Role of CaMKII in hydrogen peroxide activation of ERK1/2, p38 MAPK, HSP27 and actin reorganization in endothelial cells

Andrew Nguyen, Peng Chen, Hua Cai*

Section of Cardiology, Department of Medicine, The University of Chicago, Chicago, IL, USA

Received 22 March 2004; revised 10 May 2004; accepted 9 June 2004

Available online 6 July 2004

Edited by Lukas Huber

Abstract **Growing evidence suggests that reactive oxygen species such as hydrogen peroxide (H_2O_2) can function as important signaling molecules in vascular cells. H_2O_2-activated redox-sensitive pathways mediate both physiological and pathological responses given the location and concentration of H_2O_2. We showed previously for the first time that calcium/calmodulin-dependent protein kinase II (CaMKII) is redox-sensitive in endothelial cells, mediating H_2O_2 upregulation of endothelial nitric oxide synthase. This response is always accompanied by an elongation phenotype of endothelial cells, implying modulation of actin cytoskeleton. In the present study, we investigated the role of CaMKII in H_2O_2 activation of p38 MAPK/heat shock protein 27 (HSP27) pathway and ERK1/2, both of which have been known to regulate actin reorganization in endothelial cells. Addition of H_2O_2 to bovine aortic endothelial cells increased ERK1/2 phosphorylation and activity, which was attenuated by a specific inhibitor of CaMKII, KN93. KN93 also prevented H_2O_2 activation of p38 MAPK. Transfection of endothelial cells with a CaMKII-specific inhibitory peptide (AA 281–309) reduced H_2O_2 phosphorylation of p38 MAPK and ERK1/2. Furthermore, blockade of CaMKII or janus kinase 2 (JAK2, downstream of CaMKII) prevented H_2O_2 activation of HSP27. KN93 attenuated, whereas AG490 (JAK2 inhibitor) abolished, H_2O_2-induced formation of actin stress fibers. Blockade of ERK1/2 inhibited H_2O_2 phosphorylation of HSP27 transiently. It also partially prevented H_2O_2 induction of actin stress fibers. In summary, redox-sensitive activation of p38 MAPK/HSP27 pathway or ERK1/2 in endothelial cells requires CaMKII. These pathways are at least partially responsible for H_2O_2 induced reorganization of actin cytoskeleton.**

Keywords: Hydrogen peroxide; Calcium/calmodulin-dependent protein kinase II; Janus kinase 2; ERK1/2; p38 MAPK; Heat shock protein 27; Actin cytoskeleton

1. Introduction

Reactive oxygen species such as hydrogen peroxide (H_2O_2) can function as important signaling molecules in vascular cells [1–3]. In particular, H_2O_2 is involved in modulating redox-sensitive mitogenic responses and gene regulation in the endothelium [2,4–6]. For example, we have recently shown that H_2O_2 potently upregulates endothelial NO· synthase (eNOS) gene expression in endothelial cells via activation of calcium/calmodulin-dependent kinase II (CaMKII) and janus kinase 2 (JAK2) [5,7]. This was the first characterization of CaMKII as a mediator of redox-sensitive gene regulation in the endothelium. Interestingly, upregulation of eNOS gene by H_2O_2 is always accompanied by a morphology change of endothelial cell phenotype from cobblestone to elongation, suggesting that modulation of actin cytoskeleton occurs during this response. Consistent with our observations, earlier work by Liu et al. [8] demonstrated alignment of actin filament in H_2O_2 (same low concentrations employed)-stimulated endothelial cells. p38 MAPK phosphorylation of heat shock protein 27 (HSP27), an actin binding protein, has been previously shown to mediate H_2O_2 reorganization of actin cytoskeleton [9–11]. The inter-relationship between CaMKII/JAK2 and H_2O_2 activation of p38 MAPK/HSP27, and the consequence of this interaction regarding actin cytoskeleton were investigated in the present study. Borbiev et al. [12,13] have previously shown that CaMKII activation of ERK1/2 is involved in thrombin induction of actin stress fibers in endothelial cells. The role of CaMKII in H_2O_2 activation of ERK1/2 and its relation to H_2O_2 regulation of actin cytoskeleton was also studied.

2. Materials and methods

2.1. Materials

KN93, AG490, PD98059, SB202190, and the CaMKII inhibitory peptide (AA 281–309, Cat#208711) were purchased from Calbiochem, San Diego, CA. Antibodies against phospho-ERK1/2, phospho-p38 MAPK and phospho-HSP27 were obtained from Cell Signaling Technology, Beverly, MA. Other chemicals were obtained from Sigma in highest purity.

2.2. Cell culture

Bovine aortic endothelial cells (Cell Systems) were cultured in Media 199 (Invitrogen Life Technologies, Carlsbad, CA) containing 10% fetal calf serum (FCS, Hyclone Laboratories, Logan, UT) as described previously [5]. One-day post confluent cells, starved with 5% media overnight, were used for experiments [5].

2.3. Western blot analysis of ERK1/2, p38 MAPK and HSP27 phosphorylation

Activation of ERK1/2 in H_2O_2-stimulated endothelial cells was assessed by its phosphorylation at threonine 202 and tyrosine 204, and by its ability to phosphorylate myelin basic protein (MBP) (see below for the kinase assay). For Western blot analysis of ERK1/2 phosphorylation, 20 μg of cellular proteins was separated in 10%

* Corresponding author. Fax: +1-773-702-2681.
E-mail addresses: hcai@medicine.bsd.uchicago.edu, lcai@medicine.bsd.uchicago.edu (H. Cai).

