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Akt-Dependent Phosphorylation of Serine 1179 and Mitogen-Activated Protein Kinase Kinase/Extracellular Signal-Regulated Kinase 1/2 Cooperatively Mediate Activation of the Endothelial Nitric-Oxide Synthase by Hydrogen Peroxide

HUA CAI, ZONGMING LI, MICHAEL E. DAVIS, WILLIAM KANNER, DAVID G. HARRISON, and SAMUEL C. DUDLEY JR.

Division of Cardiology, Emory University School of Medicine, Atlanta, Georgia (H.C., M.E.D., D.G.H., S.C.D.); and Atlanta Veterans Administration Medical Center, Atlanta, Georgia (Z.L., W.K., D.G.H., S.C.D.)

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ABSTRACT

Hydrogen peroxide mediates vasodilation, but the mechanisms responsible for this process remain undefined. We examined the effect of H_2O_2 on nitric oxide ($NO^{\bullet}$) production and the signaling events involved. $NO^{\bullet}$ release from bovine aortic endothelial cells was detected with an $NO^{\bullet}$-specific microelectrode. The addition of H_2O_2 caused a potent dose-dependent increase in $NO^{\bullet}$ production. This was partially Ca^{2+}-dependent because BAPTA/AM reduced $NO^{\bullet}$ production at low (<50 μM) but not high (>100 μM) concentrations of H_2O_2. Phosphatidylinositol (PI) 3-kinase inhibition [with wortmannin or 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride], infection with a dominant-negative mutant of Akt, or mitogen-activated protein kinase kinase/extracellular signal-regulated kinase ½ (MEK/ERK1/2) inhibition (with PD98059 or U0126) partially attenuated, whereas inhibition of both PI 3-kinase and MEK1/2 abolished H_2O_2-dependent $NO^{\bullet}$ production. ERK1/2 seemed necessary for $NO^{\bullet}$ production early (<5 min) after H_2O_2 addition, whereas PI 3-kinase/Akt was more important at later time points. Phosphorylation of endothelial nitric-oxide synthase (eNOS) at serine 1179 was observed >10 min after the addition of H_2O_2, and this was prevented by wortmannin but not by PD98059. c-Src family tyrosine kinase(s) was found to be upstream of H_2O_2-dependent Akt and eNOS serine 1179 phosphorylation and subsequent $NO^{\bullet}$ production. In summary, H_2O_2 causes endothelial $NO^{\bullet}$ release mediated by cooperative effects between PI 3-kinase/Akt-dependent eNOS serine 1179 phosphorylation and activation of MEK/ERK1/2. This may represent an acute cellular adaptation to an increase in oxidant stress.

Several pathophysiological conditions are associated with increased vascular production of superoxide anion ($O_2^{\bullet-}$) (Cai and Harrison, 2000). Superoxide in turn reacts with nitric oxide ($NO^{\bullet}$) in a diffusion-limited fashion to form peroxynitrite. This results in the loss of many of the beneficial effects of $NO^{\bullet}$, including vasodilation. Furthermore, $O2^{\bullet-}$ serves as a source of other reactive oxygen species, which may contribute to vascular disease and, in some cases, may have specific signaling properties. In particular, the dismutation product of $O_2^{\bullet-}$, H_2O_2, may mediate compensatory responses. For example, we showed recently that H_2O_2 potently induces endothelial nitric-oxide synthase (eNOS) gene expression in endothelial cells via a calcium/calmodulin-dependent protein kinase II (Ca^{2+}/CaM kinase II)/Janus tyrosine kinase-2–dependent pathway (Drummond et al., 2000; Cai et al., 2001). This phenomenon may represent an important compensatory response to increased oxidant stress.

In addition to this long-term effect on eNOS expression, there may be short-term effects of H_2O_2 on eNOS function. Earlier in vitro studies suggest that H_2O_2 produces both endothelium-dependent and -independent vasodilation; however, the underlying mechanisms remain controversial (Rubanyi and Vanhoutte, 1986; Thomas and Ramwell, 1986). H_2O_2 has been shown to directly activate cyclic GMP via a

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ABBREVIATIONS: eNOS, endothelial nitric-oxide synthase; MEK, mitogen-activated protein kinase kinase; ERK1/2, extracellular signal-regulated kinase 1/2; PI, phosphatidylinositol; CaM kinase II, calcium/calmodulin-dependent protein kinase II; ERK5, extracellular signal-regulated kinase 5; AMPK, AMP-dependent protein kinase; BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid/acetoxymethyl ester; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; PD98059, 2′-amino-3′-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene; MOI, multiplicity of infection; PP1, protein phosphatase 1.

compound I/H_2O_2 complex (Burke and Wolin, 1987; Wolin and Burke, 1987). Recently, it was reported that H_2O_2 functions as an endothelium-derived hyperpolarizing factor (Matoba et al., 2000) in small arteries and activates potassium channel opening in large cerebral arteries (Iida and Katusic, 2000). On the other hand, the vasodilation caused by H_2O_2 seems to depend on eNOS, because N^{ω}-nitro-L-arginine methyl ester prevents it (Zembowicz et al., 1993; Bharadwaj and Prasad, 1995; Yang et al., 1999). In the present study, we sought to determine whether H_2O_2 (20–200 μM) is able to directly stimulate NO$^{\cdot}$ release from endothelial cells and to examine the potential signaling mechanisms involved.

