Cyclic AMP Response Element–Binding Protein Mediates Reactive Oxygen Species–Induced c-fos Expression

Toshihiro Ichiki, Tomotake Tokunou, Kae Fukuyama, Naoko Iino, Satoko Masuda, Akira Takeshita

Abstract—Although the cyclic AMP response element–binding protein (CREB) plays an important role in the survival of neuronal cells and T lymphocytes, the role of CREB in vascular smooth muscle cells (VSMCs) is incompletely characterized. We examined the role of CREB in VSMCs stimulated with reactive oxygen species. Activation of CREB was examined by Western blot analysis with an antibody that specifically recognizes phosphorylation at serine 133 of CREB, which is a critical marker of activation. Hydrogen peroxide (H$_2$O$_2$) time-dependently induced phosphorylation of CREB, with a peak at 15 minutes. The H$_2$O$_2$-induced phosphorylation of CREB was partially blocked by inhibition of either extracellular signal–regulated protein kinase kinase by PD98059 or of p38 mitogen-activated protein kinase (MAPK) by SB203580. AG1478, an epidermal growth factor receptor (EGFR) inhibitor, suppressed the H$_2$O$_2$-induced phosphorylation of CREB and tyrosine phosphorylation of EGFR. Overexpression of the dominant-negative form of CREB by an adenovirus vector suppressed H$_2$O$_2$-induced c-fos expression. These findings suggest that H$_2$O$_2$ induces CREB phosphorylation through EGFR transactivation and mitogen-activated protein kinase pathways. CREB might be a novel redox-sensitive transcription factor involved in the regulation of VSMC gene expression. (Hypertension. 2003; 42:177-183.)

Key Words: free radicals ■ cyclic AMP ■ kinase ■ epidermal growth factor receptor

A growing body of evidence suggests that reactive oxygen species (ROS) such as hydrogen peroxide (H$_2$O$_2$), superoxide, and hydroxy radical play a critical role in the pathogenesis of atherosclerosis, hypertension, and heart failure.$^1$ ROS are also involved in signal transduction of receptors for growth factors and vasoactive peptides, such as platelet-derived growth factor$^2$ and Angiotsensin (Ang) II.$^3$ ROS added exogenously to cells also elicit intracellular signaling events similar to those activated by growth factors or cytokines. H$_2$O$_2$ activates extracellular signal–regulated protein kinase (ERK),$^4$ p38 mitogen-activated protein kinase (MAPK),$^5$ and c-Jun NH$_2$-terminal kinase.$^6$ Recent studies have shown that H$_2$O$_2$ induces phosphorylation of epidermal growth factor receptor (EGFR)$^7,8$ as induced by Ang II$^7$ and thrombin.$^8$

The cyclic AMP response element–binding protein (CREB)$^9$ is a 43-kDa nuclear transcription factor belonging to the CREB/activated transcription factor (ATF) family. CREB binds to the octanucleotide sequence TGACGTCA as a homodimer or a heterodimer in association with other members of the CREB/ATF family.$^{10,11}$ Previous studies have demonstrated that neurotransmitters, hormones, and growth factors in different cell types could activate CREB.$^{10,12}$ Phosphorylation of a serine residue at position 133 (Ser-133) is necessary for transcriptional activation by CREB. Phosphorylation of CREB at Ser-133 allows access for CREB-binding protein (CBP), which is a transcriptional coactivator and has histone acetylation activity.$^{13}$ Phosphorylation of Ser-133 is mediated by a variety of protein kinases, such as p90$^{Rsk2}$ in response to activation of a ras-dependent ERK pathway$^{14}$ and MAPK–activated protein (MAPKAP) kinase 2 in response to activation of p38 MAPK.$^{15}$

Overexpression of a dominant-negative CREB transgene that has a mutation at Ser-133 and thus, is unable to bind to CBP induces apoptosis in T cells after growth factor stimulation.$^{16}$ Transgenic mice that overexpress a dominant-negative CREB in cardiomyocytes developed dilated cardiomyopathy.$^{17}$ These studies suggest that CREB might contribute to cell survival and development in these cell types.