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.06.061

SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk at room temperature for 2 h and then incubated with the phospho-specific antibody for ERK1/2 (1:1000 dilutions) at 4 °C overnight. After washing with PBST (0.1% Tween in PBS) and subsequent incubation with the goat anti-rabbit secondary antibody (1:2000), the membranes were washed again with PBST and ERK1/2 phosphorylation determined by chemiluminescence detection with ECL reagent (Amersham Biotechnology). The intensity of the target bands was analyzed with a Bio-Rad Gel Doc 1000 system. The phosphorylation of p38 MAPK and HSP27 was examined following identical procedure with the phospho-specific antibodies against Threonine 180-Tyrosine 182 at p38 MAPK (1:1000) or Serine 82 at HSP27 (1:2000), respectively.

2.4. In-gel kinase assay of ERK1/2

To examine ERK1/2 activity, 20 μg of cellular proteins was separated in 10% polyacrylamide gel containing myelin basic protein (MBP, Sigma Chemical, St. Louis, MO) as a specific substrate [14]. 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 $MgCl_2$, and 0.1 μmol/L cold ATP). After being washed 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 ERK1/2, 44/42 kDa, and reflective of the ERK1/2 activity as described by Liu et al. [14].

2.5. Transient transfection with CaMKII inhibitory peptide

Endothelial cells at 90% confluence were transiently transfected with a CaMKII-specific (281–309) inhibitory peptide (1–2 ng/ml) or β-gal in Chariot reagent (Active Motif, Carlsbad, CA) according to the manufacturer's manual. Transfections were performed in serum-free media 199 for 2 h and then 10% serum was added. Protein phosphorylation by H_2O_2 in peptide-transfected cells was examined 48 h later. In the β-gal-transfected plate, blue cells were counted and the number divided by the total cell number to calculate the transfection efficiency. The effects on kinase activation of CaMKII inhibitory peptide were normalized against this ratio.

2.6. Analysis of actin cytoskeleton by confocal microscopy

Endothelial cell monolayers cultured on glass coverslips were transfected with a DNA construct of pEYFP-actin (BD Biosciences Clontech, Palo Alto, CA) using Effectene reagent (Qiagen Inc., Valencia, CA) following the instructions of the manufacturer. The pEYFP-actin construct expresses yellow-green fluorescent protein-actin fusion protein within the cells and the fluorescent images reflect real-time structure of actin cytoskeleton. Cells were used for H_2O_2 experiments 48 h later. The redistributed actin cytoskeleton in control and 3-h H_2O_2 treated cells in successfully transfected yellow-green fluorescent cells were captured and analyzed with a Zeiss Confocal Microscope at excitation and emission wavelengths of 513 and 527 nm, respectively.

In separate experiments, actin stress fibers or polymerized actin filaments in H_2O_2 stimulated endothelial cells were stained with Alexa Fluro 488-Phalloidin (Cat# A12379, Molecular Probes, Eugene, OR). In brief, one-day post confluent endothelial cells cultured on glass coverslips were treated with different kinase inhibitors for 1 h prior to stimulation with H_2O_2 for 3 h. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton, blocked with BSA/PBS and then incubated with Phalloidin for 30 min in dark. The coverslips were mounted using the ProLong antifade mounting media (Molecular Probes, Eugene, OR) and images of actin filaments captured using a confocal microscope as described above.

2.7. Statistical analysis

Differences in ERK1/2 or p38MAPK phosphorylation among control cells and cells treated with H_2O_2 in the presence or absence of CaMKII peptide inhibitor were compared with one way ANOVA. When differences were indicated, the Dunnet's post hoc test was employed. Statistical significance was set for $P < 0.05$. All grouped data shown in the figures were presented as means ± S.E.M.

3. Results

3.1. CaMII/JAK2 is upstream of hydrogen peroxide activation of p38 MAPK/HSP27

We have previously shown that H_2O_2 activates CaMKII in endothelial cells under identical experimental protocols [5]. In this earlier study, we demonstrated that H_2O_2 increased CaMKII autophosphorylation (examined by a phospho-specific antibody) and activity (assessed by an in-gel kinase assay) [5]. In this study, we also demonstrated that CaMKII/JAK2 activation is required for H_2O_2 upregulation of eNOS [5], which is always accompanied by an elongation phenotype of endothelial cells. We thus examined effects on p38 MAPK activation of HSP27, the pathway known to modulate actin cytoskeleton in endothelial cells [9], of CaMKII/JAK2. To examine a role of CaMKII or JAK2 in H_2O_2 activation of p38 MAPK, endothelial cells were pretreated with KN93 (10 μmol/L), a selective inhibitor for CaMKII, or AG490 (10 μmol/L), a specific antagonist of JAK2, for 1 h prior to H_2O_2 (100 μmol/L unless otherwise stated) stimulation and subsequent analysis of p38 MAPK phosphorylation. KN93 inhibits CaMKII by competitive binding to the calmodulin-binding domain of the enzyme. We have previously shown that KN93 inhibits CaMKII activity in endothelial cells [5]. Others have also demonstrated that KN93 inhibits CaMKII specifically in hepatocytes [15], fibroblasts [16], neurons [17] and vascular smooth muscle cells [18,19] in the concentration we employed.

