/L) was added to the suspension (400 μL) containing lucigenin (5 μmol/L). This concentration of lucigenin does not appear to be involved in redox cycling and specifically detects $\cdot O_2^-$.[31] Luminescence was measured every 18 sec for 3 min in a luminometer (AutoLumat LB 953, Berthold Technologies, Bad Wildbad, Germany). A buffer blank was subtracted from each reading. The amount of $\cdot O_2^-$ generated was calculated by comparison with a standard curve using xanthine/xanthine oxidase.[32] Activity is expressed as nanomoles of $\cdot O_2^-$ per minute per milligram of protein. To verify the specificity of the lucigenin assay for $\cdot O_2^-$ in our models, we examined effects of superoxide dismutase (SOD) (120 U/mL) (enzymatic scavenger of $\cdot O_2^-$) and tiron (10 mmol/L) (nonenzymatic scavenger of $\cdot O_2$) on Ang II-stimulated activation of NAD(P)H oxidase.

Western Blotting of ERK1/2

Quiescent cells from adult rats were stimulated with Ang II for 5 min. In some experiments cells were pre-exposed for 30 min to AG1420. Cells were washed with cold PBS, and 800 μL of lysis buffer (Na pyrophosphate 50 mmol/L, NaF 50 mmol/L, NaCl 50 mmol/L, EDTA 5 mmol/L, EGTA 5 mmol/L, Na_3VO_4 2 mmol/L, HEPES [at pH 7.4] 10 mmol/L, Triton X-100 0.1%, PMSF 50 mmol/L) was added. The plates were placed on dry ice for 1 min. Cells were scraped off, transferred to Eppendorf tubes, and sonicated for 1 sec. The protein supernatant was separated by centrifugation and protein concentrations were determined with the Bio-Rad Protein Assay reagent (Bio-Rad Laboratories). Equal amounts of proteins were loaded on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane (Boehinger Mannheim, Laval, PQ) for 1 h at 100 V. Membranes were blocked with blocking buffer, containing tris-buffered saline, 0.1% Tween-20 with 5% w/v nonfat dry milk and incubated for 24 h at 4°C. Membranes were incubated for 24 h at 4°C with a phospho-specific ERK1/2 antibody (Calbiochem, La Jolla, CA) (diluted 1:1000). Membranes were washed, incubated with a goat antirabbit HRP conjugated antibody (Bio-Rad Laboratories) diluted 1:2000 for 1 h at room temperature, and washed extensively. Membranes were then incubated with Blotting Substrate (POD) (Boehinger Mannheim) following the manufacture's protocol, exposed to film, and developed. The film was scanned by ScanJet 6100C/T (Hewlett Packard, Greeley, NC) and computer-saved.

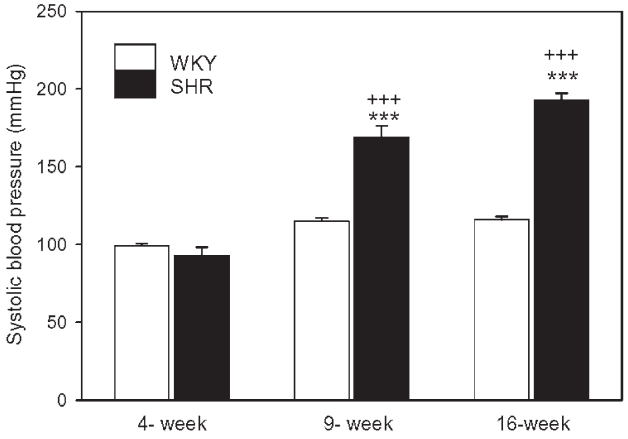

FIG. 1 Systolic blood pressure in 4-, 9-, and 16- week-old SHR and WKY. Bars represent mean ± SEM of 10 animals in each group. A significant difference was assessed by one-way ANOVA and Newman-Keuls post-test. ***$P < .001$ versus WKY counterpart. +++$P < .001$ versus 4-week-old SHR.

Statistical Analyses

Experiments were repeated 4 to 6 times in duplicate. Results are presented as mean ± SEM and compared by ANOVA or by Student *t* test where appropriate. $P < .05$ was considered significant.

Results

The systolic BP of 9-week-old SHR and 16-week-old SHR was significantly greater than that of age-matched WKY ($P < .001$; Fig. 1). The systolic BP of 9- and 16-week-old SHR was significantly higher than in 4-week-old SHR.

The systolic BP was not significantly different between 4-week-old SHR and WKY.

