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NAD(P)H Oxidase-derived Hydrogen Peroxide Mediates Endothelial Nitric Oxide Production in Response to Angiotensin II*

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Recently, it has been shown that the exogenous addition of hydrogen peroxide (H_2O_2) increases endothelial nitric oxide ($NO^{\cdot}$) production. The current study is designed to determine whether endogenous levels of H_2O_2 are ever sufficient to stimulate $NO^{\cdot}$ production in intact endothelial cells. $NO^{\cdot}$ production was detected by a $NO^{\cdot}$-specific microelectrode or by an electron spin resonance spectroscopy using Fe^{2+}-$(DETC)_2$ as a $NO^{\cdot}$-specific spin trap. The addition of H_2O_2 to bovine aortic endothelial cells caused a potent and dose-dependent increase in $NO^{\cdot}$ release. Incubation with angiotensin II (10^{-7} mol) elevated intracellular H_2O_2 levels, which were attenuated with PEG-catalase. Angiotensin II increased $NO^{\cdot}$ production by 2-fold, and this was prevented by Losartan and by PEG-catalase, suggesting a critical role of AT1 receptor and H_2O_2 in this response. In contrast, $NO^{\cdot}$ production evoked by either bradykinin or calcium ionophore A23187 was unaffected by PEG-catalase. As in bovine aortic endothelial cells, angiotensin II doubled $NO^{\cdot}$ production in aortic endothelial cells from C57BL/6 mice but had no effect on $NO^{\cdot}$ production in endothelial cells from $p47^{phox-/-}$ mice. In contrast, A23187 stimulated $NO^{\cdot}$ production to a similar extent in endothelial cells from wild-type and $p47^{phox-/-}$ mice. In summary, the present study provides direct evidence that endogenous H_2O_2, derived from the NAD(P)H oxidase, mediates endothelial $NO^{\cdot}$ production in response to angiotensin II. Under disease conditions associated with elevated levels of angiotensin II, this response may represent a compensatory mechanism. Because angiotensin II also stimulates $O_2^{\cdot-}$ production from the NAD(P)H oxidase, the H_2O_2 stimulation of $NO^{\cdot}$ may facilitate peroxynitrite formation in response to this octapeptide.

Growing evidence indicates that angiotensin II activates the vascular NADPH oxidase, leading to increased production of superoxide anion ($O_2^{\cdot-}$)[1] (1–4). Superoxide, in turn, reacts with the nitric oxide radical ($NO^{\cdot}$) in a diffusion-limited fashion to form peroxynitrite. This results in the loss of many of the beneficial effects of $NO^{\cdot}$, including vasodilatation (5). We and others have shown that angiotensin II contributes to endothelial dysfunction *in vivo* by this mechanism (6–8). On the other hand, $O_2^{\cdot-}$ serves as a source of other reactive oxygen species, which may contribute to vascular disease and, in some cases, may have specific signaling properties (9). In particular, the dismutation product of $O_2^{\cdot-}$, H_2O_2, may mediate compensatory responses. For example, we have shown that H_2O_2 potently induces endothelial nitric oxide synthase (eNOS) gene expression in endothelial cells via a Ca^{2+}/calmodulin-dependent protein kinase II/Janus kinase 2-dependent pathway (10, 11).

Recently, is has been shown that exogenous H_2O_2 acutely activates eNOS to cause endothelial $NO^{\cdot}$ release (12). These responses were only observed when micromolar concentrations of H_2O_2 were employed. It remains unclear, therefore, whether endogenous levels of H_2O_2 are ever sufficiently high to stimulate $NO^{\cdot}$ production in response to either physiological or pathophysiological stimuli. One such pathophysiological stimulus, angiotensin II, is known to activate the NADPH oxidase and could lead to H_2O_2 formation. Previous studies have suggested that angiotensin II increases $NO^{\cdot}$ production, although the precise signaling mechanisms have not been defined (13–15). The present study is designed to examine whether endogenous accumulation of H_2O_2 in response to angiotensin II and other stimuli is sufficient to stimulate endothelial $NO^{\cdot}$ production.

MATERIALS AND METHODS

Cell Culture—Bovine aortic endothelial cells (Cell Systems) were cultured in Media 199 (Invitrogen) containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT) as described previously (10, 11). Mouse aortic endothelial cells (MAECs) from C57BL/6 and $p47^{phox-/-}$ mice were isolated by heparinization and matrigel culture and were maintained in 10% fetal calf serum-containing Media 199 supplemented with L-glutamine (2 mmol/liter, Invitrogen), 1× MEM vitamin solution (Hyclone Laboratories, Logan, UT), streptomycin (20 μg/ml), and penicillin (20 units/ml) from Invitrogen. On the day prior to the study, the fetal calf serum concentration was reduced to 5%. Human aortic endothelial cells (Biowhittaker, Walkersville, MD) were cultured

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[1] The abbreviations used are: $O_2^{\cdot-}$, superoxide anion; $NO^{\cdot}$, endothelial nitric oxide; eNOS, endothelial nitric oxide synthase; MAECs, mouse aortic endothelial cells, MEM, minimum Eagle's medium; PEG, polyethylene glycol; SOD, superoxide dismutase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DETC, diethyl dithiocarbamate; L-NAME, L-nitroarginine methyl ester; ESR, electron spin resonance.

in 2% fetal calf serum-endothelial growth medium-2 supplemented with growth factors according to the manufacturer's instructions.