The p38 MAPK phosphorylation, assessed by Western blotting with a phospho-specific antibody, was increased by H_2O_2 (Fig. 1A). This response was abolished by KN93 and markedly attenuated by AG490 (Fig. 1A). Furthermore, p38 MAPK phosphorylation in responses to different concentrations of H_2O_2 (100, 200, and 400 μmol/L) or at different time points post H_2O_2 exposure (5, 20, and 40 min) was examined. As is evident, H_2O_2 dose- and time-dependently increased p38 MAPK phosphorylation and these responses were attenuated by KN93 (Fig. 1B). In addition, transfection of endothelial cells with a CaMKII inhibitory peptide (AA 281–309) markedly reduced H_2O_2 phosphorylation of p38 MAPK by approximately 70% after normalization for transfection efficiency against β-gal (Fig. 1C, β-gal was provided by the manufacturer for transfection control). The inhibitory peptide blocks calcium/calmodulin activation of CaMKII, and has been previously shown to inhibit CaMKII activation in neurons and myocytes [20–22]. Take together, these data suggest that CaMKII/JAK2 functions as a novel redox-sensitive activator of p38 MAPK in endothelial cells via direct or indirect mechanisms.

As is obvious in Fig. 2A, H_2O_2 treatment of endothelial cells led to a time-dependent phosphorylation of HSP27. Pretreatment with KN93 for 1 h markedly reduced HSP27 phosphorylation (Fig. 2A). Likewise, AG490 pretreatment significantly prevented H_2O_2 phosphorylation of HSP27 (Fig. 3B), together suggesting that CaMKII and JAK2 are upstream of redox-regulation of the actin binding protein HSP27. The p38 MAPK/HSP27 pathway has been shown previously to mediate actin reorganization in response to H_2O_2 in human endothelial cells [9]. Consistent with this previous observation, H_2O_2 phosphorylation of HSP27 was found attenuated by SB202190 (10 μmol/L), a selective inhibitor of p38 MAPK (Fig. 2B).

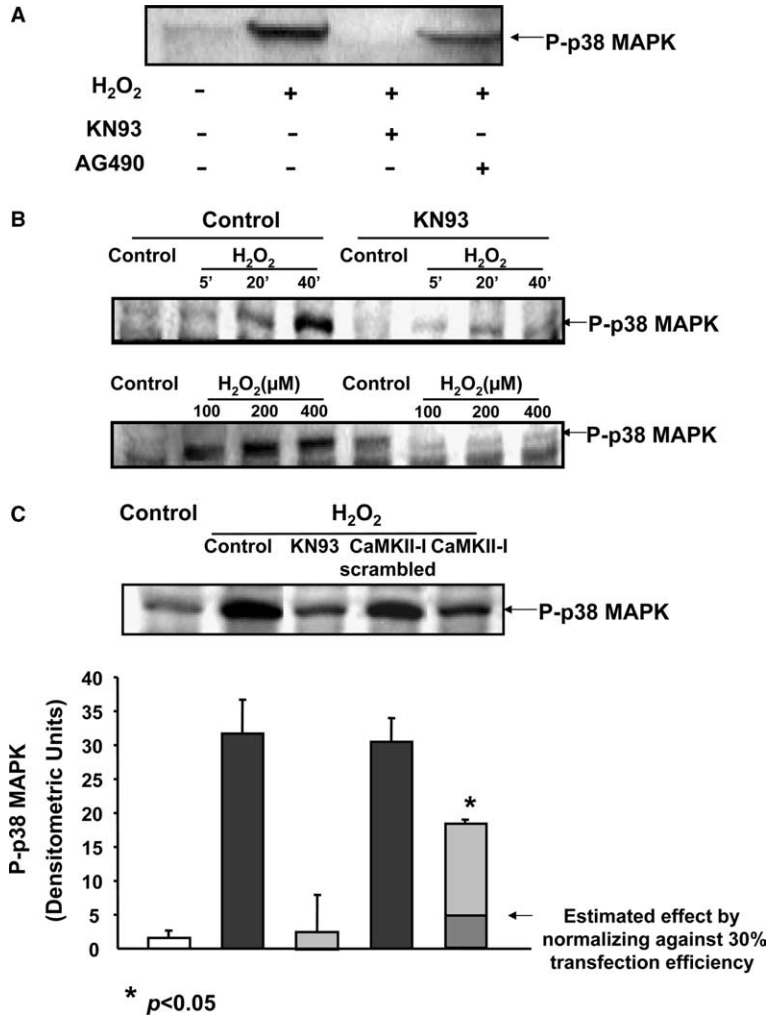

Fig. 1. H_2O_2 activation of p38 MAPK requires CaMKII. (A) Post-confluent endothelial cells were pretreated with a selective CaMKII inhibitor KN93 (10 μmol/L) or AG490, a specific inhibitor for janus kinase 2 (JAK2) for 1 h prior to stimulation by H_2O_2 (100 μmol/L) for 40 min. Cellular proteins were subjected to Western blotting for p38 MAPK phosphorylation with a phospho-specific antibody (Cell Signaling Technology). (B) Endothelial cells were treated with H_2O_2 (100 μmol/L) for 5, 20 and 40 min or different concentrations of H_2O_2 (100, 200, and 400 μmol/L) for 40 min in the presence or absence of KN93 pretreatment. Phosphorylation of p38 MAPK was examined as described. (C) Endothelial cells were transfected with an inhibitory peptide of CaMKII (AA208-319, Calbiochem) for 48-h prior to stimulation with H_2O_2 (100 μmol/L). Phosphorylation of p38 MAPK was analyzed as described above. Each experiment was repeated four times and the representative Western blot and grouped densitometric data are illustrated (means ± S.E.M.).