Materials and Methods

Materials. BAPTA/AM, PD98059, wortmannin, and LY294002 were purchased from Calbiochem (San Diego, CA). U0126 and PP1 were obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Antibodies against Akt, phospho-Akt at serine 473, phospho-eNOS at serine 1179, ERK1/2, phospho-ERK1/2, phospho-ERK5, phospho-AMP–dependent protein kinase (AMPK) were obtained from Cell Signaling Technology Inc. (Beverly, MA). Monoclonal anti-eNOS antibody was obtained from BD Biosciences (San Jose, CA). Other chemicals were obtained from Sigma (St. Louis, MO) in the highest purity available.

Cell Culture. Bovine aortic endothelial cells (Cell Systems, Kirkland, WA) were cultured in medium 199 (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum (Hyclone Laboratories, Logan, UT) as described previously (Drummond et al., 2000; Cai et al., 2001). One-day-postconfluence cells, starved with 5% medium overnight, were used for experiments.

Detection of NO$^{\cdot}$ Using a Selective Microelectrode. Bare carbon-fiber electrodes (100 μm long × 30 μm optical density) were coated with nafion and *o*-phenylenediamine for specific detection of NO$^{\cdot}$ as described by Friedemann et al. (1996). Control experiments showed that in the voltage-clamp mode, these coatings effectively eliminated electrode responsiveness to other oxidizable species, including nitrate, nitrite, nitroxyl, and H_2O_2. To detect NO$^{\cdot}$ from endothelial monolayers, bovine aortic endothelial cells were cultured on 35-mm dishes and studied 1 day after confluence. The culture dishes were mounted on a plate, and temperature was maintained at 37°C. The electrode tip was advanced to the surface of the cell monolayer and then withdrawn precisely at 5 μm. NO$^{\cdot}$-dependent oxidation currents were recorded in the voltage-clamp mode immediately after the addition of H_2O_2 using an Axopatch 200B amplifier (Axon Instruments, Union City, CA). Recordings were made at 0.65 V, which was the approximate voltage for peak NO$^{\cdot}$ oxidation, against a silver/silver chloride reference electrode. NO$^{\cdot}$ release after H_2O_2 stimulation was recorded, and the average concentration of NO$^{\cdot}$ released in the first 5 min was calculated from a standard curve obtained using dilutions of a deoxygenated solution saturated with pure NO$^{\cdot}$ gas. In additional experiments, individual measurements of NO$^{\cdot}$ release were made 5, 10, and 15 min after H_2O_2 stimulation. The pCLAMP 7.0 (Axon Instruments) was used to deliver voltage protocols and to 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.

Examination of Protein Phosphorylation by H_2O_2. Phosphorylation of eNOS and protein kinases was examined using phosphospecific antibodies and Western blot analysis as described previously (Cai et al., 2001). A Gelcode blue stain reagent (Pierce, Rockford, IL) was used to monitor protein loading and quality of separation in the SDS/polyacrylamide gel electrophoresis.

Infection of Endothelial Cells with Adenovirus. Endothelial cells at 90% confluence were incubated with 50 MOI adenovirus containing either a dominant-negative mutant of Akt, Akt-AAA, or a β-glacatosidase (Ad-LacZ). Akt-AAA is a negative mutant of Akt in which the phosphate transfer residue in the catalytic site (Lys^{179}) and the two major regulatory phosphorylation sites (Thr^{308} and Ser^{473}) are all replaced with Ala. It was a generous gift from Dr. Kenneth Walsh (Boston University, Boston, MA). It has been shown to inhibit Akt specifically in a dominant-negative manner (Morales-Ruiz et al., 2001; Boo et al., 2002). Infections were performed in serum-free medium 199 for 2 h, and then 10% serum was added. NO$^{\cdot}$ production and eNOS phosphorylation in response to H_2O_2 was examined 48 h later.

Statistical Analysis and Data Interpretation. Unless indicated, NO$^{\cdot}$ production was monitored for 5 min immediately after the addition of H_2O_2. Average NO$^{\cdot}$ concentration during this period was determined in the presence or absence of drug interventions. H_2O_2-stimulated NO$^{\cdot}$ production, in the absence or presence of pharmacological inhibitors, was measured five times unless otherwise indicated for each condition, and the differences among groups were analyzed using one-way analysis of variance. When differences were indicated upon analysis of variance, a Dunnett's post hoc test was used. Statistical significance was assumed at $p < 0.05$. All grouped data shown in the figures were presented as means ± S.E.M.

Results

Characterization of the NO$^{\cdot}$-Specific Electrode. In cyclic voltammetry experiments (253 mV/s) using a 1 μM NO$^{\cdot}$ solution, the oxidation current displayed a characteristic peak at 0.65 V versus an Ag/AgCl reference electrode. A standard current-concentration curve for NO$^{\cdot}$ was generated using dilutions of an NO$^{\cdot}$ saturated deoxygenated solution. The response of the electrode was linearly related to the concentration of NO$^{\cdot}$ present, and the detection limit was ~5 nM.