It was reported that CREB was activated by hydrogen peroxide (H$_2$O$_2$) through the p38 MAPK pathway in a macrophage cell line.$^{18}$ We previously reported that AngII$^{19}$ and thrombin$^{20}$ activated CREB in an ERK- and a p38 MAPK–dependent manner and that CREB played an important role in the hypertrophy of vascular smooth muscle cells (VSMCs). Although ROS play an important role in the signaling of AngII and thrombin, the role of ROS in the activation of CREB in VSMCs has not been determined. In the present study, we examined the signaling pathway of H$_2$O$_2$-induced CREB activation and the role of ROS in AngII-induced CREB activation.

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From the Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, Fukuoka, Japan.
Correspondence to Toshihiro Ichiki, MD, Department of Cardiovascular Medicine, Kyushu University Graduate School of Medical Sciences, 3-1-1 Maidashi, Higashi-ku, 812-8582 Fukuoka, Japan. E-mail ichiki@cardiol.med.kyushu-u.ac.jp
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Methods

Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum were purchased from Gibco BRL, PD98059, KN93, KN92, and wortmannin were obtained from Research Biochemicals International. SB203580 was a generous gift from GlaxoSmithKline Pharmaceuticals. H89 was obtained from Biomol Research Laboratories, Inc. AG1478, catalase, and N-acetylcysteine (NAC) were obtained from Sigma. Antibodies against CREB, ERK, p38 MAPK, and their phosphorylated forms were obtained from New England Biolabs, Inc. Horseradish peroxidase-conjugated secondary antibodies (anti-rabbit or anti-mouse immunoglobulin G) were from Vector Laboratories, Inc. [32 P]dCTP was obtained form Du Pont (University of Pittsburgh, Pittsburgh, Pa). An adenovirus vector expressing Lac Z (AdLacZ) under gentle agitation for 2 hours at room temperature. After infection, the cells were washed 3 times, cultured in DMEM with 0.1% bovine serum albumin for 2 days, and then used for the experiments. Multiplicity of infection (MOI) indicates the number of viruses per cell added to the culture dish.

Statistical Analysis

Statistical analyses were performed by 1-way ANOVA and multiple-comparison (Fisher) tests when appropriate. A value of P<0.05 was considered significant. Data were expressed as mean±SE.

Results

Phosphorylation of CREB at Ser-133 by H2O2

To investigate whether CREB is phosphorylated in response to H2O2, we performed Western blot analysis with an antibody that recognizes CREB species phosphorylated at Ser-133. H2O2 strongly stimulated phosphorylation of CREB (Figure 1A, upper panel), with a peak at 15 minutes of stimulation. H2O2 dose-dependently induced phosphorylation of CREB at 15 minutes of stimulation (Figure 1B, upper panel). However, higher concentrations of H2O2 (104 to 106 μmol/L) reduced CREB phosphorylation (data not shown), suggesting that H2O2 at higher concentration might be toxic to our VSMCs. Incubation with xanthine and xanthine oxidase also induced phosphorylation of CREB (data not shown). Catalase (1000 U/mL) abolished the H2O2-induced CREB phosphorylation, confirming that H2O2 is responsible for the phosphorylation of CREB (Figure 1C). The basal phosphorylation level of CREB was slightly increased by preincubation with catalase; however, the mechanism is not known at this point. NAC, a potent antioxidant, also inhibited H2O2-induced CREB phosphorylation (Figure 1D) without affecting the basal CREB phosphorylation level. The total level of CREB protein expression as detected by Western blot analysis with an antibody against CREB was unchanged after H2O2 stimulation (Figure 1A through 1D, lower panels).