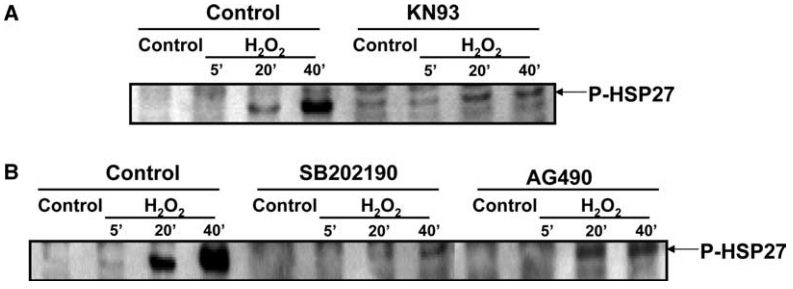

Fig. 2. Role of CaMKII/JAK2 in H_2O_2 activation of heat shock protein 27 (HSP27). (A) Endothelial cells were exposed to H_2O_2 (100 μmol/L) for 5, 20, and 40 min in the presence or absence of one-hour KN93 (10 μmol/L) pretreatment. HSP27 phosphorylation was analyzed by Western blotting using a phospho-specific antibody (Cell Signaling Technology). (B) Endothelial cells were exposed to H_2O_2 (100 μmol/L) for 5, 20, and 40 min in the presence or absence of one-hour AG490 (10 μmol/L) or SB202190 (10 μmol/L) pretreatment. HSP27 phosphorylation was analyzed as described above.

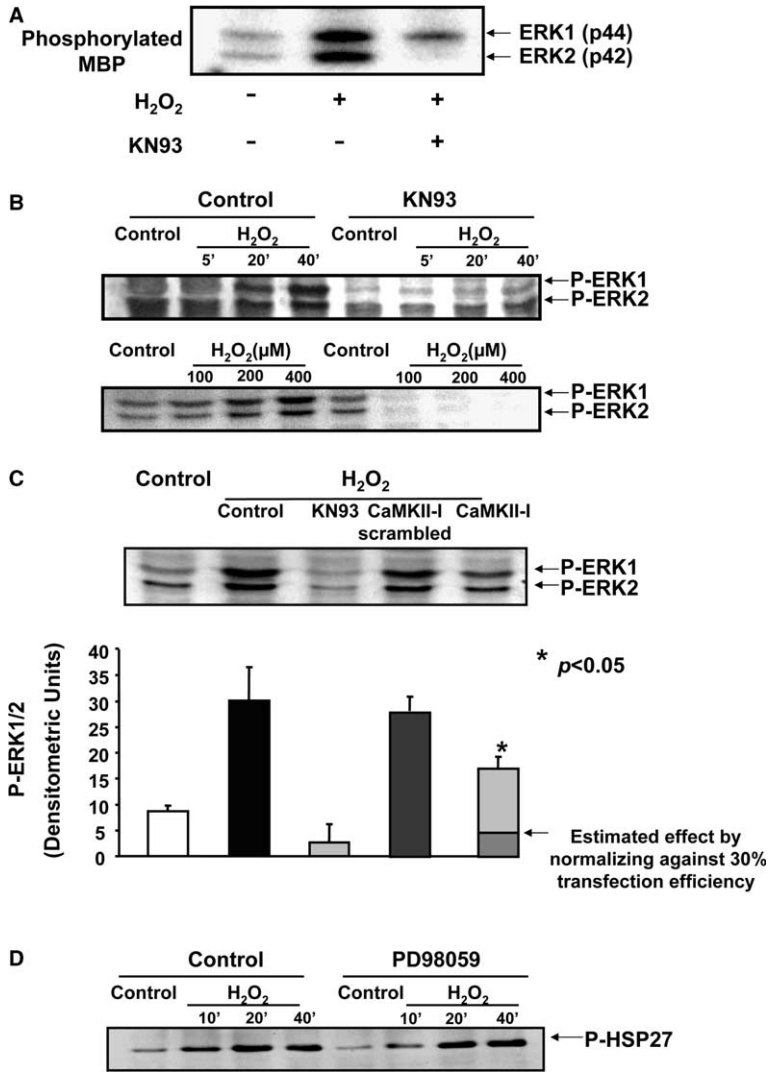

Fig. 3. H_2O_2 activation of ERK1/2 requires CaMKII. (A) Post-confluent endothelial cells were pretreated with a selective CaMKII inhibitor KN93 (10 μmol/L) for 1 h prior to stimulation by H_2O_2 (100 μmol/L) for 40 min. In-gel kinase assay was performed for analysis of ERK1/2 activity. (B) Endothelial cells were treated with H_2O_2 (100 μmol/L) for 5, 20 and 40 min or different concentrations of H_2O_2 (100, 200, and 400 μmol/L) for 40 min in the presence or absence of KN93 pretreatment. Cellular proteins were subjected to Western blotting for ERK1/2 phosphorylation with a phospho-specific antibody (Cell Signaling Technology). (C) Endothelial cells were transfected with an inhibitory peptide of CaMKII (AA208-319, Calbiochem) for 48-h prior to stimulation with H_2O_2 (100 μmol/L). ERK1/2 phosphorylation was analyzed as described above. Each experiment was repeated four times and the representative Western blot and grouped densitometric data are illustrated (means ± S.E.M.). (D) Endothelial cells were exposed to H_2O_2 (100 μmol/L) for 5, 20, and 40 min in the presence or absence of one-hour PD98059 (50 μmol/L) pretreatment. HSP27 phosphorylation was analyzed using a phospho-specific antibody (Cell Signaling Technology).

