Induction of Endothelial NO Synthase by Hydrogen Peroxide via a Ca^{2+}/Calmodulin-Dependent Protein Kinase II/Janus Kinase 2–Dependent Pathway

Hua Cai, Michael E. Davis, Grant R. Drummond, David G. Harrison

Abstract—We have recently demonstrated that hydrogen peroxide (H_2O_2) is an extremely potent stimulus of endothelial NO synthase (eNOS) gene expression. The present study was designed to identify the signaling mechanisms mediating this response. Induction of eNOS expression by H_2O_2 was found to be Ca^{2+} dependent, inasmuch as it was blocked by BAPTA-AM. Further studies have indicated that Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) plays a critical role in mediating this response. Immunocytochemical staining with an anti-CaM kinase II antibody confirmed the expression of CaM kinase II in cultured bovine aortic endothelial cells. H_2O_2 induced autophosphorylation of CaM kinase II and increased the activity of the enzyme, as assessed by an in-gel kinase assay. A specific inhibitor for CaM kinase II, KN93, and a calmodulin antagonist, W-7, attenuated eNOS induction by H_2O_2. Further studies have indicated that janus kinase 2 is important in mediating increased eNOS expression in response to H_2O_2 and likely is downstream from CaM kinase II. In conclusion, these data provide the first evidence that CaM kinase II plays a critical role in endothelial redox signaling. Regulation of eNOS via this pathway may represent an important vascular adaptation to oxidant stress. **(*Arterioscler Thromb Vasc Biol*. 2001;21:1571-1576.)**

Key Words: Ca^{2+}/calmodulin-dependent protein kinase II ■ janus kinase 2 ■ endothelial NO synthase ■ hydrogen peroxide ■ gene regulation

It has recently become apparent that a variety of common pathological conditions, including hypercholesterolemia, hypertension, diabetes, and heart failure, are associated with an increase in the vascular production of reactive oxygen species. In these conditions, there is often observed a loss of nitric oxide ($NO^{\cdot}$) biological activity. One explanation for this is that $NO^{\cdot}$ reacts with a number of reactive oxygen species, including superoxide anion ($O_2^{\cdot -}$), hydroxyl radical, peroxynitrite, and lipid radicals, resulting in a decrease in the half-life of $NO^{\cdot}$.[1]

Recently, we have found that hydrogen peroxide (H_2O_2), the product of $O_2^{\cdot -}$ dismutation, potently stimulates endothelial NO synthase (eNOS) expression via an increase in transcription and an increase in mRNA half-life.[2] Of note, even a short exposure of endothelial cells to H_2O_2 led to a sustained increase in eNOS expression for up to 72 hours. This phenomenon did not seem to be due to the formation of hydroxyl radical or other potential derivatives of H_2O_2, because it was unaffected by inhibiting or enhancing the Fenton reaction. Induction of eNOS by H_2O_2 may represent an important compensatory response to vascular oxidant stress that occurs in diseases in which vascular oxidant stress is increased.

H_2O_2 is potentially an important endogenous signaling molecule.[3,4] Like $NO^{\cdot}$, it is relatively stable and can diffuse freely from one cell to the next. Furthermore, a number of intracellular targets have been identified that are sensitive to H_2O_2. Some of these include protein tyrosine kinases (PTKs)[5,6] and phosphatases,[7] mitogen-activated protein kinases (MAPKs),[8,9] protein kinase C,[10] phospholipases,[11] and guanylate cyclase.[12] Recent studies have suggested that H_2O_2 mediates the agonist-induced activation of protein kinases. For example, by preincubating cells with catalase, H_2O_2 is shown to be required for epidermal growth factor–induced phosphorylation of its receptor and phospholipase C,[13] platelet-derived growth factor stimulation of signal transducers and activators of transcription,[14] and activation of Akt by angiotensin II.[15] Given the myriad of potential intracellular targets for H_2O_2, defining the signaling cascade mediating eNOS induction by H_2O_2 seems particularly important, and the present study examined this process in greater detail. Our data suggest that upregulation of eNOS by H_2O_2 is mediated by a previously undefined Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}/CaM kinase II)/janus kinase 2 (JAK2)–dependent pathway. CaM kinase II plays a critical role in the process, and the data suggest a novel role of this enzyme in endothelial cell function.

Received June 25, 2001; revision accepted July 16, 2001.