MAPK Pathways Mediate H2O2-Induced CREB Phosphorylation

Downstream kinases of ERK14 and p38 MAPK15 have been reported to phosphorylate CREB. H2O2-induced CREB phosphorylation was partially blocked by PD98059 (30 μmol/L), an ERK kinase inhibitor, or SB203580 (10 μmol/L), a p38 MAPK inhibitor (Figure 2A). PD98059 and SB203580 showed an additive effect. The same concentrations of PD98059 or SB203580 completely blocked H2O2-induced ERK or p38 MAPK activation, respectively, suggesting that partial inhibition might not be due to insufficient doses of the inhibitors (data not shown). Recent studies have shown that H2O2 activates MAPK through EGFR transactivation.6,25 AG1478, an EGFR inhibitor, almost completely inhibited CREB phosphorylation by H2O2 (Figure 2B). Immunoprecipitation with an anti-EGFR antibody, followed by Western blot analysis with an anti-phosphotyrosine antibody, revealed that H2O2 phosphorylated at the tyrosine residue of EGFR and AG1478 completely inhibited H2O2-induced tyrosine phosphorylation (Figure 2C). AG1478 also inhibited H2O2-induced ERK and p38 MAPK activation.
Phosphorylation of CREB by H2O2. A, VSMCs were stimulated with H2O2 (100 μmol/L) for the indicated periods. B, VSMCs were stimulated for 15 minutes with varying concentrations of H2O2 as indicated in the figure. C, VSMCs were preincubated with catalase (1000 U/mL) for 30 minutes and then stimulated with H2O2 (100 μmol/L) for 15 minutes. D, VSMCs were preincubated with NAC (10 mmol/L) for 30 minutes and then stimulated with H2O2 (100 μmol/L) for 15 minutes. Phosphorylation of CREB was detected by Western blot analysis with a phospho-specific CREB antibody (upper panel). The membrane was stripped and reprobed with an anti-CREB antibody (lower panel). A representative autoradiograph is shown. Right-hand graph shows densitometric analysis of Western blots (n=6). The ratio of phosphorylated CREB to total CREB (pCREB/CREB) is indicated as a percentage of unstimulated controls. *P<0.005 vs control.

Discussion

A growing body of evidence suggests that oxidative stress plays an important role in the progression of cardiovascular diseases, such as unstable angina, myocardial infarction, and heart failure. Therefore, it is important to understand the intracellular signals that are elicited by ROS to obtain insights into the pathogenesis of cardiovascular diseases. It is also generally accepted that ROS mimic the signaling of cytokines or growth factors, indicating that studying ROS signaling might contribute to understanding the signaling pathway of CREB.
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Figure 2. Effects of MAPK and EGFR inhibitors on \( \text{H}_2\text{O}_2 \)-mediated CREB phosphorylation. A, VSMCs were preincubated with PD98059 (30 \( \mu \text{mol/L} \)) and/or SB203580 (10 \( \mu \text{mol/L} \)) for 30 minutes and then stimulated with \( \text{H}_2\text{O}_2 \) (100 \( \mu \text{mol/L} \)) for 15 minutes. B–E, VSMCs were preincubated with AG1478 (2.5 \( \mu \text{mol/L} \)) for 30 minutes and then stimulated with \( \text{H}_2\text{O}_2 \) (100 \( \mu \text{mol/L} \)) for 15 minutes. Western blot analyses of CREB (A and B) were performed as described in the legend to Figure 1. C, Cell lysates were subjected to immunoprecipitation with an anti-EGFR antibody, followed by Western blot analysis (Blot) with an anti-phosphotyrosine antibody (upper panel). The membrane was stripped and reprobed with an anti-EGFR antibody (lower panel). Western blot analyses of ERK (D) and p38MAPK (E) was performed with the same procedure as described in the legend to Figure 1. A representative autoradiograph is shown. Right-hand panels (A and B) show densitometric analysis of Western blots (n=6). \( *P<0.05 \) vs control; \( \#P<0.05 \) vs \( \text{H}_2\text{O}_2 \) alone.

Cytokines and growth factors. \( \text{H}_2\text{O}_2 \) activates CREB in macrophage cells line but inhibits it in neuronal cells, suggesting that ROS differentially regulate CREB function in different cell types. We therefore studied the role of ROS in the activation of CREB in VSMCs, and this study is, to our knowledge, the first report showing that \( \text{H}_2\text{O}_2 \)-induced CREB phosphorylation and CRE-dependent gene transcription involve transactivation of EGFR.

Recently, nerve growth factor (NGF) and EGF were reported to phosphorylate and activate CREB through an ERK-p90Rsk2-dependent pathway. In contrast to EGF or NGF, fibroblast growth factor (FGF)-induced CREB phosphorylation is mediated by MAPKAP kinase-2, which lies immediately downstream from p38 MAPK. PD98059 or SB203580 suppressed \( \text{H}_2\text{O}_2 \)-induced CREB phosphorylation. Therefore, p90Rsk2 and MAPKAP kinase-2 might be responsible for CREB phosphorylation and CRE-dependent gene transcription. A recent report showed that the CREB-CBP complex induced by \( \text{H}_2\text{O}_2 \) might activate matrix metalloproteinase and induce EGFR transactivation, which are followed by CREB phosphorylation.