Endothelial NO$^{\cdot}$ Production in Response to H_2O_2 is Partially Calcium-Independent. To determine the effect of H_2O_2 on endothelial NO$^{\cdot}$ production, cells were exposed to different concentrations of H_2O_2 and NO$^{\cdot}$ production monitored with the NO$^{\cdot}$-specific electrode. As shown in Fig. 1, H_2O_2 caused a dose-dependent increase in average NO$^{\cdot}$ production over 5 min (shown as ♦). In addition to its conventional Ca^{2+}-dependent activation, eNOS can be activated via Ca^{2+}-independent mechanisms in response to a variety of

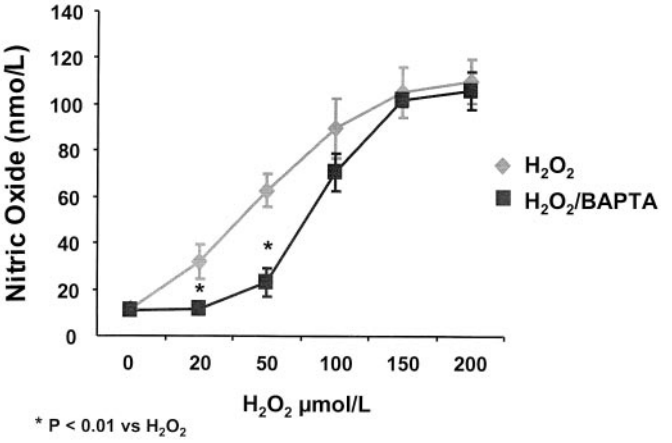

Fig. 1. Calcium-dependent and independent effects of H_2O_2 on endothelial NO$^{\cdot}$ production. Postconfluent bovine aortic endothelial cells were exposed to different concentrations of H_2O_2, and NO$^{\cdot}$ production was monitored using the NO$^{\cdot}$-specific electrode. Calcium chelation was achieved by pretreating endothelial cells with BAPTA/AM (10 μM) for 1 h. Lines show the concentration-response relationship between the applied H_2O_2 concentrations and the 5-min average of NO$^{\cdot}$ production in the absence (♦) or presence (■) of BAPTA/AM.

stimuli (Corson et al., 1996; Dimmeler et al., 1999; Fulton et al., 1999; Gallis et al., 1999; Michell et al., 1999; Fisslthaler et al., 2000). Thus, the calcium-dependence of the H_2O_2-dependent NO$^\bullet$ production was examined. Intracellular and extracellular Ca^{2+} buffering was achieved by 1-h pretreatment of endothelial cells with BAPTA/AM (10 μM). We and others have shown previously that this concentration of BAPTA/AM specifically blocks calcium without affecting other metal ions (Golconda et al., 1993; Cai et al., 2001). BAPTA/AM significantly reduced NO$^\bullet$ production in response to 20 to 50 μM H_2O_2 ($p < 0.01$), although having no effect when 100 to 200 μM H_2O_2 was used to stimulate cells (Fig. 1, shown as ■). Thus, NO$^\bullet$ production in response to the highest concentrations of H_2O_2 was calcium-independent, whereas responses to <50 μM H_2O_2 was entirely calcium-dependent.

Role of PI 3-kinase and Akt in H_2O_2-Dependent NO$^\bullet$ Production. Recent studies have shown that eNOS can be activated by PI 3-kinase–dependent phosphorylation at serine 1179 (Dimmeler et al., 1999; Fulton et al., 1999; Michell et al., 1999). To determine whether this pathway was involved in NO$^\bullet$ stimulation by H_2O_2, endothelial cells were pretreated for 1 h with inhibitors selective for PI 3-kinase, wortmannin (100 nM) or LY294002 (10 μM), before H_2O_2 (150 μM) stimulation. As demonstrated in Fig. 2A, H_2O_2 caused a 12.8-fold increase in NO$^\bullet$ production ($n = 12$, $p < 0.001$). Both wortmannin and LY294002 blocked H_2O_2-stimulated NO$^\bullet$ production by approximately 46% ($p < 0.05$). Western blot analysis using phosphospecific antibodies indicated that Akt serine 473 and eNOS serine 1179 phosphorylation were increased by H_2O_2 exposure; however, these responses were delayed and only appreciable at 10 and 15 min after addition (Fig. 2B). Of note, wortmannin (100 nM) prevented H_2O_2-dependent Akt phosphorylation and serine 1179 phosphorylation of eNOS at all time points, as shown in the representative Western blots and mean data (Fig. 2, B and C). Expression of unphosphorylated Akt and eNOS was not affected by H_2O_2 or the protein kinase inhibitors during the time periods examined (Fig. 2B).

To further examine the role of Akt in eNOS activation by H_2O_2, endothelial cells were infected with an adenoviral construct containing a dominant-negative mutant of Akt (Akt-AAA) or β-glacatosidase (Ad-LacZ) at 50 MOI before H_2O_2 treatment. As shown in Fig. 3A, H_2O_2 stimulated NO$^\bullet$ production by 9-fold in Ad-LacZ infected cells, and this response was decreased by approximately 60% in cells infected with Akt-AAA ($n = 3$, $p < 0.01$). H_2O_2-dependent serine 1179 phosphorylation of eNOS was also prevented by Akt-AAA (Fig. 3B). Taken together, these data show that the phosphorylation of eNOS at serine 1179 by Akt is partially responsible for the activation of eNOS by H_2O_2, but this phenomenon occurs >10 min after the addition of the peroxide.

Role of the MEK/ERK1/2 Activation in H_2O_2-Dependent NO$^\bullet$ Production. In view of the relatively delayed activation of Akt and eNOS serine 1179 phosphorylation in response to H_2O_2, and the incomplete inhibition of NO$^\bullet$ production by wortmannin and LY294002, we considered the possibility that another protein kinase may be participating in the activation of eNOS at earlier time points after exposure to H_2O_2. Because there are multiple putative ERK1/2 phosphorylation sites present in the eNOS sequence, we examined the possible role of this kinase in the H_2O_2 response. Before H_2O_2 stimulation, endothelial cells were pretreated for 1 h with PD98059, a selective inhibitor of MEK1/2 (the direct upstream kinase activator of ERK1/2). PD98059 (50 μM) significantly reduced NO$^\bullet$ stimulation in response to 150 μM H_2O_2 by 53% (Fig. 4A; $p < 0.01$). The specific MEK1/2 inhibitor U0126 (10 μM) also attenuated H_2O_2-dependent NO$^\bullet$ to a similar extent (Fig. 4A; $p < 0.01$). Both inhibitors have been shown to be highly specific for the MEK/ERK1/2 pathway (Davies et al., 2000).