3.2. CaMKII is upstream of H_2O_2 activation of ERK1/2

Previous work by Borbiev et al. [12,13] demonstrated that ERK1/2 activation is involved in thrombin induced actin reorganization in endothelial cells. To examine a role of CaMKII in H_2O_2 activation of ERK1/2, endothelial cells were pretreated with KN93 (10 μmol/L) for 1 h prior to H_2O_2 stimulation and subsequent analysis of ERK1/2 phosphorylation or activity. The activity of ERK1/2, analyzed by the in-gel kinase assay, was robustly increased in endothelial cells treated with H_2O_2 for 60 min and this response was completely prevented by KN93 (Fig. 3A). Likewise, ERK1/2 phosphorylation in responses to different concentrations of H_2O_2 (100, 200, and 400 μmol/L) or at different time points post H_2O_2 exposure (5, 20, and 40 min) was determined by Western blot analysis. Of note, H_2O_2 dose- and time-dependently increased ERK1/2 phosphorylation and these responses were attenuated by KN93 (Fig. 3B). These experiments were repeated for 3–4 times and overall KN93 has little effect on basal phosphorylation of ERK1/2.

Furthermore, transient transfection of endothelial cells with a CaMKII inhibitory peptide (AA 281–309) markedly reduced H_2O_2 phosphorylation of ERK1/2 after normalization for transfection efficiency against β-gal (Fig. 3C). Taken together, these data suggest that CaMKII functions as a novel redox-sensitive activator of ERK1/2 in endothelial cells. Our data seem to be consistent with earlier observations that JAK2, the downstream effector of CaMKII, lies upstream of ERK1/2 in response to a variety of stimuli in different cell types [23–27].

3.3. Role of ERK1/2 in hydrogen peroxide activation of HSP27

It has been shown that CaMKII mediates thrombin activation of ERK1/2 and formation of actin stress fibers [12,13]. The potential role of HSP27 in this response, however, remained obscure. Endothelial cells were treated with ERK1/2 inhibitor PD98059 (50 μmol/L) for 1 h prior to H_2O_2 stimulation. As is evident, PD98059 reduced HSP27 phosphorylation at early time point (<10 min) post H_2O_2 stimulation while having no effect at later time points (20–40 min) (Fig. 3D). By contrast, as described earlier, blockade of CaMKII, JAK2 or p38 MAPK attenuated H_2O_2 phosphorylation of HSP27 at all time points examined (longest 40 min, Fig. 1A and B). Take together, these data seem to suggest that while ERK1/2 is transiently involved in HSP27 activation, CaMKII/JAK2/P38 MAPK pathway is the dominant cascade that is responsible for H_2O_2 activation of HSP27 in endothelial cells.