From the Division of Cardiology, Department of Medicine, Emory University School of Medicine, Atlanta, Ga.

Presented in part at the 72nd Scientific Sessions of the American Heart Association, Atlanta, Ga, November 7–10, 1999, and at the 73rd Scientific Sessions of American Heart Association, New Orleans, La, November 12–15, 2000.

Correspondence to David G. Harrison, MD, Division of Cardiology, Emory University School of Medicine, Suite 319, Woodruff Memorial Research Building, 1639 Pierce Dr, Atlanta, GA 30322. E-mail dharr02@emory.edu

Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org**

Methods

Materials

Pharmacological inhibitors and anti–CaM kinase II were purchased from Calbiochem. Anti–phospho-CaM kinase II was purchased from Promega. Anti–phospho-JAK2 was obtained from Upstate Biotechnology. Rhodamine red X–conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories.

Cell Culture

Bovine aortic endothelial cells (Cell Systems) were cultured in medium 199 (Life Technologies) containing 10% FCS (Hyclone Laboratories), as described previously.[2]

Measurement of eNOS mRNA and Kinase Activation

To examine the effects on eNOS mRNA expression of specific pharmacological kinase inhibitors, endothelial cells were pretreated with drugs (for 1 hour unless indicated otherwise) before 1-hour H_2O_2 (100 μmol/L unless indicated otherwise) stimulation. The inhibitors used were present during the incubation time with H_2O_2. Cells were harvested 24 hours later for RNA extraction. Northern analysis of eNOS mRNA was conducted as described previously.[16] Cell lysates, harvested in 1× SDS sample buffer (62.5 mmol/L Tris, 2% SDS, 10% glycerol, 0.005% bromphenol blue, and 15% of 2-mercaptoethanol), were separated in 7.5% to 12.5% SDS-PAGE, and protein phosphorylation was detected with phospho-specific antibodies. To examine the activity of CaM kinase II, 20 μg cell lysate was separated in 10% polyacrylamide gel containing myelin basic protein (MBP, Sigma Chemical Co) as a specific substrate.[17] The gel was washed with 40 mmol/L HEPES (pH 7.4) to renature the proteins before incubation with 250 μCi [γ-^{32}P]ATP in phosphorylation buffer (25 mmol/L HEPES, pH 7.4, 10 mmol/L $MgCI_2$, and 0.1 μmol/L cold ATP). After a wash with tetrasodium pyrophosphate (1% [wt/vol])–containing HEPES buffer, the gel was dried and subjected to autoradiography. A phosphorylated MBP band was clearly visible at the expected molecular weight of CaM kinase II, ≈56 kDa, and the amount of ^{32}P incorporated into MBP in the gel was quantified with a Bio-Rad Gel Doc 1000 system. In separate experiments, cell lysates were immunoprecipitated with a specific antibody against CaM kinase II before being subjected to the in-gel kinase assay with the use of an identical approach.

Immunocytochemical Staining of CaM Kinase II

To confirm the presence of CaM kinase II in endothelial cells, a primary antibody against CaM kinase II and a rhodamine red X–conjugated goat anti-rabbit secondary antibody were used to stain endothelial cells cultured on glass coverslips. In brief, postconfluent endothelial cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde. Cells were permeabilized with PBS/BSA solution containing 0.1% Triton and blocked with diluted goat serum overnight. The next day, endothelial cells were incubated with a solution containing the anti–CaM kinase II antibody (1:100 dilution) for 2 hours, washed with PBS, and then incubated with rhodamine red X–conjugated goat anti-rabbit secondary antibody for 60 minutes. The coverslips were then mounted to a glass microscope slide with ProLong antifade mounting media (Molecular Probes), and cells were examined with use of a Zeiss LSM 510 confocal laser scanning microscope.

Statistical Analysis

Densitometry was used to quantify the intensities of bands on Northern and Western blots. Representative blots and grouped densitometric data from 3 separate experiments were presented. Differences in band intensities were subsequently analyzed by using *t* tests, followed by a Bonferroni test to correct for multiple comparisons. To compare the CaM kinase II phosphorylation and kinase activity in H_2O_2-treated groups at different time points with control group values, ANOVA, followed by a Dunnett ad hoc test, was used. Statistical significance was assumed at $P<0.05$.