ERK1/2 phosphorylation in response to H_2O_2 was examined by Western blotting using a phosphospecific antibody recognizing ERK1/2 phosphorylated at threonine 202 and tyrosine 204. As shown in Fig. 4B, ERK1/2 activation occurred within 30 s after the addition of H_2O_2. ERK1/2 phos-

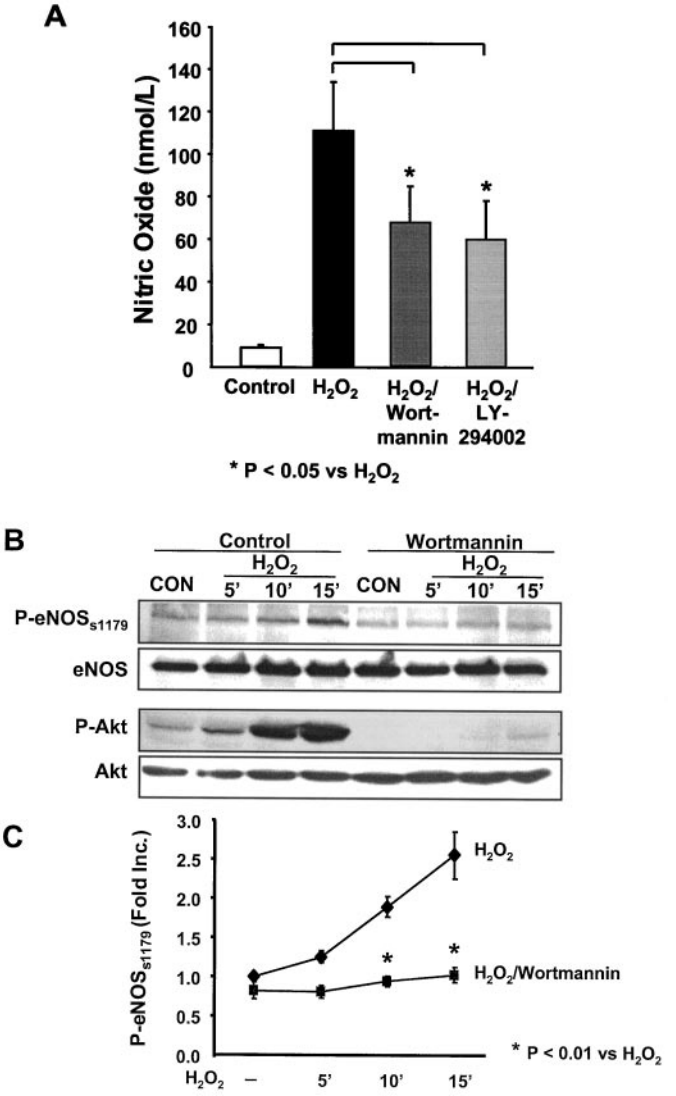

Fig. 2. Role of PI 3-kinase–dependent eNOS serine 1179 phosphorylation in NO$^\bullet$ stimulation by H_2O_2. A, effect of PI 3-kinase/Akt pathway inhibition on H_2O_2-stimulated NO$^\bullet$ production. Endothelial cells were pretreated with wortmannin (100 nM) or LY294002 (10 μM) for 1 h before exposure to H_2O_2 (150 μM), and NO$^\bullet$ production was determined with the use of the NO$^\bullet$ electrode. B, effect of wortmannin on H_2O_2-dependent Akt serine 473 and eNOS serine 1179 phosphorylation. Endothelial cells were pretreatment with wortmannin for 1 h before H_2O_2 stimulation and Akt; eNOS serine 1179 phosphorylation was determined by Western blot analysis using phosphospecific antibodies. C, mean data on H_2O_2-dependent eNOS serine 1179 phosphorylation normalized by native eNOS protein expression in the presence or absence of wortmannin.

phorylation by H_2O_2 was completely prevented by PD98059 at all time points examined (Fig. 4B). To determine whether MEK/ERK1/2 inhibition has any effect on PI 3-kinase/Akt-dependent eNOS phosphorylation at serine 1179, cells were pretreated with PD98059 before stimulation with H_2O_2 (150 μM). Akt and eNOS serine 1179 phosphorylation was determined as described above. As shown in representative Western blots in Fig. 4C and mean data in Fig. 4D, PD98559 had no effect on either Akt or eNOS phosphorylation by H_2O_2, indicating that the PI 3-kinase/Akt phosphorylation of eNOS is not dependent on ERK1/2. Likewise, pretreatment of endothelial cells with wortmannin had no effect on H_2O_2-dependent ERK1/2 activation (Fig. 4E).