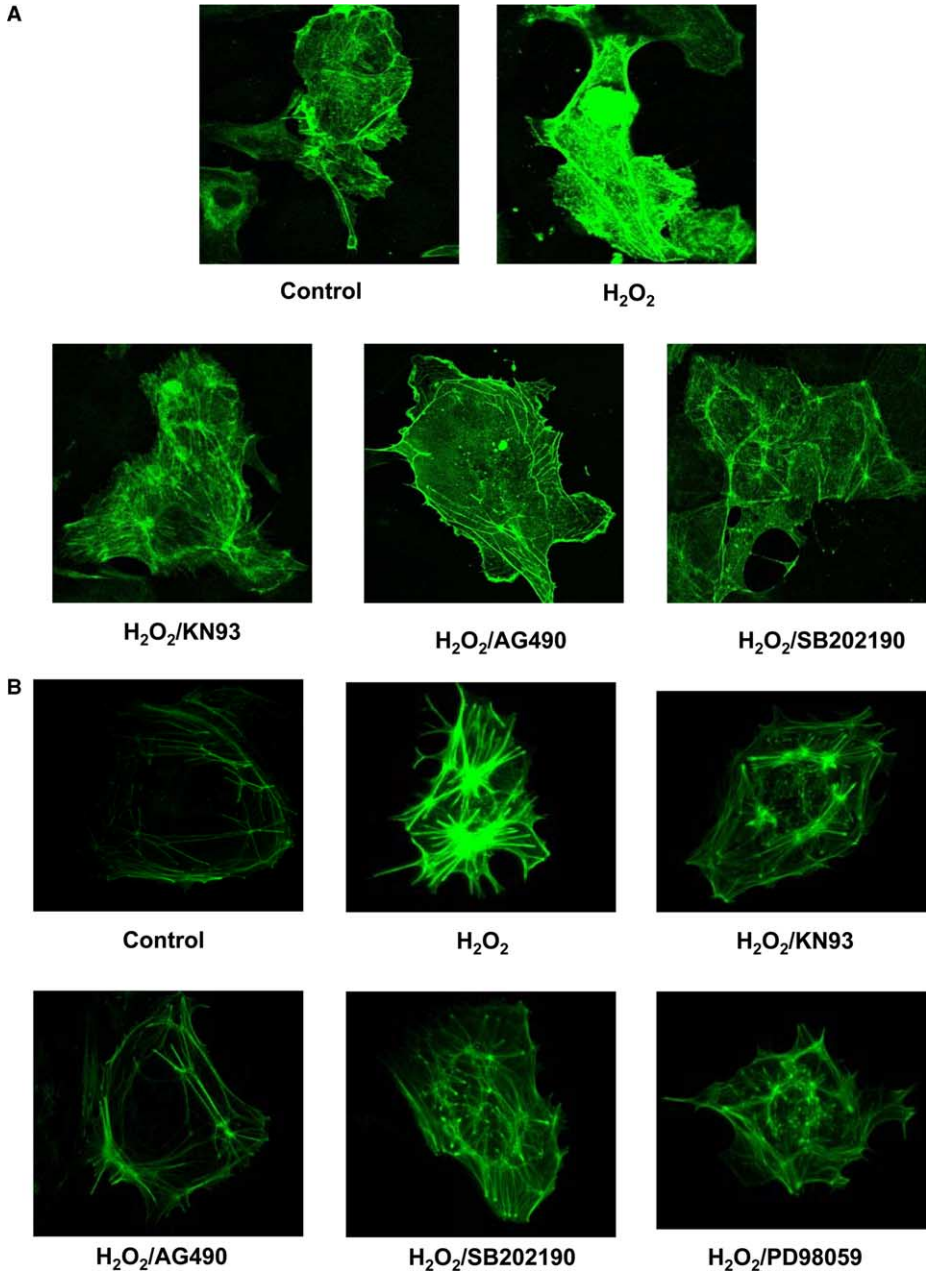

Fig. 4. (A) Role of CaMKII and JAK2 in H_2O_2 modulation of actin cytoskeleton. Endothelial cells were transfected with a DNA construct of pEYFP-actin for 48 h. Cells were stimulated with H_2O_2 (100 μmol/L) in the presence or absence of one-hour pretreatment with KN93, AG490, or SB202190, specific inhibitors of CaMKII, JAK2 or p38 MAPK, respectively. The redistributed actin cytoskeleton in control and 3 h-H_2O_2 treated cells in successfully transfected yellow-green fluorescent cells were captured and analyzed with a Zeiss Confocal Microscope at excitation and emission wavelengths of 513 and 527 nm, respectively. (B) Role of CaMKII, JAK2 and ERK1/2 in H_2O_2 modulation of actin cytoskeleton – Alexa Fluro 488–Phalloidin assay. One-day post confluent endothelial cells cultured on glass coverslips were treated with different kinase inhibitors for 1 h prior to stimulation with H_2O_2 for 3 h. Cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton, blocked with BSA/PBS and then incubated with Rhodamine–Phalloidin for 30 min in dark. The coverslips were mounted using the ProLong antifade mounting media (Molecular Probes, Eugene, OR) and images of actin filaments captured using a confocal microscope as described above.

3.4. Role of CaMKII, JAK2 and ERK1/2 in H_2O_2 induced reorganization of actin cytoskeleton

To examine the effect on H_2O_2-induced reorganization of actin cytoskeleton of CaMKII or JAK2 blockade, endothelial cells were transfected with a construct of pEYFP-actin as described in Section 2. Exposure of endothelial cells to H_2O_2 for 3 h caused a robust increase in actin stress fiber formation (Fig. 4A). This response was prevented by KN93 and abolished by AG490 pretreatment (Fig. 4A). SB202190 also prevented this response to serve as a positive control (Fig. 4A). Taken together, these data seem to suggest that H_2O_2 activation of CaMKII and JAK2 is involved in actin cytoskeleton reorganization in endothelial cells, presumably via HSP27-dependent mechanisms. This response may at least partially explain the elongation morphology accompanying H_2O_2 upregulation of eNOS mRNA.

In separate experiments, polymeric actin filaments were stained using Alexa Fluro 488–Phalloidin. Consistent to findings using pEYFP-actin transfection, three-hour treatment with H_2O_2 potently increased the formation of actin stress fibers (Fig. 4B). This response was apparently prevented by blockade of p38 MAPK, CaMKII and JAK2 with SB202190, KN93 and AG490, respectively. Inhibition of ERK1/2 with PD98059, however, also reduced actin stress fiber formation in H_2O_2 treated cells (Fig. 4B). In view of the transient inhibition of HSP27 phosphorylation by PD98059, these data seem to suggest that HSP27 is unlikely the major downstream effector of ERK1/2 with regards to modulation of actin cytoskeleton.

4. Discussion

The present study has defined a novel signaling pathway whereby redox-sensitive activation of CaMKII is required for ERK1/2 and p38 MAPK activation in endothelial cells. Furthermore, CaMKII and its downstream effector JAK2 are upstream of H_2O_2 activation of p38 MAPK/HSP27 pathway and may subsequently be responsible for H_2O_2 induction of actin stress fibers. Though ERK1/2 is only transiently involved in HSP27 phosphorylation, it appears partially involved in actin reorganization induced by H_2O_2.

Importantly, these data provide new insights into the signaling functions of CaMKII in endothelial cells. CaMKII has been well characterized in neuronal tissues and is a critical mediator of long term memory [14,28,29]. Its function in the endothelium, however, has remained mostly unknown. We have previously shown that one of the important functions of CaMKII in the endothelium is to mediate H_2O_2 upregulation of eNOS [5]. Recent studies demonstrate that CaMKII is also involved in acute production of $NO^{\cdot}$ from endothelial cells as well as relaxation responses to anesthetics of blood vessels [30,31]. Interestingly, the long-term eNOS upregulation response is always accompanied with elongation morphology of endothelial cells, implicating that the signaling pathways mediating eNOS gene regulation might also regulate actin cytoskeleton. Indeed our new data demonstrated that CaMKII and its downstream effector JAK2, both of which are required for H_2O_2 upregulation of eNOS, are upstream of p38 MAPK/HSP27 pathway that was found important for actin cytoskeleton reorganization in endothelial cells. Inhibition of CaMKII or JAK2 with KN93 or AG490, respectively, prevented H_2O_2 induced actin stress fiber formation in endothelial cells. This seems to share some similarity with recent reports that CaMKII, by altering phosphorylation of non-muscle filamin, is responsible for enhanced endothelial permeability in response to thrombin [12]. It is also consistent with earlier studies where JAK2 was found upstream of p38 MAPK in response to growth hormone stimulation [32].