Detection of NO· Using a Selective Microelectrode—Bare carbon fiber electrodes (100-μm length × 30-μm outer diameter) were coated with nafion and *o*-phenylenediamine for the specific detection of NO· as described by Friedemann *et al.* (16). Control experiments showed that these coatings effectively eliminated electrode responsiveness to other oxidizable species, including nitrate, nitrite, and H_2O_2. To detect NO· from endothelial monolayers, cells were cultured on 35-mm dishes and studied 1 day post confluence. Cells were maintained at 37 °C. The electrode tip was advanced to the surface of an individual cell and then withdrawn precisely 5 μm. NO·-dependent oxidation currents were recorded (voltage clamp mode) immediately post addition of H_2O_2 using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Recordings were made at 0.65 V, approximately the voltage for peak NO· oxidation, and the current generated against a silver/silver chloride reference electrode was recorded. The average concentration of NO· released within 5 min after H_2O_2 stimulation was calculated from a standard curve obtained using dilutions of de-oxygenated, saturated NO· gas solutions. In additional experiments, individual measurements of NO· release were made at 5, 10, and 15 min following H_2O_2 stimulation. The pCLAMP 7.0 (Axon Instruments) was used to deliver voltage protocols and acquire and analyze data. The signal obtained in response to H_2O_2 was corrected for background using media containing H_2O_2 in the absence of cells.

Detection of NO· by ESR Using a NO·-specific Spin Trap Fe^{2+}-

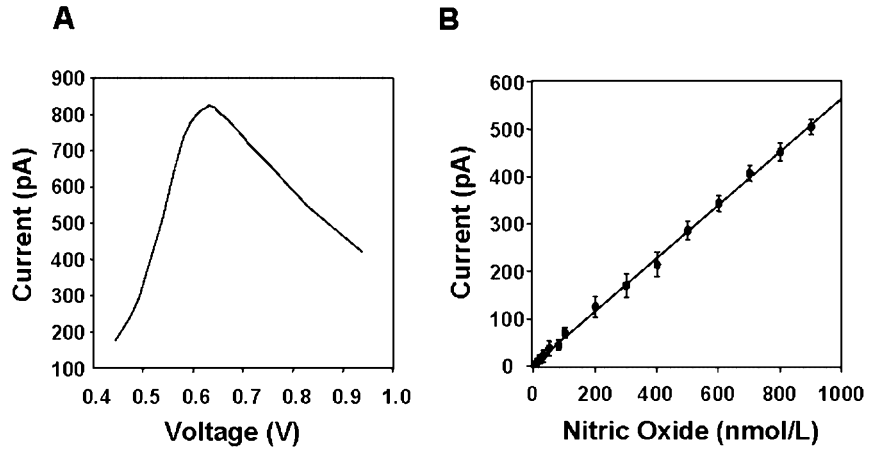

FIG. 1. **Characterization of the NO·-specific microelectrode.** *Panel A* shows that in a cyclic voltammetry ramp (253 mV/s) in the presence of 1 μmol/liter NO·, the oxidation current displays a peak characteristic of NO· at 0.6–0.7 V *versus* an Ag/AgCl reference electrode. *Panel B* demonstrates that the response of the electrode was linearly related to the concentration of NO· present (r = 1.0). The detection limit was ~5 nmol/liter. NO· standards were produced by diluting a deoxygenated, saturated solution made from pure NO· gas.