PI 3-kinase/Akt-Dependent eNOS Serine 1179 Phosphorylation and MEK/ERK1/2 Cooperate in Mediating H_2O_2-dependent NO$^\cdot$ Production. To determine whether ERK1/2 and PI 3-kinase have cooperative effects on eNOS activation, endothelial cells were cotreated with PD98059 (50 μM) and wortmannin (100 nM) before H_2O_2 treatment. As shown in Fig. 5A, coinhibition with these inhibitors of MEK1/2 and PI 3-kinase completely prevented NO$^\cdot$ stimulation by H_2O_2 (150 μM, $p < 0.001$). In view of the gradual activation of Akt but the immediate activation of ERK1/2 by H_2O_2, we sought to determine whether ERK1/2 and Akt affected NO$^\cdot$ production at different times after exposure to H_2O_2. Endothelial cells were therefore pretreated with either PD98059, wortmannin, or a combination of both, and NO$^\cdot$ production was tracked for 15 min. Whereas PD98059 largely prevented NO$^\cdot$ release by H_2O_2 at early time points (<5 min), it had little effect at later time points. In contrast, wortmannin inhibited NO$^\cdot$ release by H_2O_2 at later time points more so than at early time points (Fig. 5B). These results are in keeping with the concept that parallel signaling pathways affect eNOS activation with different time courses. Of note, the combination of both wortmannin and PD98059 prevented H_2O_2-stimulated NO$^\cdot$ production at all time points examined, suggesting that the activation of both PI 3-kinase/Akt and MEK/ERK1/2 pathways are sufficient to fully activate eNOS. In these experiments, the NO$^\cdot$ concentrations reported are maximal responses at selective time points to indicate the temporal regulation by kinase inhibitors.

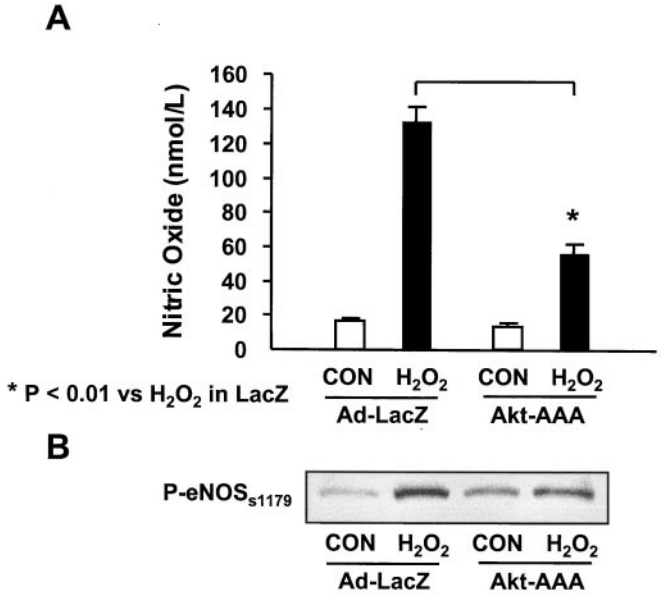

Fig. 3. Role of Akt in NO$^\cdot$ production and eNOS serine 1179 phosphorylation in response to H_2O_2. A, effect of infection with an adenovirus containing a dominant-negative mutant of Akt (Akt-AAA) on H_2O_2-dependent NO$^\cdot$ production. Endothelial cells at 90% confluence were infected with 50 MOI Akt-AAA or Ad-LacZ before exposure to H_2O_2 (150 μM). NO$^\cdot$ production was measured by use of the NO$^\cdot$-specific electrode 48 h later. B, effect of infection with Akt-AAA on H_2O_2-dependent eNOS serine1179 phosphorylation. Endothelial cells were infected as described above, and Western analysis using a phosphospecific antibody against eNOS phosphorylated at serine 1179 was used to determine eNOS phosphorylation. A representative blot of H_2O_2-dependent eNOS serine 1179 phosphorylation in the Ad-LacZ or Akt-AAA infected cells is presented.