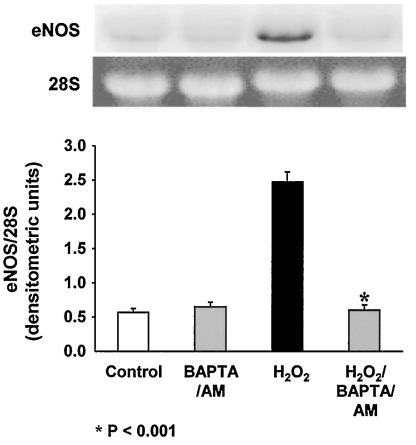

Figure 1. Northern analysis showing effect of Ca^{2+} chelator on eNOS induction by H_2O_2. Cells were pretreated with 10 μmol/L BAPTA-AM for 1 hour before exposure to either control medium or medium containing H_2O_2 for 1 hour. Cells were harvested at the 24-hour time point. The top panel is a representative Northern blot, the middle panel shows the corresponding 28S bands, and the bottom panel represents grouped densitometric data (mean±SEM) from 3 experiments.

Results

eNOS Induction by H_2O_2 Is a Ca^{2+}-Dependent Process

It has been shown that H_2O_2 induces intracellular Ca^{2+} oscillations in endothelial cells.[18] To examine whether Ca^{2+} is required for H_2O_2-dependent eNOS induction, BAPTA-AM (10 μmol/L) was used to pretreat endothelial cells before H_2O_2 stimulation. This concentration of BAPTA-AM specifically binds Ca^{2+} but does not interact with transition metals.[19,20] As shown in Figure 1, H_2O_2 increased eNOS mRNA steady-state levels by >4-fold, and chelating intracellular free Ca^{2+} with BAPTA-AM abolished this response ($P<0.001$). Of note, BAPTA-AM itself had no effect on eNOS mRNA expression in cells that were not treated with H_2O_2.

CaM Kinase II Is Involved in eNOS Induction by H_2O_2

CaM kinase II is a member of the multifunctional CaM kinase family that is rapidly activated by Ca^{2+}/calmodulin.[21] In particular, it functions as a decoder of oscillated Ca^{2+} signals.[22] Because chelation of intracellular Ca^{2+} blocked the effect of H_2O_2 on eNOS expression, we sought to determine whether CaM kinase II is involved in this response. To confirm the presence of CaM kinase II in endothelial cells, a specific anti–CaM kinase II antibody and a rhodamine red X–conjugated secondary antibody were used to stain for CaM kinase II in endothelial cells cultured on glass coverslips. As shown in Figure I (see online at http://atvb.ahajournals.org), strong staining for CaM kinase II was detected in the cytoplasm. No immunoreactivity was observed in the absence of the primary antibody. CaM kinase II autophosphorylation, detected with a phospho-specific antibody recognizing phospho-Thr286, was increased by H_2O_2 in a time-dependent fashion (Figure IIA; see online at http://atvb.ahajournals.org). CaM kinase II phosphorylation occurred as early as 2 minutes, peaked at 5 minutes, and persisted up to 20 minutes after H_2O_2 exposure.

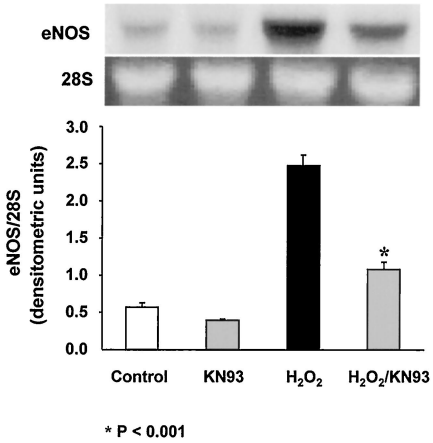

Figure 2. Northern analysis showing the effect of KN93, a specific inhibitor of CaM kinase II, on eNOS induction by H_2O_2. KN93 (10 μmol/L) was used to pretreat endothelial cells for 1 hour before exposure to control medium or medium containing H_2O_2 for 1 hour. Cells were harvested at the 24-hour time point. The top panel shows a representative Northern blot, the middle panel shows the corresponding 28S bands, and the bottom panel represents grouped densitometric data (mean±SEM) from 3 experiments.