Although \( \text{H}_2\text{O}_2 \) stimulated CREB phosphorylation by several-fold, \( \text{H}_2\text{O}_2 \) increased CRE luciferase activity only 2-fold. The reason for this discrepancy is unclear. One explanation is that the basal luciferase activity might be relatively high in our VSMCs. Another possibility is that competition of activated CREB between endogenous CRE sites and CRE luciferase might occur and that most of the activated CREB might participate in the activation of endogenous genes, resulting in the weak activation of CRE luciferase by \( \text{H}_2\text{O}_2 \). Previously, Brindle et al. and Ginty et al. reported that the ability to activate CRE-dependent gene transcription is different among signaling pathways despite the similar level of CREB phosphorylation. A recent report by Mayr et al. might explain this differential effect on CREB phosphorylation and CRE-dependent gene transcription. They showed that the CREB-CBP complex induced by mitogenic signals such as NGF or EGF is less stable than that induced by cAMP in the nucleus. Therefore, the relative instability of the \( \text{H}_2\text{O}_2 \)-induced CREB/CBP complex might account for the weak activation of CRE-dependent gene transcription by \( \text{H}_2\text{O}_2 \).

Overexpression of wild-type CREB increased c-fos mRNA induction, and overexpression of dominant-negative CREB decreased c-fos mRNA expression in response to \( \text{H}_2\text{O}_2 \), suggesting the critical and essential role of CREB for c-fos gene expression. Overexpression of wild-type CREB strongly enhanced basal and \( \text{H}_2\text{O}_2 \)-induced phosphorylation of CREB. However, basal c-fos mRNA expression is not so prominent in wild-type, CREB-overexpressing cells. The mechanism is presently unknown. An explanation could be that activation of CREB alone is not sufficient for c-fos gene expression, and

loproteins but not through Pyk2 in VSMCs. We have not examined the role of Pyk2 or matrix metalloproteins in CREB activation. However, our results suggest that H2O2 might activate matrix metalloproteins and induce EGFR transactivation, which are followed by CREB phosphorylation.
simultaneous activation of an additional transcription factor(s) is necessary for induction.

The mechanism by which CREBM1 inhibits CREB function is believed to be the replacement of endogenous CREB with the mutated CREB, rather than inhibition of phosphorylation of endogenous CREB. Because CREB can dimerize with ATF-1, it is possible that the effect of CREBM1 might be ascribed to sequestration of ATF-1. This possibility cannot be excluded at this point.

Although H₂O₂ and AngII use similar signaling mechanisms in terms of CREB activation, AngII but not H₂O₂ requires PKA activity for the activation of CREB. Recently, Impey et al reported that PKA activity is necessary for the translocation of ERK activated by NGF and that basal PKA activity might be required for AngII-induced ERK and CREB activation but not for H₂O₂-induced ERK activation. Inhibition of CREB function suppressed H₂O₂-induced c-fos mRNA expression. Although NAC inhibited AngII-induced MAPK activation and CREB phosphorylation, AngII-induced c-fos mRNA induction was not inhibited by NAC. These data suggest that AngII-induced c-fos mRNA induction might involve a ROS-independent pathway, although ROS play an important role in AngII-induced MAPK activation. Because NAC did not completely inhibit AngII-induced activation of MAPKs and CREB, the remaining activity of these pathways might be sufficient to induce c-fos mRNA. ROS seem to play a critical role in AngII signaling; however, signaling mechanisms are not identical between AngII and H₂O₂.

**Perspective**
We have shown that H₂O₂ activates ERK and p38 MAPK through EGFR transactivation. These MAPKs mediate phos-
Figure 5. Effect of NAC on AngII-induced ERK, p38 MAPK, and CREB activation and on c-fos mRNA induction. VSMCs were preincubated with or without NAC (10 mmol/L) for 1 hour and then stimulated with AngII (100 nmol/L) for 5 minutes. Western blot analyses of (A) phosphorylated ERK and ERK, (B) phosphorylated p38 MAPK and p38 MAPK, and (C) phosphorylated CREB and CREB were performed as described in the legend to Figure 1. D, VSMCs were preincubated with or without NAC (10 mmol/L) for 1 hour and then stimulated with AngII (100 nmol/L) for 30 minutes. Northern blot analysis of c-fos mRNA was performed as described in the legend to Figure 4. Right-hand panel shows densitometric analyses of Northern and Western blots, (n=4). *P<0.05 vs control; #P<0.05 vs H2O2 alone.

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References


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