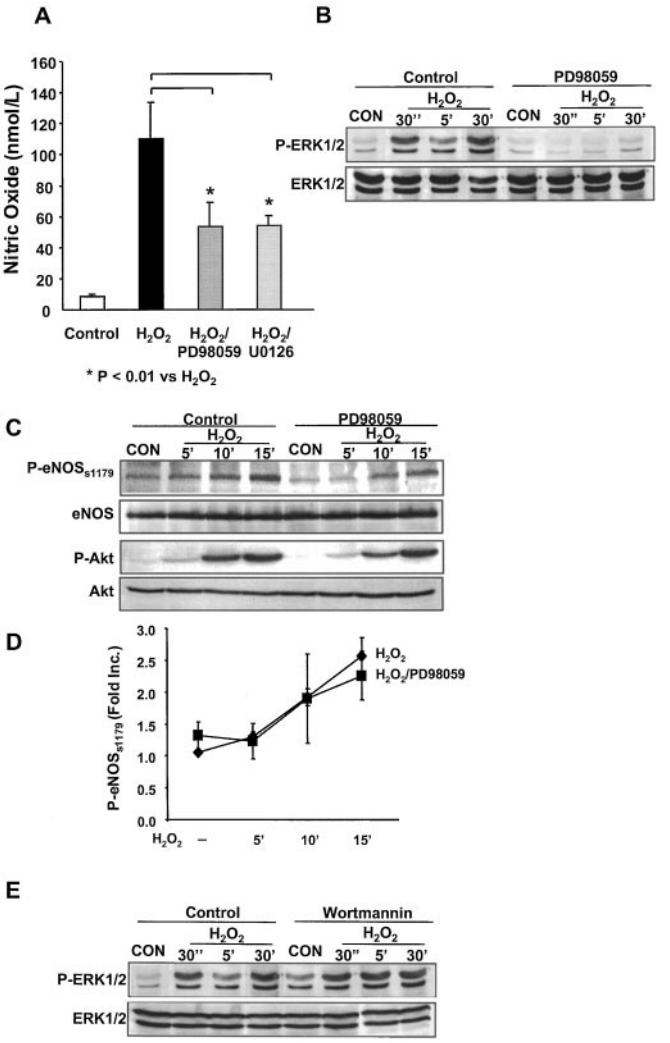

Fig. 4. Role of MEK/ERK1/2 activation in NO$^\cdot$ stimulation by H_2O_2. A, effect of MEK/ERK1/2 inhibition on H_2O_2-stimulated NO$^\cdot$ production. Endothelial cells were pretreated with either PD98059 (50 μM) or U0126 (10 μM) for 1 h before stimulation with H_2O_2 (150 μM), and NO$^\cdot$ production was determined by use of the NO$^\cdot$-specific electrode. B, effect of PD98059 on H_2O_2-dependent ERK1/2 phosphorylation. Endothelial cells were pretreated with PD98059 for 1 h before H_2O_2 stimulation and ERK1/2 activation determined by Western blot analysis using the phosphospecific antibody. C, effect of PD98059 on H_2O_2-dependent Akt and eNOS serine 1179 phosphorylation. Endothelial cells were pretreated with PD98059 for 1 h before H_2O_2 stimulation and Akt; eNOS phosphorylation was determined by Western analysis using phosphospecific antibodies. D, mean data on H_2O_2-dependent eNOS serine 1179 phosphorylation normalized by native eNOS protein expression in the presence or absence of PD98059. E, effect of wortmannin on H_2O_2-dependent ERK1/2 phosphorylation. Endothelial cells were pretreated with wortmannin (100 nM) for 1 h before H_2O_2 stimulation, and ERK1/2 activation was determined by Western blot analysis using the phosphospecific antibody.

Role of c-Src Family Tyrosine Kinase(s) in PI 3-Kinase/Akt-Dependent eNOS Phosphorylation at Serine 1179 and NO• Production in Response to H_2O_2. It has been shown previously that the tyrosine kinase c-Src can be activated by H_2O_2 (Yoshizumi et al., 2000). The possible role of c-Src family tyrosine kinase(s) in mediating NO• stimulation by H_2O_2 was examined by pretreatment of endothelial cells with a selective c-Src family tyrosine kinase(s) inhibitor, PP1. As shown in Fig. 6A, 1-h treatment with PP1 (10 μM) significantly attenuated H_2O_2-dependent NO• production by 44% ($p < 0.01$), supporting a role of c-Src family tyrosine kinase(s) in this response. Additional experiments suggested that c-Src was upstream of Akt, because PP1 prevented the time-dependent Akt and eNOS serine 1179 phosphorylation by H_2O_2 (Fig. 6B) but had no effect on H_2O_2-dependent ERK1/2 activation (Fig. 6C).

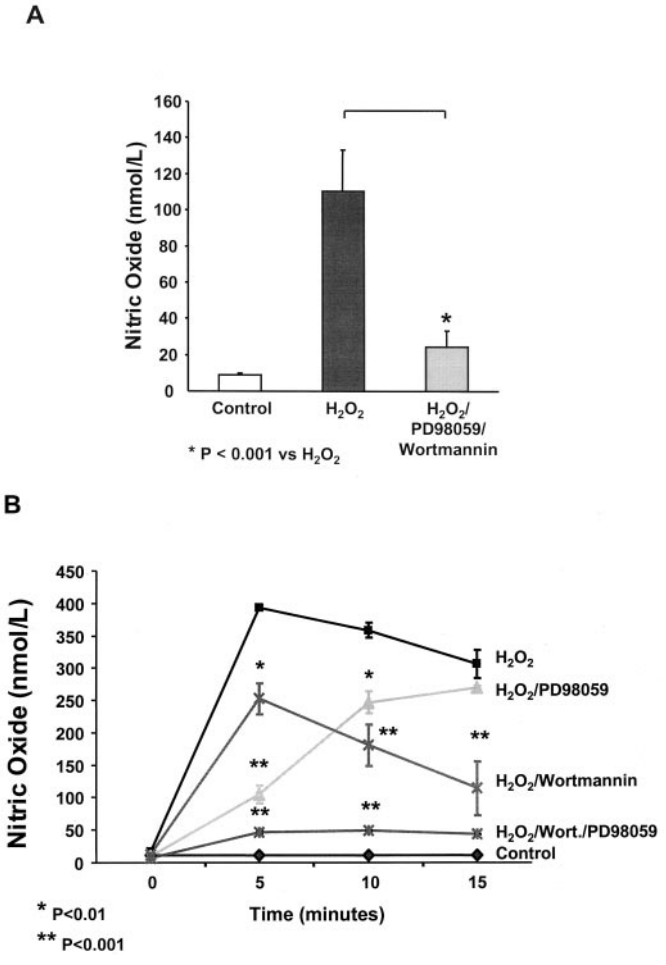

Fig. 5. Cumulative effects of PI 3-kinase/Akt-dependent eNOS serine 1179 phosphorylation and MEK/ERK1/2 activation on H_2O_2-dependent NO• production. A, effect of coinhibition of PI 3-kinase/Akt and ERK1/2 pathways on NO• stimulation by H_2O_2 (150 μM). Endothelial cells were cotreated with wortmannin (100 nM) and PD98059 (50 μM) for 1 h before H_2O_2 stimulation, and NO• production was determined with use of the NO• electrode. B, effect of inhibition of MEK/ERK1/2 and/or PI-3K/Akt pathways on endothelial NO• release at various time points after the addition of H_2O_2. Endothelial cells were pretreated with wortmannin, PD98059, or a combination of both for 1 h before stimulation with H_2O_2, and NO• production followed for 15 min.