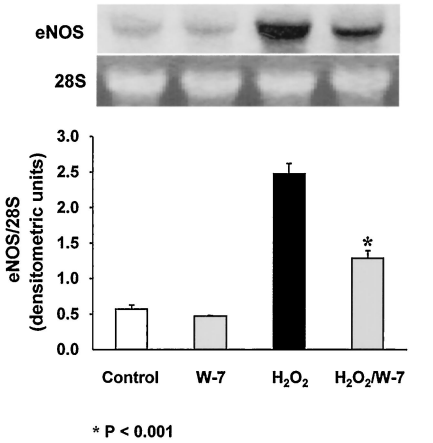

Figure 3. Northern analysis showing the effect of W-7, a specific inhibitor of calmodulin, on eNOS induction by H_2O_2. W-7 (3 μmol/L) was used to pretreat endothelial cells for 1 hour before exposure to control medium or medium containing H_2O_2 for 1 hour. Cells were harvested at the 24-hour time point. The top panel shows a representative Northern blot, the middle panel shows the corresponding 28S bands, and the bottom panel represents grouped densitometric data (mean±SEM) from 3 experiments.

The activity of CaM kinase II, as reflected by its ability to phosphorylate the specific substrate MBP in an in-gel kinase assay, was also increased by H_2O_2 (Figure IIB, $P<0.001$ at 60 minutes). The sustained increase in CaM kinase II activity was expected because the enzyme becomes fully active independent of Ca^{2+}/calmodulin after autophosphorylation at Thr286 by Ca^{2+}/calmodulin binding.[21,23] Figure IIC shows results from an in-gel kinase assay by using cell lysates immunoprecipitated with anti–CaM kinase II antibody. Pretreatment of endothelial cells with the Ca^{2+} chelator, BAPTA-AM, and a known specific inhibitor for CaM kinase II, KN93, abolished the activation of CaM kinase II by H_2O_2 (Figure IIC). More important, pretreatment of endothelial cells with KN93 (10 μmol/L) significantly inhibited the induction of eNOS by H_2O_2 by 77% ($P<0.001$, Figure 2). Furthermore, pretreatment of endothelial cells with a specific calmodulin antagonist, W-7 (3 μmol/L), also reduced eNOS upregulation by H_2O_2 by 53% ($P<0.001$, Figure 3). In summary, the data described in this paragraph strongly suggest that CaM kinase II is involved in the signaling cascade linking H_2O_2 to eNOS induction.

CaM Kinase II Modulates eNOS Expression Transcriptionally

We have previously shown that eNOS induction by H_2O_2 involves an increase in eNOS gene transcription and an increase in the stability of the eNOS mRNA.[2] To determine whether CaM kinase II affects transcriptional activation of eNOS by H_2O_2, endothelial cells were pretreated with KN93 before exposure to H_2O_2 for 1 hour. Nuclei were harvested 5 hours later, and nuclear run-on analyses were performed. As shown in Figure 4, H_2O_2 increased the eNOS gene transcription rate by >4-fold. This response was attenuated by 10 μmol/L KN93. We also examined the role of CaM kinase II in modulating eNOS mRNA half-life in response to H_2O_2. Endothelial cells pretreated with control medium or medium containing 10 μmol/L KN93 were incubated with H_2O_2 for 6 hours before the addition of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB, 60 μmol/L), a DNA polymerase II inhibitor. Northern analysis was then performed at various time points to follow the rate of mRNA decay. In contrast to the effect of KN93 on transcription rate, KN93 had no effect on the H_2O_2-induced increase in eNOS mRNA stability (Figure 5). These findings suggest that CaM kinase II mediates transcriptional regulation of eNOS yet does not affect eNOS mRNA half-life.

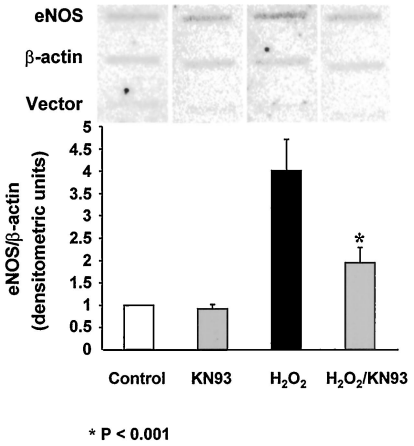

Figure 4. Nuclear run-on experiment examining eNOS gene transcription in the presence of H_2O_2 and KN93. Cells were pretreated with control medium or medium containing 10 μmol/L KN93 for 1 hour and exposed to H_2O_2 for 1 hour. Nuclei from each group were harvested at the 6-hour time point, and a nuclear run-on experiment was conducted as previously shown.[16] The top panel is a representative slot blot, and the bottom panel shows grouped densitometric data (mean±SEM) from 3 separate experiments.