The Ang II stimulation of VSMCs from 4-, 9-, and 16-week-old SHR resulted in a slow and sustained increase in CM-H_2DCFDA fluorescence, similar to our previous findings.[21] Maximal responses were obtained within 15 to 20 min after Ang II addition. It has been previously demonstrated that the CM-H_2DCFDA fluorescence signal derives primary from H_2O_2, because catalase, which reduces H_2O_2, to H_2O and O_2, abolished the increase in DCFDA fluorescence.[7] The Ang II induced a dose-dependent increase in H_2O_2 (Fig. 2), with maximal responses at 10^{-7} mol/L in VSMCs from both SHR and WKY at all ages studied. Responses to Ang II were significantly greater in SHR compared to WKY at 9 and 16 weeks ($P <$ .05) (Fig. 2). The increase induced by Ang II was greater in 9- and 16-week-old SHR versus 4-week-old SHR. The Ang II-induced responses were inhibited by DPI, indicating that NAD(P)H oxidase is a possible source of Ang II-stimulated ROS generation.

To investigate whether IGF-1R plays a role in enhanced Ang II-mediated ROS generation in SHR, we examined the effects of AG 1024 on Ang II-induced CM-H_2DCFDA fluorescence. AG 1024 did not influence CM-H_2DCFDA fluorescence in VSMCs from prehypertensive SHR, but significantly attenuated responses in cells from 9- and 16-week-old SHR ($P < .05$; Fig. 3).

Basal NAD(P)H-oxidase activity, as assessed by low-dose lucigenin chemiluminescence, was significantly higher in VSMCs from 9-week-old SHR compared to cells from 4-week-old rats (Fig. 4). Although there was a trend for basal NAD(P)H oxidase activity to be increased in VSMCs from 16-week-old SHR, significance was not achieved. Enzyme activity in VSMCs from 9- and 16-week-old SHR increased when cells were stimulated with Ang II, but there was no effect on cells from 4-week-old rats. Pretreatment of 9- and 16- week-old SHR cells with AG 1024 significantly reduced Ang II-mediated activation of NAD(P)H oxidase (Fig. 5). Responses in VSMCs from 4-week-old rats were not influenced by AG 1024. Superoxide dismutase and tiron attenuated Ang II-induced activation of NAD(P)H oxidase, demonstrating that the lucigenin signal derives from NAD(P)H-driven $\cdot O_2^-$ generation (data not shown).

To confirm that IGF-1R influences Ang II-mediated signaling, we assessed effects of Ang II on ERK1/2 phosphorylation in the absence and presence of AG1024. As demonstrated in Fig. 6, Ang II stimulation significantly increased ERK1/2 phosphorylation in VSMCs, with enhanced actions in cells from SHR. In the presence of AG1024, Ang II-induced responses were significantly reduced.