Discussion

In the present study, we found that H_2O_2 is a potent stimulus for endothelial NO• release. This response is partially Ca^{2+}-dependent, but it also involves additive actions of the PI 3-kinase/Akt-dependent eNOS serine 1179 phosphorylation and MEK/ERK1/2 activation. The c-Src family tyrosine kinase(s) is required for H_2O_2-dependent eNOS serine 1179 phosphorylation by Akt and subsequent NO• production.

Endothelial production of NO• was initially considered to be dependent on increases in intracellular calcium and binding of calcium/calmodulin to eNOS. It has become apparent that eNOS activation is also regulated by its phosphorylation status. In particular, phosphorylation of serine 1179 and dephosphorylation of threonine 495 seem to be particularly important in the activation of eNOS in response to several stimuli, including shear stress, insulin, and the vascular endothelial cell growth factor. In keeping with this concept, we found that H_2O_2 stimulation of NO• production was only partially Ca^{2+}-dependent. The release of NO• by low doses of H_2O_2 (<50 μM) was largely prevented by the chelation of calcium with BAPTA/AM. These findings are consistent with data from Yang et al. (1999), who demonstrated that H_2O_2 concentrations of less than 44 μM relaxed phenylephrine-precontracted rat aorta in a Ca^{2+}-dependent manner. In our study, higher concentrations of H_2O_2 (>100 μM) stimulated NO• in a Ca^{2+}-independent manner, whereas NO• release in response to intermediate concentrations of H_2O_2 (50–100 μM) seemed to be partially calcium-dependent.

Because phosphorylation of eNOS seems to allow the enzyme to produce NO• in the absence of an increase in intracellular calcium, we examined the role of several potential phosphorylation cascades that might be activated by H_2O_2 and stimulate NO• production. These experiments suggest that both ERK1/2 and Akt are involved in the response to H_2O_2, and that these two signaling pathways evoke NO• production at different times after H_2O_2 addition. Our studies indicate that H_2O_2 produces a very rapid activation of ERK1/2 and that the inhibition of this blunts the NO• production at time points <5 min after the addition of the peroxide. After this (>10 min), Akt activation occurs, resulting in the phosphorylation of serine 1179 and prolonged activation of eNOS. These two signaling pathways do not seem dependent on one another, and they seem to elegantly regulate NO• production after stimulation with H_2O_2.

To our knowledge, these studies are the first to demonstrate that ERK1/2 can stimulate the production of NO• by eNOS. Bernier et al. (2000) have shown that ERK1/2 is associated with eNOS but that stimulation with bradykinin diminished this association approximately 5 min after its addition. The authors interpreted these data as suggesting that ERK1/2 inhibited eNOS, in contrast to our current findings. At first glance, our findings would seem at odds with the observation by Bernier et al. (2000); however, our data suggest that the stimulatory effect of ERK1/2 is lost at approximately the time they observe disassociation of the ERK1/2 and eNOS. The mechanism whereby ERK1/2 is involved in eNOS activation remains unclear. There are numerous potential ERK1/2 phosphorylation sites in eNOS; however, their role in eNOS activation by H_2O_2 remains undefined. It is also possible that ERK1/2 acts indirectly on eNOS. For

example, ERK1/2 might alter interactions between eNOS and 90-kDa heat shock protein, a phenomenon reported to enhance eNOS enzyme activity (Brouet et al., 2001). In preliminary experiments, however, we found little effect of H_2O_2 on 90-kDa heat shock protein binding to eNOS. Alternatively, ERK1/2 might alter the inhibitory interaction of eNOS with caveolin-1. In preliminary experiments, we saw no consistent effect of H_2O_2 on the interactions between eNOS and caveolin-1. In PC12 cells, the ERK1/2 inhibitors PD98059 and U0126 have been shown to inhibit ERK5 activation by epidermal growth factor and oxidant stress (Kamakura et al., 1999; Suzaki et al., 2002). Pretreatment with PD98059, however, had little effect on H_2O_2-dependent ERK5 activation in cultured endothelial cells, excluding a role of the ERK5 in mediating NO$^\cdot$ stimulation in response to H_2O_2.

In preliminary studies, the involvement of several other signaling pathways was excluded. Serine 1179 of eNOS can be phosphorylated by protein kinases other than Akt, including protein kinase A, AMPK, and Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) (Skepper et al., 1998; Chen et al., 1999; Fleming et al., 2001; Michell et al., 2001; Boo et al., 2002). None of these seemed to be responsible for H_2O_2 stimulation of eNOS serine 1179 phosphorylation. Pretreatment of endothelial cells with HA89 (10 μM), a selective inhibitor of protein kinase A, or KN93 (10 μM), a specific CaM kinase II inhibitor, had no effect on eNOS serine 1179 phosphorylation or NO$^\cdot$ stimulation by H_2O_2. AMPK was robustly phosphorylated by H_2O_2 but not affected by wortmannin, implying that AMPK was unlikely involved in H_2O_2 stimulation of NO$^\cdot$ production that is sensitive to PI 3-kinase inhibition. Bradykinin stimulation has been shown to decrease protein kinase C (PKC)-dependent eNOS threonine 495 phosphorylation, leading to the activation of the enzyme. This effect is associated with a concomitant increase in eNOS phosphorylation at serine 1179 (Fleming et al., 2001). Nevertheless, we found that H_2O_2 had no effect on eNOS threonine 495 phosphorylation, and the PKC inhibitor GF1092303X (2 μM) did not inhibit NO$^\cdot$ production in response to H_2O_2.