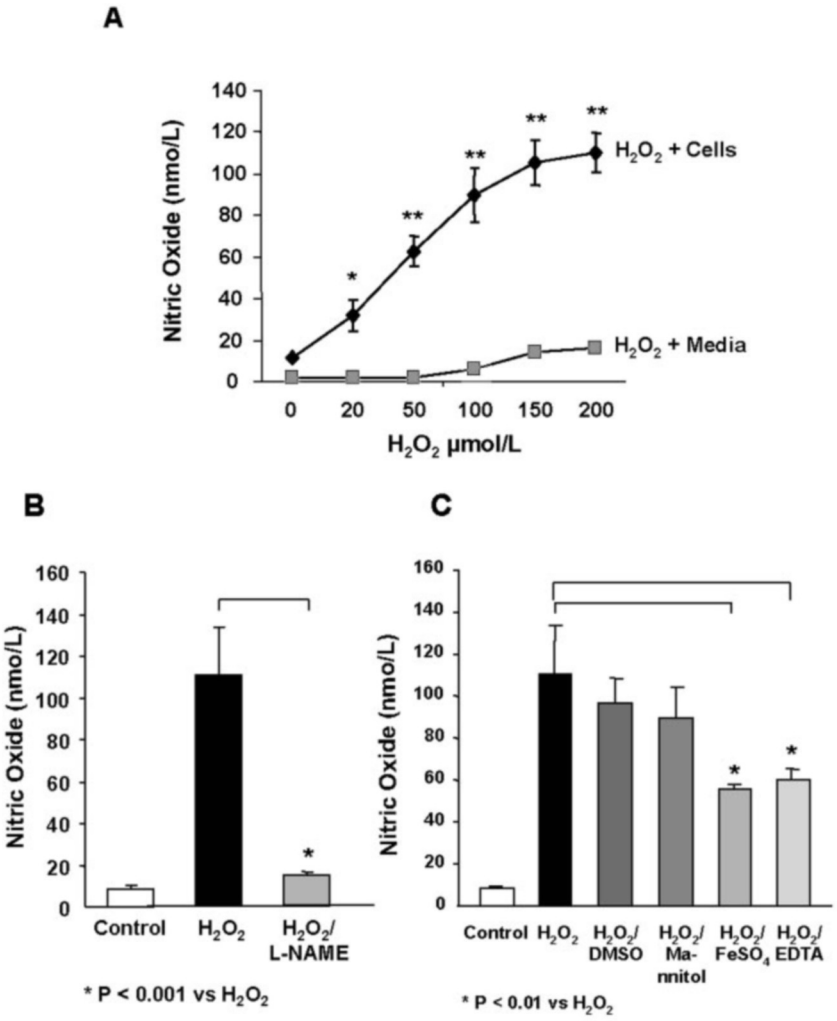

FIG. 2. **Effect of H_2O_2 on endothelial NO· production.** *A*, the concentration-response relationship between the applied H_2O_2 concentrations and NO· production. Postconfluent bovine aortic endothelial cells were exposed to H_2O_2, and the 5-min average NO· concentration was detected using the NO·-specific microelectrode. The electrode response in a cell-free system was used as a control. *B*, effect of NOS inhibition on H_2O_2-dependent NO· production. Endothelial cells were pretreated for one-hour with L-NAME (1 mmol/liter) and the NOS inhibitor prior to H_2O_2 stimulation, and NO· production was determined with the NO·-specific microelectrode. *C*, the role of the hydroxyl radical in H_2O_2-dependent NO· production. Endothelial cells were pretreated with the hydroxyl radical chelators Me_2SO (0.3%) and mannitol (20 mmol/liter) or the Fenton reaction enhancers EDTA (100 μmol/liter) and $FeSO_4$ (100 μmol/liter) for one h prior to H_2O_2 stimulation, and NO· production was determined with the NO·-specific microelectrode.

(DETC)$_2$—Nitric oxide was also detected using a Fe^{2+}-$(DETC)_2$ colloid (17). In brief, post-confluent endothelial cells were rinsed with phosphate-buffered saline and modified Krebs/HEPES buffer (99.01 mmol/liter NaCl, 4.69 mmol/liter KCl, 1.87 mmol/liter $CaCl_2$, 1.20 mmol/liter $MgSO_4$, 25 mmol/liter $NaHCO_3$, 1.03 mmol/liter K_2HPO_4, 20 mmol/liter sodium-HEPES, and 11.1 mmol/liter d-glucose, pH 7.35) prior to incubation with Fe^{2+}-$(DETC)_2$ (0.5 mmol) in Krebs/HEPES buffer containing agonists (angiotensin II, bradykinin, and A23187) and/or PEG-catalase. The $Fe^{2+}(DETC)_2$ colloid was prepared immediately prior to use. After incubation at 37 °C for 30 min, cells were gently scraped with a rubber policeman and collected. Cell suspensions collected in 1-ml syringes were snap-frozen with liquid N_2. The frozen sample column was then loaded into a finger Dewar and analyzed with a Bruker EMX ESR spectrometer (Bruker Instruments, Billerica, MA) at the following settings: field sweep, 160 G; microwave frequency, 9.39 GHz; microwave power, 10 milliwatts; modulation amplitude, 3 G; conversion time, 2621 ms; time constant, 328 ms; modulation amplitude, 3 G; receiver gain, 1×10^4 and 4 scans. Because the $Fe^{2+}(DETC)_2$ colloid is lipophylic, it specifically detects NO· in lipid bilayers, *i.e.* sites where NO· is relatively protected from oxidative degradation by $O_2^{\cdot-}$.

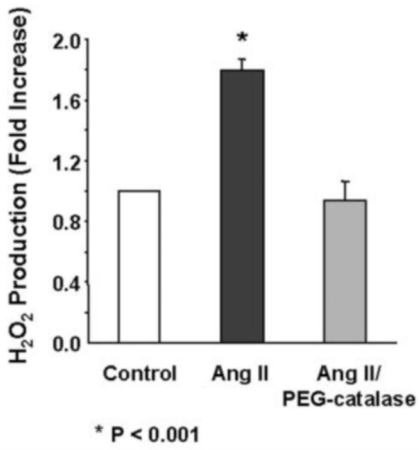

FIG. 3. **Effects of angiotensin II on endothelial H_2O_2 production detected by a DCFH-DA fluorescent assay.** Endothelial cells were pretreated with control media or media containing PEG-catalase (50 units/ml) for 18 h before being loaded with DCFH-DA (30 μmol/liter) for 1 h. After stimulation with angiotensin II (*Ang II*) for 30 min, cells were scraped and loaded into a 96-well plate and read with a fluorescent plate reader at excitation and emission wavelengths of 475 and 525 nm, respectively. Grouped data from three separate experiments are presented in -fold increase.

Detection of Intracellular H_2O_2 Using a DCFH-DA Fluorescent Assay—Intracellular H_2O_2 was measured by a 2′,7′-dichlorofluorescin diacetate (DCFH-DA)-based fluorescent assay as described previously (18). Cells were stimulated with angiotensin II for 30 min, rinsed twice with ice-cold PBS, and scraped. A 200-μl cell suspension was loaded into a 96-well plate and read with a fluorescent plate reader at excitation and emission wavelengths of 475 and 525 nm, respectively. The cells were counted in duplicates by a hemacytometer.