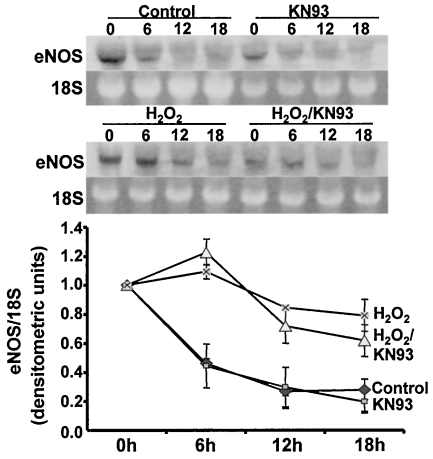

Figure 5. DRB chase experiment examining eNOS mRNA stability in the presence of H_2O_2 and KN93. Cells pretreated with control medium or medium containing 10 μmol/L KN93 for 1 hour were incubated with H_2O_2 for 6 hours before addition of DRB (60 μmol/L). Cells were harvested at the time points indicated, and Northern analysis was performed to determine the rate of mRNA decay in each group. The top panel shows a representative eNOS Northern blot and corresponding 18S bands as loading controls. The bottom panel is grouped densitometric data (mean±SEM) from 3 separate experiments. Note that the double amount of RNA was loaded for the control groups to enhance visualization of eNOS bands at the later time points.

A Tyrosine Kinase, JAK2, Is Involved in Mediating eNOS Induction by H_2O_2

Because protein tyrosine phosphorylation occurs in response to H_2O_2,[5,6] we considered the possibility that tyrosine kinase activation may be responsible for its stimulation of eNOS mRNA. Endothelial cells were pretreated with either tyrphostin A25 or herbimycin A (6 hours) before exposure to H_2O_2. These agents exhibit broad specificity for the inhibition of tyrosine kinases. As demonstrated in Figure IIIA (see online at http://atvb.ahajournals.org), at a concentration as low as 10 μmol/L, tyrphostin A25 significantly inhibited the H_2O_2-dependent eNOS induction by 74% ($P<0.001$). Likewise, herbimycin A (1 μmol/L) attenuated the upregulation of eNOS mRNA by H_2O_2 by 76% ($P<0.001$, Figure IIIB). Of note, pretreatment with either 0.1 to 10 μmol/L PP2 or PP1 (1 to 10 μmol/L), specific Src family tyrosine kinase inhibitors, had no effect on eNOS mRNA upregulation by H_2O_2. These results indicate that a non-Src tyrosine kinase, which is tyrphostin and herbimycin A sensitive, is likely involved in mediating eNOS induction by H_2O_2.

Cieslik et al[24] recently demonstrated that lysophosphatidylcholine-mediated transcriptional regulation of eNOS requires JAK2 activity.[24] It has also been shown that H_2O_2 stimulates JAK2 activity in fibroblasts.[14,25] Previous studies have suggested that herbimycin A inhibits JAK2 activation by interferon-γ.[26,27] Having a structure similar to that of tyrphostin A25, another tyrphostin family PTK inhibitor, AG490 (tyrphostin B64), is a selective inhibitor of JAK2. Therefore, we considered the possibility that JAK2 activation is required for eNOS induction by H_2O_2. Pretreatment of endothelial cells with AG490 (10 μmol/L) abolished eNOS upregulation by H_2O_2 (Figure 6).

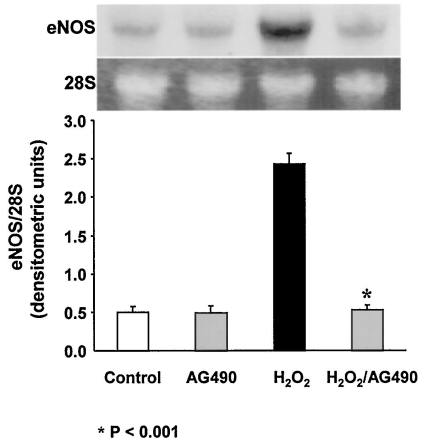

Figure 6. Northern analysis showing effects of the selective JAK2 inhibitor, AG490, on eNOS induction by H_2O_2. Endothelial cells were pretreated with 10 μmol/L AG490 for 1 hour before exposure to either control medium or medium containing H_2O_2 for 1 hour. Cells were harvested at the 24-hour time point. The top panel shows a representative Northern blot, the middle panel shows the corresponding 28S bands, and the bottom panel represents grouped densitometric data (mean±SEM) from 3 experiments.