Our present experiments indicate that the c-Src family tyrosine kinase(s) is necessary for H_2O_2-dependent eNOS serine phosphorylation by Akt and subsequent NO$^\cdot$ production. Although c-Src can be an upstream activator of ERK1/2 in response to other stimuli such as laminar shear stress (Davis et al., 2001), it was not responsible for ERK1/2 activation by H_2O_2 because the c-Src family tyrosine kinase(s) inhibitor PP1 had no effect on this response.

Recently, Jaimes et al. (2001) have shown that prolonged incubation (>30 min) of endothelial cells with H_2O_2 leads to a near complete inhibition of eNOS. These investigators provided evidence that depletion of the eNOS cofactor FMN by H_2O_2 may contribute to this phenomenon. In preliminary studies, we also found that long-term incubation of endothelial cells with H_2O_2 leads to an initial increase in NO$^\cdot$ production lasting approximately 20 min, followed by a decline to near baseline levels. After this prolonged incubation, NO$^\cdot$ production could not be elicited by either additional H_2O_2 or by the calcium ionophore A23187, in keeping with the findings of Jaimes et al. Taken together, these data would suggest that H_2O_2 may stimulate NO$^\cdot$ production during brief exposures, such as during periods of ischemia and reperfusion or during bouts of exercise, which are known to be associated with increases in H_2O_2. Over the long term, depletion of cofactors such as the flavins results in inhibition of enzymatic function.

In summary, the present study indicates that H_2O_2 causes an acute and potent NO$^\cdot$ release from endothelial cells that is mediated by additive effects of the PI 3-kinase/Akt-depen-

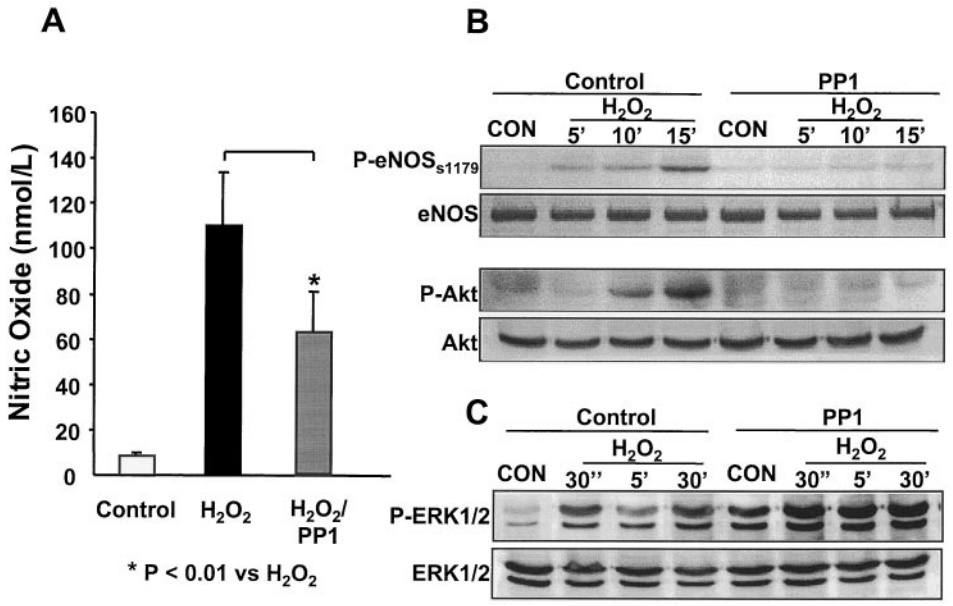

Fig. 6. Role of c-Src family tyrosine kinase(s) in H_2O_2-dependent eNOS serine 1179 phosphorylation by Akt and endothelial NO$^\cdot$ production. A, effect of c-Src family tyrosine kinase(s) inhibition on H_2O_2-stimulated NO$^\cdot$ production. Endothelial cells were pretreated with the selective c-Src family tyrosine kinase(s) inhibitor PP1 (10 μM) for 1 h before H_2O_2 (150 μM) exposure, and NO$^\cdot$ production was determined with use of the NO$^\cdot$ electrode. B, effect of c-Src family tyrosine kinase(s) inhibition on H_2O_2-dependent Akt and eNOS serine 1179 phosphorylation. Endothelial cells were pretreated with PP1 for 1 h before H_2O_2 exposure and Akt; eNOS phosphorylation was determined by Western blot analysis using phosphospecific antibodies. C, effect of c-Src family tyrosine kinase(s) inhibition on H_2O_2-dependent ERK1/2 phosphorylation. Endothelial cells were pretreated with PP1 for 1 h before H_2O_2 exposure, and ERK1/2 phosphorylation was determined by Western blot analysis using phosphospecific antibodies.

dent eNOS serine 1179 phosphorylation and MEK/ERK1/2 activation. Electrochemical detection of NO$^{\bullet}$ demonstrated that these pathways activate eNOS with distinct time courses. This response may contribute to H_2O_2-mediated endothelium-dependent vasorelaxation previously reported. In disease conditions that are associated with an increase in oxidant stress, this phenomenon may represent an immediate attempt to compensate for increased oxidant damage. Failure of this compensation may be significant in the pathogenesis of atherosclerosis.

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Address correspondence to: Dr. Hua Cai, Division of Cardiology, Emory University School of Medicine, Suite 319, Woodruff Memorial Research Building, 1639 Pierce Drive, Atlanta, GA 30322. E-mail: chua@emory.edu