Statistical Analysis—H_2O_2 or angiotensin II-stimulated NO· production, in the absence or presence of pharmacological inhibitors, was measured five times (unless indicated) for each condition, and the differences among groups were analyzed using one-way analysis of variance. When differences were indicated, a Dunnet's *post hoc* test was employed. Statistical significance was assumed for $p < 0.05$. All grouped data shown in the figures were presented as mean ± S.E.

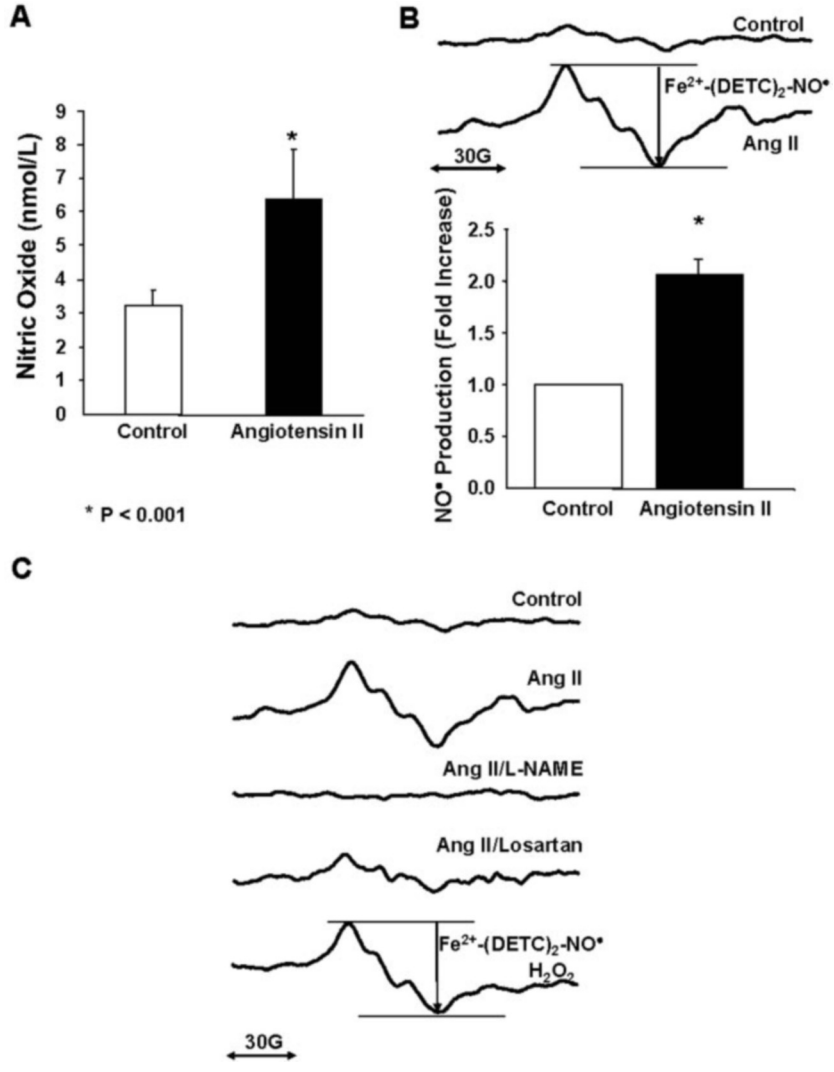

FIG. 4. **Effect of angiotensin II on endothelial NO· production.** *A*, endothelial NO· release in response to angiotensin II detection with the NO·-specific electrode. Cells were exposed to angiotensin II (10^{-7} mol), and the increase in extracellular NO· concentration was averaged over 5 min. *B*, endothelial NO· production in response to angiotensin II (*Ang II*) detection with the NO·-specific spin trap and ESR. Cells were incubated with angiotensin II (10^{-7} mol) for 30 min and collected for NO· detection following the protocol described under "Materials and Methods." The *upper panel s*hows representative NO· spectra from control and angiotensin II-treated cells. The *lower panel* is grouped data of ESR intensity expressed in -fold increase from four separate experiments. *C*, effects of NOS inhibition and AT1 receptor antagonism on angiotensin II stimulation of NO· production. Cells were pretreated with L-NAME (1 mmol/liter) or Losartan (10 μmol/liter), the AT1 receptor antagonist, for 15 min prior to stimulation with angiotensin II. Both of the drugs abolished the angiotensin II-dependent NO· production. A representative spectrum for the $Fe^{2+}(DETC)_2$-NO· signal obtained in cells treated with H_2O_2 is also presented.