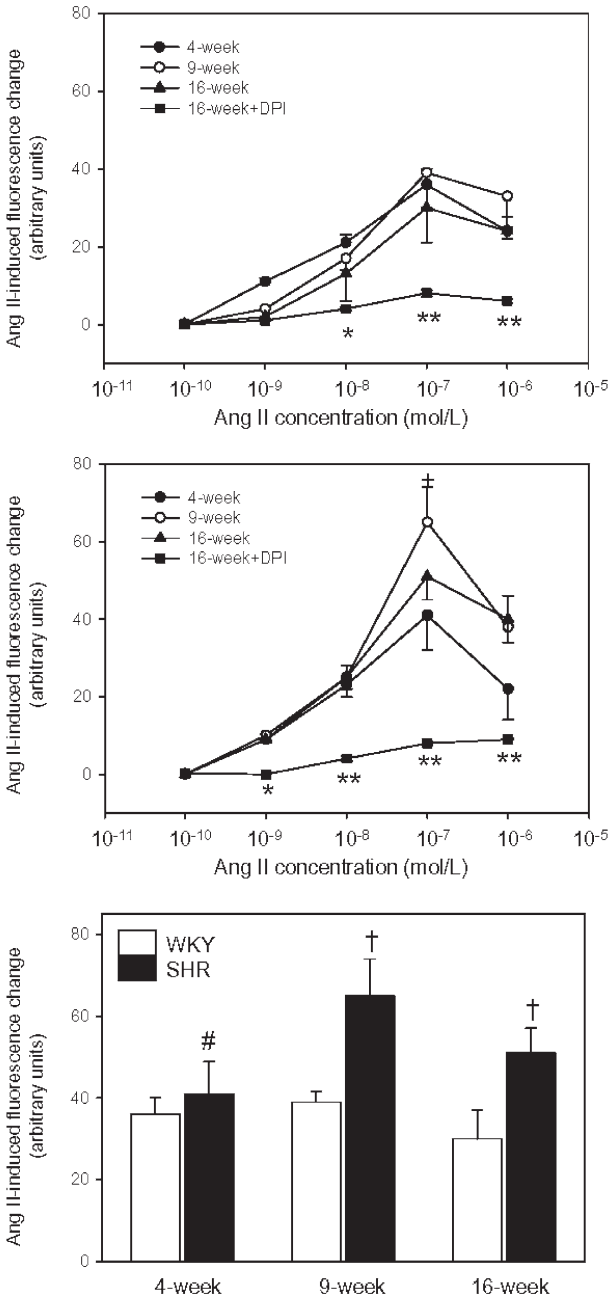

FIG. 2 The Ang II-stimulated generation of ROS in mesenteric vascular smooth muscle cells from WKY and SHR. Line graphs demonstrate effects of Ang II on CM-H2DCFDA fluorescence in the absence and presence of DPI (10^{-6} mol/L) in WKY (**upper panel**) and SHR (**middle panel**). Bar graph (**lower panel**) demontrates effects of Ang II (10^{-7} mol/L) on CM-H2DCFDA fluorescence in cells from 4-, 9-, and 16- week-old SHR and WKY. Results are presented as the Ang II-induced change in fluorescence, calculated as the difference between the stimulated response and the basal value. Experiments were repeated three times, with each experimental field comprising 12 to 24 cells. *$P < .05$, **$P < .01$ versus other groups; +$P < .05$ versus 4- and 16-week-old SHR; †$P < .05$ versus WKY, #$P < .05$ versus 9-week SHR.

Discussion

Major findings from our study demonstrate that basal and Ang II-induced NAD(P)H-driven generation of ROS are enhanced in VSMCs from SHR during the development of hypertension, but not in cells from prehypertensive rats. These processes appear to be mediated, in part, through activation of IGF-1R, as AG 1024 attenuated Ang II-

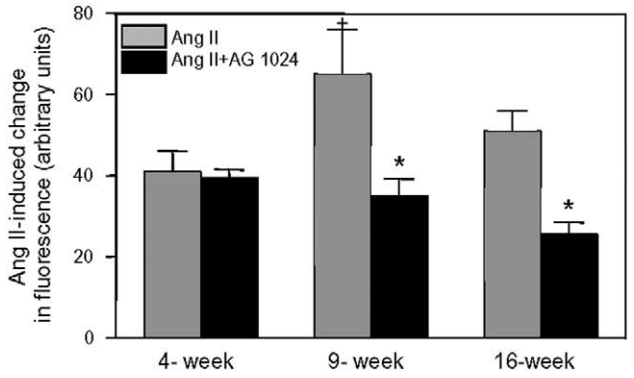

FIG. 3 Effect of AG 1024 on Ang II-stimulated generation of ROS in mesenteric smooth muscle cells from SHR. Bar graph demontrates effects of Ang II (10^{-7} mol/L) on CM-H2DCFDA fluorescence in the absence and presence of AG 1024 (5×10^{-6} mol/L). Cells were pretreated with the inhibitor for 20 min before Ang II addition. Results are presented as the Ang II-induced changes in fluorescence, calculated as the difference between the stimulated response and the basal value. Experiments were repeated three times. $+P <$ .05 versus 4- and 16-week-old SHR; $*P <$ .05 versus Ang II counterpart.

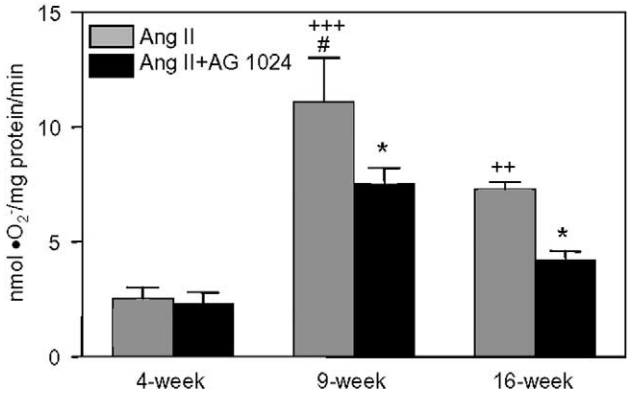

FIG. 5 Effect of IGF-1R kinase antagonist on Ang II-mediated activation of NAD(P)H oxidase in vascular smooth muscle cells from SHR. Cells were treated with AG 1024 (5×10^{-6} mol/L) for 30 min before Ang II addition. NAD(P)H (10^{-5} mol/L) was added to lucigenin (5 μmol/L)-treated cell homogenates and chemiluminiscence was measured. Enzyme activity is expressed as nanomoles of $\cdot O_2^-$/min/mg protein. Bar graphs are mean ± SEM of four experiments. $++P <$ .01 and $+++P <$ .001 versus 4-week Ang II; $*P <$ .05 versus Ang counterpart. $\#P <$ .05 versus 16-week Ang II.