JAK2 Is Downstream From CaM Kinase II

To examine the interrelationship between CaM kinase II, Ca^{2+}, and PTK, endothelial cells were pretreated with different inhibitors before the addition of H_2O_2. CaM kinase II activation was examined by using the in-gel kinase assay as described earlier. As expected, CaM kinase II activation by H_2O_2 was blocked by BAPTA-AM (10 μmol/L), suggesting that an increase in intracellular Ca^{2+} in response to H_2O_2 is upstream from CaM kinase II (Figure IVA; see online at http://atvb.ahajournals.org). In contrast, neither tyrphostin A25 (10 μmol/L), herbimycin A (1 μmol/L), nor AG490 (10 μmol/L) affected CaM kinase II activation by H_2O_2 (Figure IVA). As shown in Figure IVB, H_2O_2-dependent JAK2 phosphorylation was inhibited by KN93 and BAPTA-AM. These data indicate that CaM kinase II activation is unlikely to be dependent on JAK2 activation but more likely precedes JAK2 activation.

Discussion

The present study has defined a novel signaling pathway through which H_2O_2 increases the expression of eNOS. This pathway involves CaM kinase II and JAK2. Our studies indicate that CaM kinase II is activated by H_2O_2 and that this activation likely lies upstream of JAK2 and is critical for the induction of eNOS gene expression by H_2O_2.

Importantly, this is the first evidence that CaM kinase II mediates gene expression in endothelial cells. CaM kinase II belongs to a multifunctional CaM kinase family, which consists of CaM kinase I, II, and IV.[21] It is highly expressed in neuronal cells and is known to be the critical mediator for hippocampal long-term potentiation.[17,22] It is also expressed in the pancreatic B cell and is activated by glucose and other secretagogues.[28] Recently, CaM kinase members were identified as mediators of hypertrophy in cardiomyocytes.[29,30] However, there are very few studies of this kinase in endothelial cells. In 1993, Krizbai, Deli, and colleagues[31,32]

reported that primary cultures of cerebral endothelial cells express CaM kinase II by in situ hybridization. Two years later, Marsen et al[33] demonstrated that CaM kinase II inhibitor and the calmodulin antagonist prevented thrombin induction of preproendothelin-1 mRNA in human endothelial cells, suggesting that CaM kinase II may participate in thrombin signaling. In this prior study, CaM kinase II activation was not examined, and in fact, the presence of CaM kinase II was not directly demonstrated. Thus, our present study seems to be the first to fully demonstrate a role of this enzyme in endothelial cell signaling and, in particular, in redox signaling. Evidence from nuclear run-on and DRB chase experiments in the presence or absence of KN93 suggested that CaM kinase II predominantly contributes to the transcriptional but not posttranscriptional regulation of eNOS by H_2O_2.

A selective JAK2 inhibitor, AG490, completely attenuated eNOS induction by H_2O_2, suggesting that JAK2 is involved in transcriptional and posttranscriptional modulation of eNOS expression by H_2O_2. JAK2 is an intracellular tyrosine kinase that is activated by cytokines and growth factors via binding to the cytokine family of receptors. It is activated by autophosphorylation at tyrosine residues and also activates downstream targets by tyrosine phosphorylation. Using the in-gel kinase assay, we found that JAK2 blockade with tyrphostin A25, herbimycin A, and AG490 had no effect on H_2O_2 stimulation of CaM kinase II activity. This suggests that activation of CaM kinase II by H_2O_2 is an upstream event leading to JAK activation and that CaM kinase II activation is not dependent on JAK2 activation. Thus, this pathway seems to represent a rather unusual situation, in which a tyrosine kinase is phosphorylated, directly or indirectly, by a serine/threonine kinase. To our knowledge, this is the first evidence that CaM kinase II mediates JAK2 activation. This is analogous to a recent report showing that CaM kinase II can lead to PTK phosphorylation and ultimately phospholipase D activation in Chinese hamster ovary cells.[34] However, whether JAK2 is the direct or the indirect downstream effector of CaM kinase II remains to be elucidated. It was reported that JAK2 activation in fibroblasts is Src independent but Fyn dependent,[25] and this is consistent with our findings in present study, in which it was determined that non–Src-related tyrosine kinase activity is required for eNOS induction by H_2O_2. It is interesting to speculate that H_2O_2 activation of Fyn in endothelial cells may be CaM kinase II dependent.