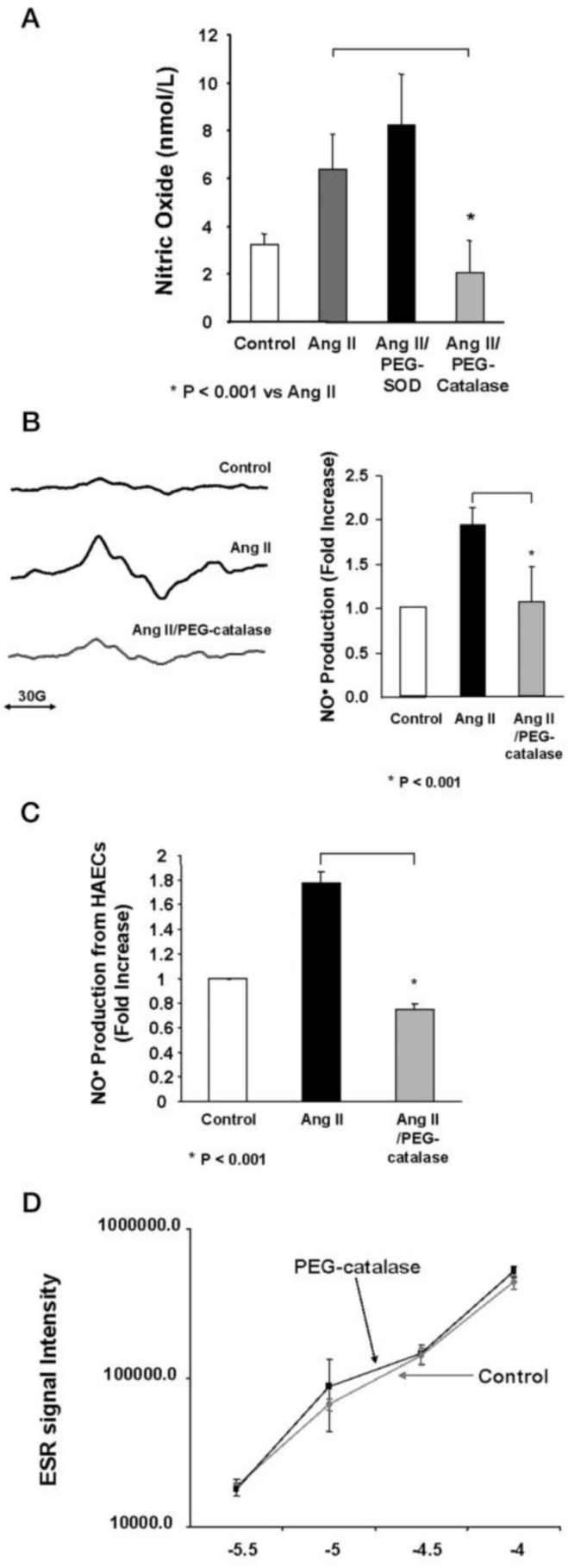

FIG. 5. **Role of the intracellular H_2O_2 in angiotensin II timulation of endothelial NO• production.** *A*, effect of H_2O_2 reduction on angiotensin II (*Ang II*)-dependent NO• production/detection with the NO•-specific microelectrode. Cells were pretreated with PEG-catalase (50 units/ml) or PEG-SOD (75 units/ml) for 18 h, and the 5-min average NO• release was determined by the NO•-specific microelectrode. *B*, effect of H_2O_2 reduction on angiotensin II-dependent NO• production/detection with the NO•-specific spin trap and ESR. Cells were pretreated with PEG-catalase (50 units/ml) for 18 h before being exposed to angiotensin II, and NO• was detected by ESR. The *left panel* demonstrates representative spectra for the NO• signal achieved from control cells or cells treated with angiotensin II in the presence or absence of pharmacological interventions. The *right panel* shows grouped data of ESR intensity expressed in -fold increase from four separate experiments. *C*, effect of PEG-catalase on angiotensin II-stimulated NO• production in human aortic endothelial cells (HAECs) were pretreated with PEG-catalase for 18 h prior to angiotensin II stimulation, and NO• was measured by ESR spin trapping. *D*, effect of PEG-catalase on ESR trapping of exogenous NO•. Cells were exposed to different concentrations of the NO• donor spermine NONOate in the presence or absence of PEG-catalase, and NO• was detected by ESR.

RESULTS

Characterization of NO•-specific Microelectrode—In cyclic voltammetry experiments (253 mV/s) using a 1 μmol/liter NO• solution, the oxidation current displayed a characteristic peak at 0.65 V *versus* an Ag/AgCl reference electrode (Fig. 1*A*). The response of the electrode was linearly related to the concentration of NO• present, and the detection limit was ~5 nmol/liter (Fig. 1*B*).

H_2O_2 Acutely Stimulates NO• Production by Endothelial Cells—H_2O_2 caused a potent, dose-dependent increase in NO• concentration directly above the endothelial monolayer, which reached a peak of 12.8-fold greater than baseline at a concentration of 150 μmol/liter H_2O_2 (Fig. 2*A*). One hour of pretreatment with L-NAME (1 mmol/liter) completely attenuated this response, supporting the specificity of the electrode and the dependence of the response on eNOS (Fig. 2*B*, $p < 0.001$). Further studies demonstrated that this response was not mediated by a hydroxyl radical. Preincubation of endothelial cells with the hydroxyl radical scavengers Me_2SO (0.3%) or mannitol (20 mmol/liter) had no effect on H_2O_2-dependent NO• production. Interestingly, treatment of cells with the Fenton reaction enhancers EDTA (100 μmol/liter) or $FeSO_4$ (100 μmol/liter) inhibited NO• stimulation by H_2O_2 by 47% (Fig. 2*C*, $p < 0.01$). Because Fenton reaction enhancers deplete H_2O_2 in the process of generating hydroxyl radical, these data support the concept that H_2O_2, but not the hydroxyl radical, activates NO• production in endothelial cells.

Angiotensin II Increases Intracellular H_2O_2 Production—In cultured endothelial cells it has been shown previously that angiotensin II stimulates reactive oxygen species production (3, 4). Moreover, H_2O_2 is known to mediate AT1 receptor-dependent angiotensin II signaling in vascular cells (9, 19). It is possible that a rise in intracellular H_2O_2 may play an important role in angiotensin II-dependent NO• production. As shown in Fig. 3, intracellular H_2O_2 detected using DCFH-DA was increased by 1.8-fold by incubating endothelial cells with angiotensin II (10^{-7} mol) for 30 min, and this rise was completely prevented by pretreating endothelial cells with PEG-catalase (50 units/ml) for 18 h prior to the experiment. As shown previously by Beckman *et al.* (20), this prolonged preincubation is necessary to increase intracellular catalase activity.