induced activation of NAD(P)H oxidase in SHR VSMCs. AG1024 also inhibited Ang II-stimulated ERK1/2 phosphorylation, further supporting the importance of IGF-1R in Ang II signaling in VSMCs. Taken together, these data suggest that transactivation of IGF-1R by Ang II may play an important role in vascular oxidative excess during the development of hypertension in SHR.

All vascular cell types generate ROS, which play a key role in the pathogenesis of vascular injury in cardiovascular disease, including hypertension.[7,32–36] It is still unclear whether increased ROS generation is a consequence of hypertension and aging. We examined VSMCs from 4-, 9-, and 16-week-old SHR corresponding to the prehypertensive phase, developing hypertension, and established hypertension, respectively. Young SHR had similar systolic BP to age-matched WKY, and systolic BP increased progressively in 9- and 16-week-old SHR, but not in age-matched WKY. Examination of VSMCs, derived from SHR at these different stages in the development of hypertension, demonstrated enhanced basal and Ang II-stimulated NAD(P)H-driven generation of ROS in hypertension, particularly impressive in the phase of developing hypertension. These findings suggest that during development of hypertension there is upregulation of NAD(P)H oxidase and enhanced ROS production. Our findings are at some variance with those of Zalba et al[24] who demonstrated that Ang II-induced NAD(P)H-driven generation of $\cdot O_2^-$ increases with aging in SHR. However, older SHR (16- and 30-week-old) were studied. Because Ang II-induced production of ROS was not altered in VSMCs from prehypertensive SHR, it seems that increased vascular oxidative stress may not be a primary phenomenon in the pathogenesis of hypertension. However, enhanced NAD(P)H oxidase activity and increased production of ROS were maintained in serially passaged VSMCs from 9- and 16-week-old SHR, suggesting an intrinsic genetic or cellular phenotype that underlies oxidative stress, independently of BP, as this occurred in cell culture.

Basal NAD(P)H oxidase activity increased in cells from SHR at the beginning of BP elevation (9 weeks), compared to the levels of the normotensive stage (4 weeks), and those of adult SHR cells (16 weeks) with a higher BP. The Ang II effects on enzyme activity paralleled these changes. Thus, there may be a peak in oxidative stress coinciding with the phase of elevation of BP, after which there is a reduction, perhaps due to compensatory mechanisms. A peak in oxidative excess occurring at a vulnerable time during which BP is increasing may

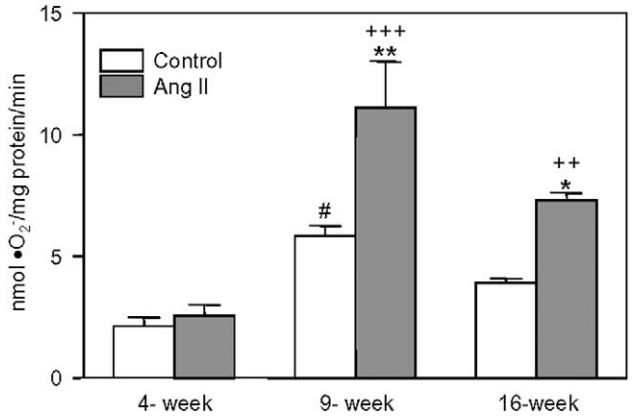

FIG. 4 Effect of Ang II on NAD(P)H oxidase activity in vascular smooth muscle cells from 4-, 9-, and 16-week-old SHR. NADPH (10^{-5} mol/L) was added to lucigenin (5 μmol/L)-treated cell homogenates and chemiluminiscence was measured. Enzyme activity is expressed as nanomoles of $\cdot O_2^-$/min/mg protein. Results are mean ± SEM of four experiments. $\#P <$ .05 versus 4-week-old control; $++P <$ .01 and $+++P <$ .001 versus 4-week Ang II; $*P <$ .05, $**P <$ .01 versus control counterpart.