Using a selective inhibitor and a specific inhibitory peptide for CaMKII, we found that activation of CaMKII is necessary for H_2O_2 activation of MAPK family member ERK1/2. Both the phosphorylation and activity of ERK1/2 were attenuated by blockade of CaMKII. This observation fits into the concept that H_2O_2 is growth-stimulating in the endothelium [1,2]. Furthermore, our findings are consistent with recent observations by Bortiev et al. [13] that thrombin-induced ERK1/2 activation is downstream of CaMKII in endothelial cells. Likewise, thrombin activation of ERK1/2 in vascular smooth muscle cells was also CaMKII-dependent [33]. In addition, a recent study reported that by binding to and activating Raf-1, CaMKII mediated integrin activation of ERK1/2 [34]. Consistent with these observations, our data further elucidated that the redox-sensitive activation of ERK1/2 in endothelial cells requires CaMKII. CaMKII/ERK1/2 pathway was found important for thrombin regulation of actin cytoskeleton [12]. Likewise, our data demonstrated that CaMKII/ERK1/2 was partially involved in H_2O_2 induced formation of actin stress fibers. HSP27, however, is unlikely the major downstream effector of ERK1/2 in modulating actin structure as blockade of ERK1/2 only transiently prevented H_2O_2 phosphorylation of HSP27. A cartoon summarizing proposed signaling mechanism is illustrated in Fig. 5.

In summary, these data characterized a novel role of CaMKII in redox-sensitive regulation of MAPK family members ERK1/2 and p38 MAPK in endothelial cells. Direct evidence was provided on the involvement of these pathways in H_2O_2 induced reorganization of actin cytoskeleton. These observations would have broad applications to situations whereby oxidant stress develops in the vasculature, including atherosclerosis, hypertension and heart failure [3,35].

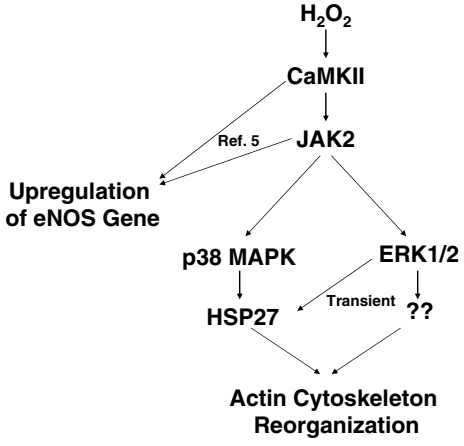

Fig. 5. Proposed signaling cascades mediating H_2O_2 induced reorganization of actin cytoskeleton.

References

[1] Stone, J.R. and Collins, T. (2002) The role of hydrogen peroxide in endothelial proliferative responses. Endothelium 9 (4), 231–238.

[2] Griendling, K.K. and Harrison, D.G. (1999) Dual role of reactive oxygen species in vascular growth. Circ. Res. 85, 562–563.

[3] Cai, H., Griendling, K.K. and Harrison, D.G. (2003) The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases. Trends Pharmacol. Sci. 24, 471–478.

[4] Griendling, K.K., Sorescu, D., Lassegue, B. and Ushio-Fukai, M. (2000) Modulation of protein kinase activity and gene expression by reactive oxygen species and their role in vascular physiology and pathophysiology. Arterioscler. Thromb. Vasc. Biol. 20, 2175–2183.

[5] Cai, H., Davis, M.E., Drummond, G.R. and Harrison, D.G. (2001) Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. Arterioscler. Thromb. Vasc. Biol. 21, 1571–1576.

[6] Cai, H., Li, Z., Davis, M.E., Kanner, W., Harrison, D.G. and Dudley Jr., S.C. (2003) 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. Mol. Pharmacol. 63, 325–331.

[7] Drummond, G.R., Cai, H., Davis, M.E., Ramasamy, S. and Harrison, D.G. (2000) Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. Circ. Res. 86, 347–354.

[8] Liu, S.M. and Sundqvist, T. (1995) Effects of hydrogen peroxide and phorbol myristate acetate on endothelial transport and F-actin distribution. Exp. Cell. Res. 217, 1–7.

[9] Huot, J., Houle, F., Marceau, F. and Landry, J. (1997) Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. Circ. Res. 80, 383–392.

[10] Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J.N., Huot, J. and Landry, J. (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J. Cell Sci. 110 (Pt 3), 357–368.

[11] Pichon, S., Bryckaert, M. and Berrou, E. (2004) Control of actin dynamics by p38 MAP kinase-Hsp27 distribution in the lamellipodium of smooth muscle cells. J. Cell Sci. 117, 2569–2577.

[12] Borbiev, T., Verin, A.D., Shi, S., Liu, F. and Garcia, J.G. (2001) Regulation of endothelial cell barrier function by calcium/calmodulin-dependent protein kinase II. Am. J. Physiol. Lung. Cell Mol. Physiol. 280, L983–L990.

[13] Borbiev, T., Verin, A.D., Birukova, A., Liu, F., Crow, M.T. and Garcia, J.G. (2003) Role of CaM kinase II and ERK activation in thrombin-induced endothelial cell barrier dysfunction. Am. J. Physiol. Lung Cell Mol. Physiol. 285, L43–L54.

[14] Liu, J., Fukunaga, K., Yamamoto, H., Nishi, K. and Miyamoto, E. (1999) Differential roles of Ca (2+)/calmodulin-dependent protein kinase II and mitogen-activated protein kinase activation in hippocampal long-term potentiation. J. Neurosci. 19, 8292–8299.

[15] Mellgren, G., Bruland, T., Doskeland, A.P., Flatmark, T., Vintermyr, O.K. and Doskeland, S.O. (1997) Synergistic antiproliferative actions of cyclic adenosine 3′, 5′-monophosphate, interleukin-1beta, and activators of Ca^{2+}/calmodulin-dependent protein kinase in primary hepatocytes. Endocrinology 138, 4373–4383.