Angiotensin II Stimulates NO• Production by Endothelial Cells—To determine whether angiotensin II has any effect on endothelial NO• production, cells were exposed to angiotensin II (10^{-7} mol), and NO• release was monitored by the NO•-specific microelectrode. As shown in Fig. 4*A*, the NO• concentration above endothelial cells doubled in response to angiotensin II ($n = 4, p < 0.001$). These results are in keeping with those of Thorup *et al.* (15), who found that in isolated renal arteries,

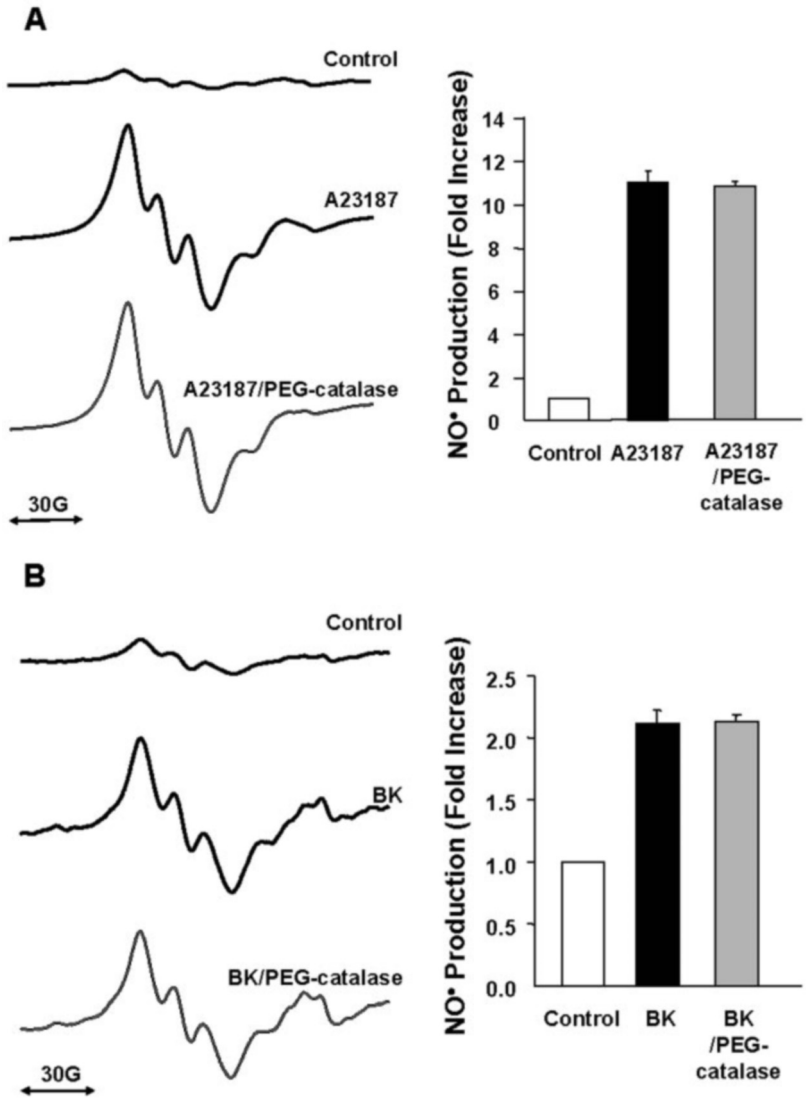

FIG. 6. **H_2O_2-mediated NO· production is restricted to angiotensin II.** *A*, effect of PEG-catalase on A23187-stimulated NO· production. Cells were pretreated with PEG-catalase (50 units/ml) for 18 h prior to stimulation with calcium ionosphere A23187 (1 μmol/liter). NO· production was determined by ESR. The *left panel* demonstrates representative spectra for NO· signals acquired from control cells or cells treated with A23187 in the presence or absence of PEG-catalase. The *right panel* shows grouped data of ESR intensity expressed in -fold increase from four separate experiments. *B*, effect of PEG-catalase on bradykinin-stimulated NO· production. Cells were pretreated with PEG-catalase (50 units/ml) for 18 h prior to stimulation with bradykinin (1 μmol/liter), and NO· production was determined by ESR. The *left panel* demonstrates representative spectra for the NO· signal achieved from control cells or cells treated with bradykinin (*BK*) in the presence or absence of PEG-catalase. The *right panel* shows grouped data of ESR intensity expressed in -fold increase from four separate experiments.

angiotensin II dose-dependently increased NO· production to similar levels (15).