There are a variety of additional signaling pathways that may interact with JAK2 and CaM kinase II in mediating eNOS expression in response to H_2O_2. In preliminary studies, we examined the roles of MAPK/extracellular signal–regulated kinase kinase 1/2, p38 MAPK, phosphatidylinositol 3-kinase, the epidermal growth factor receptor, protein kinase C, and the cAMP-response element–binding protein by using specific inhibitors for each. On the basis of these studies, none of these potential mediators seems to be involved in regulating eNOS expression in response to H_2O_2.

In conclusion, these data define a novel redox-sensitive signaling pathway that may represent an important vascular adaptation to redox stress by increasing eNOS expression and, ultimately, NO production. To our knowledge, this is first evidence that CaM kinase II is activated by oxidant stress and that it plays a critical role in modulating gene expression in the endothelium.

Acknowledgments

The study was supported by National Institutes of Health (NIH) grants HL-390006 and HL-59248, NIH Program Project grant HL-58000, and a Department of Veterans Affairs merit grant.

References

1. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*. 2000;87:840–844.
2. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG. Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. *Circ Res*. 2000;86:347–354.
3. Finkel T. Signal transduction by reactive oxygen species in nonphagocytic cells. *J Leukoc Biol*. 1999;65:337–440.
4. Wolin MS. Interactions of oxidants with vascular signaling systems. *Arterioscler Thromb Vasc Biol*. 2000;20:1430–1442.
5. Vepa S, Scribner WM, Parinandi NL, English D, Garcia JG, Natarajan V. Hydrogen peroxide stimulates tyrosine phosphorylation of focal adhesion kinase in vascular endothelial cells. *Am J Physiol*. 1999;277:L150–L158.
6. Carballo M, Conde M, El Bekay R, Martin-Nieto J, Camacho MJ, Monteseirin J, Conde J, Bedoya FJ, Sobrino F. Oxidative stress triggers STAT3 tyrosine phosphorylation and nuclear translocation in human lymphocytes. *J Biol Chem*. 1999;274:17580–17586.
7. Sullivan SG, Chiu DT, Errasfa M, Wang JM, Qi JS, Stern A. Effects of H_2O_2 on protein tyrosine phosphatase activity in HER14 cells. *Free Radic Biol Med*. 1994;16:399–403.
8. Abe J, Takahashi M, Ishida M, Lee JD, Berk BC. c-Src is required for oxidative stress-mediated activation of big mitogen-activated protein kinase 1. *J Biol Chem*. 1997;272:20389–20394.
9. Yoshizumi M, Abe J, Haendeler J, Huang Q, Berk BC. Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. *J Biol Chem*.;2000;275:11706–11712.
10. Konishi H, Tanaka M, Takemura Y, Matsuzaki H, Ono Y, Kikkawa U, Nishizuka Y. Activation of protein kinase C by tyrosine phosphorylation in response to H_2O_2. *Proc Natl Acad Sci U S A*. 1997;94:11233–11237.
11. Rao GN, Runge MS, Alexander RW. Hydrogen peroxide activation of cytosolic phospholipase A2 in vascular smooth muscle cells. *Biochim Biophys Acta*. 1995;1265:67–72.
12. Wolin MS, Burke TM. Hydrogen peroxide elicits activation of bovine pulmonary arterial soluble guanylate cyclase by a mechanism associated with its metabolism by catalase. *Biochem Biophys Res Commun*. 1987; 143:20–25.
13. Bae YS, Kang SW, Seo MS, Baines IC, Tekle E, Chock PB, Rhee SG. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide: role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem*. 1997;272:217–221.
14. Simon AR, Rai U, Fanburg BL, Cochran BH. Activation of the JAK-STAT pathway by reactive oxygen species. *Am J Physiol*. 1998; 275:C1640–C1652.
15. Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. *J Biol Chem*. 1999;274:22699–22704.
16. Ramasamy S, Parthasarathy S, Harrison DG. Regulation of endothelial nitric oxide synthase gene expression by oxidized linoleic acid. *J Lipid Res*. 1998;39:268–276.
17. Liu J, Fukunaga K, Yamamoto H, Nishi K, Miyamoto E. Differential roles of Ca(2+)/calmodulin-dependent protein kinase II and mitogen-activated protein kinase activation in hippocampal long-term potentiation. *J Neurosci*. 1999;19:8292–8299.
18. Hu Q, Corda S, Zweier JL, Capogrossi MC, Ziegelstein RC. Hydrogen peroxide induces intracellular calcium oscillations in human aortic endothelial cells. *Circulation*. 1998;97:268–275.
19. Golconda MS, Ueda N, Shah SV. Evidence suggesting that iron and calcium are interrelated in oxidant-induced DNA damage. *Kidney Int*. 1993;44:1228–1234.