[16] Fleming, I.N., Elliott, C.M. and Exton, J.H. (1998) Phospholipase C-gamma, protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II are involved in platelet-derived growth factor-induced phosphorylation of Tiam1. FEBS Lett. 429, 229–233.

[17] Liu, Q. and Berg, D.K. (1999) Actin filaments and the opposing actions of CaM kinase II and calcineurin in regulating alpha7-containing nicotinic receptors on chick ciliary ganglion neurons. J. Neurosci. 19, 10280–10288.

[18] Wang, Y.X. and Kotlikoff, M.I. (1997) Inactivation of calcium-activated chloride channels in smooth muscle by calcium/calmodulin-dependent protein kinase. Proc. Natl. Acad. Sci. USA 94, 14918–14923.

[19] Ginnan, R., Pfleiderer, P.J., Pumiglia, K.M. and Singer, H.A. (2004) PKCδ and CaMKIIδ2 mediate ATP-dependent activation of ERK1/2 in vascular smooth muscle. Am. J. Cell Physiol. 286, C1281–C1289.

[20] Waxham, M.N., Aronowski, J., Westgate, S.A. and Kelly, P.T. (1990) Mutagenesis of Thr-286 in monomeric Ca^{2+}/calmodulin-dependent protein kinase II eliminates Ca^{2+}/calmodulin-independent activity. Proc. Natl. Acad. Sci. USA 87, 1273–1277.

[21] Waxham, M.N., Malenka, R.C., Kelly, P.T. and Mauk, M.D. (1993) Calcium/calmodulin-dependent protein kinase II regulates hippocampal synaptic transmission. Brain Res. 609, 1–8.

[22] Dzhura, I., Wu, Y., Colbran, R.J., Corbin, J.D., Balser, J.R. and Anderson, M.E. (2002) Cytoskeletal disrupting agents prevent calmodulin kinase, IQ domain and voltage-dependent facilitation of L-type Ca^{2+} channels. J. Physiol. 545 (Pt 2), 399–406.

[23] Winston, L.A. and Hunter, T. (1995) JAK2, Ras, and Raf are required for activation of extracellular signal-regulated kinase/mitogen-activated protein kinase by growth hormone. J. Biol. Chem. 270, 30837–30840.

[24] Wang, L.H., Kirken, R.A., Erwin, R.A., Yu, C.R. and Farrar, W.L. (1999) JAK3, STAT, and MAPK signaling pathways as novel molecular targets for the tyrphostin AG-490 regulation of IL-2-mediated T cell response. J. Immunol. 162, 3897–3904.

[25] Takaoka, A., Tanaka, N., Mitani, Y., Miyazaki, T., Fujii, H., Sato, M., Kovarik, P., Decker, T. and Schlessinger, J., et al. (1999) Protein tyrosine kinase Pyk2 mediates the Jak-dependent activation of MAPK and Stat1 in IFN-gamma, but not IFN-alpha, signaling. EMBO J. 18, 2480–2488.

[26] Madamanchi, N.R., Li, S., Patterson, C. and Runge, M.S. (2001) Thrombin regulates vascular smooth muscle cell growth and heat shock proteins via the JAK-STAT pathway. J. Biol. Chem. 276, 18915–18924, Epub 2001 Mar 16.

[27] Ho, J.M., Nguyen, M.H., Dierov, J.K., Badger, K.M., Beattie, B.K., Tartaro, P., Haq, R., Zanke, B.W. and Carroll, M.P., et al. (2002) TEL-JAK2 constitutively activates the extracellular signal-regulated kinase (ERK), stress-activated protein/Jun kinase (SAPK/JNK), and p38 signaling pathways. Blood 100, 1438–1448.

[28] Bach, M.E., Hawkins, R.D., Osman, M., Kandel, E.R. and Mayford, M. (1995) Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the theta frequency. Cell 81, 905–915.

[29] Silva, A.J., Paylor, R., Wehner, J.M. and Tonegawa, S. (1992) Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science 257, 206–211.

[30] Su, J.Y. and Vo, A.C. (2003) Role of protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, and mitogen-activated protein kinases in volatile anesthetic-induced relaxation in newborn rabbit pulmonary artery. Anesthesiology 99, 131–137.

[31] Fleming, I., Fisslthaler, B., Dimmeler, S., Kemp, B.E. and Busse, R. (2001) Phosphorylation of Thr (495) regulates Ca (2+)/calmodulin-dependent endothelial nitric oxide synthase activity. Circ. Res. 88, E68–E75.

[32] Zhu, T. and Lobie, P.E. (2000) Janus kinase 2-dependent activation of p38 mitogen-activated protein kinase by growth hormone. Resultant transcriptional activation of ATF-2 and CHOP, cytoskeletal re-organization and mitogenesis. J. Biol. Chem. 275, 2103–2114.

[33] Ginnan, R. and Singer, H.A. (2002) CaM kinase II-dependent activation of tyrosine kinases and ERK1/2 in vascular smooth muscle. Am. J. Physiol. Cell. Physiol. 282, C754–C761.

[34] Illario, M., Cavallo, A.L., Bayer, K.U., Di Matola, T., Fenzi, G., Rossi, G. and Vitale, M. (2003) Calcium/calmodulin-dependent protein kinase II binds to Raf-1 and modulates integrin-stimulated ERK activation. J. Biol. Chem 278, 45101–45108.

[35] Cai, H. and Harrison, D.G. (2000) Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ. Res. 87, 840–844.