To confirm that angiotensin II stimulates endothelial NO· production, we employed electron spin resonance spectroscopy (ESR) with the NO·-specific spin trap Fe^{2+}-$(DETC)_2$. The Fe^{2+}-$(DETC)_2$ colloid is hydrophobic and partitions in the lipid bilayers of cell membranes where it reacts with NO· to form a Fe^{2+}-$(DETC)_2$-NO· complex that can be detected by ESR. This approach has the advantage that NO· is concentrated in the lipid bilayers (21, 22), and at this site it is protected from inactivation by O_2^-. As demonstrated in Fig. 4*B*, 30 min of incubation with angiotensin II caused a 2-fold increase in endothelial NO· production ($n = 4$, $p < 0.001$), qualitatively confirming the NO· electrode findings. Pretreatment with L-NAME (1 mmol/liter for 15 min) completely attenuated this response (Fig. 4*C*). Further studies showed that NO· stimulation by angiotensin II was dependent on the AT1 receptor, because pretreatment with the AT1 receptor antagonist Losartan (10 μmol/liter for 15 min) abolished angiotensin II-dependent NO· production (Fig. 4*C*). A representative spectrum for H_2O_2 (150 μmol/liter)-stimulated NO· production is illustrated in Fig. 4*C*.

Intracellular H_2O_2 Mediates Angiotensin II Stimulation of Endothelial NO· Production—To investigate the potential role of intracellular H_2O_2 in angiotensin II-stimulated NO· production, endothelial cells were pretreated with PEG-catalase (50 units/ml) or PEG-SOD (75 units/ml) for 18 h prior to exposure to angiotensin II. Acute NO· release was monitored by the NO·-specific electrode for 5 min. As shown in Fig. 5*A*, the average NO· concentration was consistently increased by angiotensin II, and this response was completely prevented by PEG-catalase ($n = 4$, $p < 0.001$). PEG-SOD tended to further enhance this response, although statistical significance was not reached. Supporting the electrode findings, an 18-hour pretreatment with PEG-catalase abolished the angiotensin II-stimulated NO· production detected by ESR and the NO·-specific spin trap Fe^{2+}-$(DETC)_2$ (Fig. 5*B*, $n = 4$, $p < 0.001$). Taken together, data from two distinct methodologies for direct NO· detection suggested that the scavenging of intracellular H_2O_2 prevents NO· stimulation by angiotensin II, implying a critical role of H_2O_2 in this response. These experiments were repeated in cultured human aortic endothelial cells, and the results were identical (Fig. 5*C*).

It was reported recently that catalase can direct bind NO· (23). To exclude the possibility that PEG-catalase inhibited the Fe^{2+}-$(DETC)_2$-NO· signals by direct binding of NO·, endothelial cells were exposed to different concentrations of the exogenous NO· donor, spermine-NONOate, in the presence or absence of PEG-catalase. ESR was used to evaluate the resultant NO· signals. PEG-catalase did not affect the NO· detected from the exogenous NO· donor as shown in Fig. 5*D*. These results indicate that the low concentrations of PEG-catalase used in these

experiments were insufficient to alter NO· trapping by the $Fe^{2+}(DETC)_2$ colloid and that the reduction of NO· we observed after treatment with PEG-catalase was likely due to diminished activation of eNOS by H_2O_2.

Additional experiments demonstrated that H_2O_2-mediated NO· production appeared specific for angiotensin II. The endothelial production of NO· evoked by 30 min of treatment with either the calcium ionosphere A23187 (1 μmol/liter) or bradykinin (1 μmol/liter) was not affected by PEG-catalase (Fig. 6, *A* and *B*).

NAD(P)H Oxidase-Derived H_2O_2 Mediates Endothelial NO· Production in Response to Angiotensin II—To confirm the above studies and to determine the source of H_2O_2 that mediates angiotensin II-stimulated NO· production, MAECs from wild type and $p47^{phox-/-}$ mice were studied. In recent studies, we have shown that endothelial cells from these mice have normal expression of the angiotensin II AT1 receptor and an absence of $p47^{phox}$ and fail to produce O_2^- in response to angiotensin II (24). Angiotensin II had no effect on NO· production in MAECs from $p47^{phox-/-}$ mice (Fig. 7). In contrast, angiotensin II doubled NO· production in endothelial cells cultured from wild-type C57BL/6 mice. Of note, the calcium ionophore A23187 stimulated NO· production similarly in the $p47^{phox-/-}$ and wild-type endothelial cells, indicating that that the lack of $p47^{phox}$ did not alter the ability of cells to produce NO· to other stimuli (Fig. 7). These observations strongly suggest that NAD(P)H oxidase-derived H_2O_2 plays a pivotal role in mediating angiotensin II-induced endothelial NO· production.

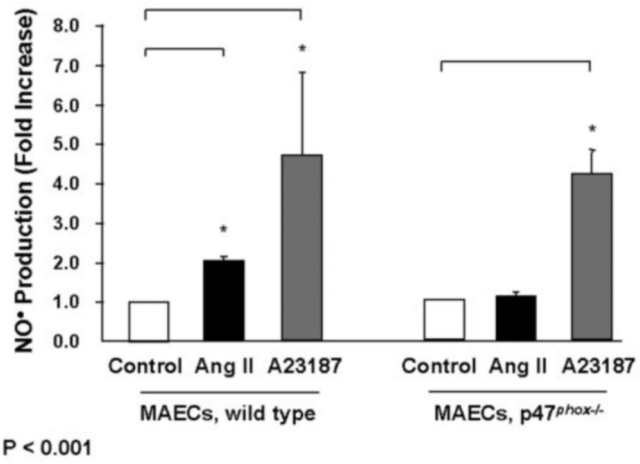

FIG. 7. **Role of the NAD(P)H oxidase-derived H_2O_2 in angiotensin II signaled endothelial NO· production.** Mouse aortic endothelial cells from wild type or $p47^{phox-/-}$ animals were treated with either angiotensin II or A23187, and NO· production was determined by ESR. Grouped data of ESR intensity from four separate experiments were expressed as -fold increase.