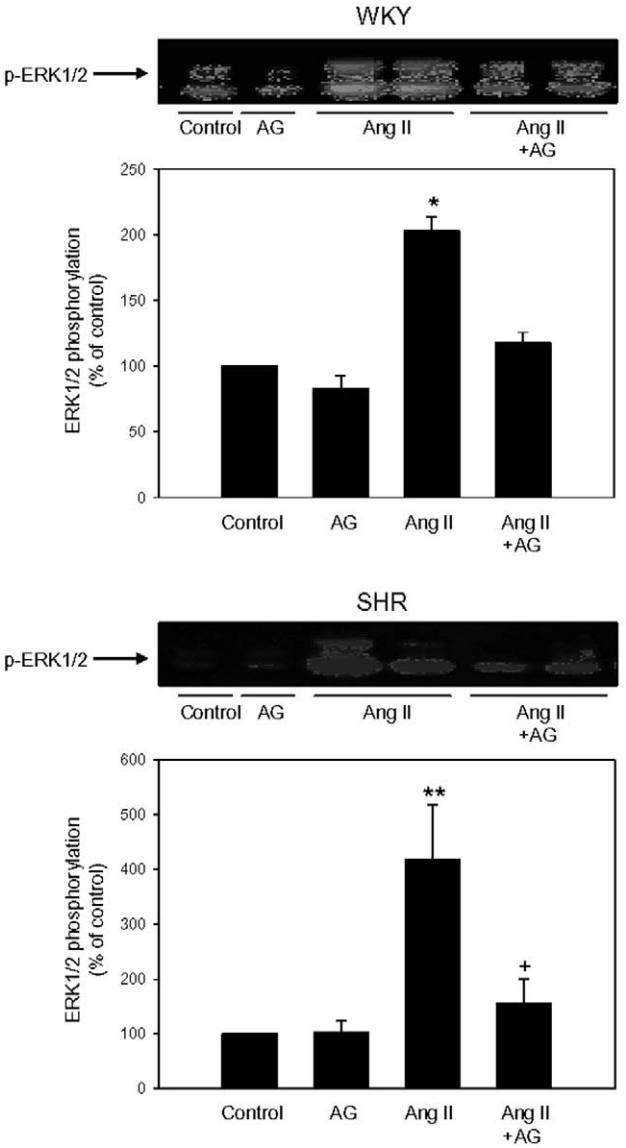

FIG. 6 Effects of IGF-1R antagonist on Ang II-mediated ERK1/2 phosphorylation in vascular smooth muscle cells (VSMC) from 16-week WKY and SHR. The VSMCs were treated with AG1024 (5×10^{-6} mol/L) for 20 to 30 min before Ang II addition (10^{-7} mol/L, 5 min). (**Upper panels**) Representative Western blots. Data are presented as percentage phosphorylation relative to control conditions, taken as 100%. Bar graphs are means ± SEM of three experiments. $*P < .05$, $**P < .01$ versus other groups, $+P < .05$ versus Control. p-ERK1/2 = phosphorylated ERK1/2; AG = AG1024.

contribute to the continued development and maintenance of hypertension.

Mechanisms whereby Ang II regulates activation of NAD(P)H oxidase in VSMCs have not been fully elucidated, but transactivation of growth factor receptors may be important.[19,37] We recently demonstrated the involvement of EGFR and IGF-1R in Ang II-induced ROS production in VSMCs from WKY rats.[21] In the present study we have extended these findings demonstrating that IGF-1R plays an important role in enhanced Ang II-mediated NAD(P)H-driven generation of ROS in VSMCS from SHR during the development of hypertension. This may relate to increased phosphorylation and activation of VSMC growth factor receptors in hypertension, as we previously reported.[20] Because receptor tyrosine kinases themselves are regulated by ROS,[19] we cannot exclude the possibility that increased oxidative stress may also influence IGF-1R in hypertension. Nevertheless, our data suggest that IGF-1R transactivation by Ang II influences NAD(P)H oxidase activity in VSMCs from SHR during the development of hypertension. Because AG 1024 only partially decreased Ang II actions, IGF-1R-independent processes also contribute to Ang II-induced ROS production in SHR VSMCs.

In conclusion, findings from the present study demonstrate that Ang II enhances generation of NAD(P)H-inducible ROS in VSMCs from SHR during the development of hypertension, but not in the prehypertensive phase. These Ang II-dependent processes are regulated, in part, through IGF-1R transactivation. Our data suggest that Ang II-induced, IGF-1R-mediated oxidative stress in VSMCs from SHR is associated with elevation of BP but may not be a primary phenomenon in the pathogenesis of hypertension in this genetic model.

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