20. Jornot L, Petersen H, Junod AF. Hydrogen peroxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions. *Biochem J*. 1998;335:85–94.
21. Hanson PI, Schulman H. Neuronal Ca^{2+}/calmodulin-dependent protein kinases. *Annu Rev Biochem*. 1992;61:559–601.
22. Fukunaga K, Miyamoto E. A working model of CaM kinase II activity in hippocampal long-term potentiation and memory. *Neurosci Res*. 2000; 38:3–17.
23. Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG. Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res*. 1996;79:984–991.
24. Cieslik K, Abrams CS, Wu KK. Up-regulation of endothelial nitric-oxide synthase promoter by the phosphatidylinositol 3-kinase gamma/Janus kinase 2/MEK-1-dependent pathway. *J Biol Chem*. 2001;276:1211–1219.
25. Abe J, Berk BC. Fyn and JAK2 mediate Ras activation by reactive oxygen species. *J Biol Chem*. 1999;274:21003–21010.
26. Nishiya T, Uehara T, Nomura Y. Herbimycin A suppresses NF-kappa B activation and tyrosine phosphorylation of JAK2 and the subsequent induction of nitric oxide synthase in C6 glioma cells. *FEBS Lett*. 1995; 371:333–336.
27. Kitamura Y, Takahashi H, Nomura Y, Taniguchi T. Possible involvement of Janus kinase Jak2 in interferon-gamma induction of nitric oxide synthase in rat glial cells. *Eur J Pharmacol*. 1996;306:297–306.
28. Easom RA. CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis. *Diabetes*. 1999;48:675–684.
29. Zhu W, Zou Y, Shiojima I, Kudoh S, Aikawa R, Hayashi D, Mizukami M, Toko H, Shibasaki F, Yazaki Y, et al. Ca^{2+}/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. *J Biol Chem*. 2000;275:15239–15245.
30. Passier R, Zeng H, Frey N, Naya FJ, Nicol RL, McKinsey TA, Overbeek P, Richardson JA, Grant SR, Olson EN. CaM kinase signaling induces cardiac hypertrophy and activates the MEF2 transcription factor in vivo. *J Clin Invest*. 2000;105:1395–1406.
31. Krizbai I, Deli M, Lengyel I, Maderspach K, Pakaski M, Joo F, Wolff JR. In situ hybridization with digoxigenin labeled oligonucleotide probes: detection of CAMK-II gene expression in primary cultures of cerebral endothelial cells. *Neurobiology (Bp)*. 1993;1:235–240.
32. Deli MA, Joo F, Krizbai I, Lengyel I, Nunzi MG, Wolff JR. Calcium/calmodulin-stimulated protein kinase II is present in primary cultures of cerebral endothelial cells. *J Neurochem*. 1993;60:1960–1963.
33. Marsen TA, Simonson MS, Dunn MJ. Thrombin-mediated ET-1 gene regulation involves CaM kinases and calcineurin in human endothelial cells. *J Cardiovasc Pharmacol*. 1995;26:S1–S4.
34. Min DS, Cho NJ, Yoon SH, Lee YH, Hahn SJ, Lee KH, Kim MS, Jo YH. Phospholipase C, protein kinase C, Ca(2+)/calmodulin-dependent protein kinase II, and tyrosine phosphorylation are involved in carbachol-induced phospholipase D activation in Chinese hamster ovary cells expressing muscarinic acetylcholine receptor of *Caenorhabditis elegans*. *J Neurochem*. 2000;75:274–281.