DISCUSSION

Previous studies have indicated that H_2O_2 is a potent stimulus for NO· production by endothelial cells and that this seems to involve activation of eNOS by phosphorylation of serine 1179 and dephosphorylation of threonine 495 (12). In the present studies, we addressed the question of whether or not H_2O_2 produced endogenously could activate endothelial cell NO· production. To address this question, we examined responses to angiotensin II, which has previously been shown to increase both O_2^- and peroxynitrite in endothelial cells (14). We found that angiotensin II stimulates NO· production from endothelial cells as detected by two distinct methodologies for detection of the NO· radical and that H_2O_2 seems to function as an intracellular second messenger in this response. The source of H_2O_2 is identified to be the endothelial NADPH oxidase, based on experiments with endothelial cells from $p47^{phox-/-}$ mice. This H_2O_2-dependent eNOS signaling pathway seemed to be specific for angiotensin II, because it was not observed for other agonists, including bradykinin and A23187.

As shown previously, the application of exogenous H_2O_2 to endothelial cells caused an acute and potent increase in endothelial NO· production. This seemed to be a direct effect of H_2O_2, and it was not mediated by Fenton products of H_2O_2, because hydroxyl radical chelators had no effect on this response. Data from NO·-specific microelectrode and ESR with NO·-specific spin trap $Fe^{2+}(FETC)_2$ strongly support the belief that angiotensin II increases endothelial NO· production. Of note, this response was prevented by Losartan, suggesting that it was mediated by the AT1 receptor. It has been reported that angiotensin IV, a cleavage product of angiotensin II, can stimulate endothelium-dependent vasodilatation and release NO· in some endothelial cells (25–28). In addition, activation of the AT2 receptor by angiotensin II has been associated with the activation of kininogen, bradykinin production, and, ultimately, NO· release via activation of the bradykinin receptor (29–32). The fact that Losartan inhibited the response to angiotensin II in the current studies does not discount these other mechanisms, as the AT2 and the angiotensin IV receptor may not be expressed at high levels in bovine aortic endothelial cells but provides yet another mechanism whereby angiotensin II can stimulate NO· production. *Prima facie*, the ability of angiotensin II to stimulate NO· release seems at odds with the notion that angiotensin II causes endothelial dysfunction by increasing reactive oxygen species production and thereby inactivating NO·. In fact, it is likely, based on previous studies, that angiotensin II is simultaneously stimulating both O_2^- and NO·, facilitating peroxynitrite production.

Our current findings are in keeping with the concept that H_2O_2 can serve as an intracellular signaling molecule. Early work demonstrated that H_2O_2 can both activate tyrosine kinases and inhibit tyrosine phosphatases (33, 34). Mitogen-activated protein kinase (MAPK) family members extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, ERK5, and c-Jun NH2-terminal kinase (JNK) have been shown to be targets of H_2O_2 (35–38). H_2O_2 also mediates epidermal growth factor (EGF)-induced phosphorylation of its receptor and phospholipase C (39), platelet-derived growth factor (PDGF) stimulation of signal transducers and activators of transcription (STATs) (40), activation of Akt by angiotensin II (41), and tyrosine phosphorylation of protein kinase C (42). Activation of Ras by reactive oxygen species is also potentially quite important (43). It has been shown that catalase, after reacting with H_2O_2, can activate guanylate cyclase. This seems to occur via a unique mechanism that is quite different from the heme-mediated activation of guanylate cyclase by nitric oxide (44). Recent data suggest that H_2O_2 can activate phosphatidylinositol 3-kinase, leading to phosphorylation of eNOS at serine 1179, which may contribute to the activation of the enzyme in response to angiotensin II.

In the present studies, we found that the NADPH oxidase was essential for angiotensin II stimulation of endothelial cell NO· production. Cells from $p47^{phox-/-}$ mice demonstrated no response to angiotensin II, although they produce NO· normally when stimulated with the calcium ionophore A23187. This finding is consistent with the concept that angiotensin II is a potent stimulus for NAD(P)H oxidase activation (1–4). Whereas most studies have focused on the O_2^- production by this oxidase, either spontaneous or superoxide dismutase-mediated dismutation of O_2^- could lead to increased levels of intracellular H_2O_2. Furthermore, recent preliminary studies from our laboratory have suggested that membranes of vascular smooth muscle and endothelial cells produce about 4-fold

more H_2O_2 than $O_2^{\cdot-}$ when stimulated by the addition of NAD(P)H. Although the Fe^{2+} center of this enzyme would be expected to mediate only a one-electron reduction of oxygen and thus produce only $O_2^{\cdot-}$, it may be that the release of the $O_2^{\cdot-}$ is electrostatically hindered so that H_2O_2, after spontaneous dismutation, is favored. Nevertheless, our data would indicate that this enzyme clearly releases sufficient H_2O_2 to modulate eNOS activation upon stimulation of cells with angiotensin II

In summary, the present study provides direct evidence that endogenous H_2O_2, derived from the NAD(P)H oxidase, mediates $NO^{\cdot}$ production in response to angiotensin II in endothelial cells. In disease conditions associated with elevated angiotensin II levels, this response may represent a compensatory mechanism. Because angiotensin II also stimulates $O_2^{\cdot-}$ production from the NAD(P)H oxidase, the H_2O_2 stimulation of $NO^{\cdot}$ may facilitate peroxynitrite formation in response to this octapeptide.

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