

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

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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.

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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 potentially 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 μ g of cellular proteins was separated in 10%

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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 -³²P]ATP in phosphorylation buffer (25 mmol/L HEPES, pH 7.4, 10 mmol/L MgCl₂, 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 H₂O₂ 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 H₂O₂ experiments 48 h later. The redistributed actin cytoskeleton in control and 3-h H₂O₂ 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 H₂O₂ stimulated endothelial cells were stained with Alexa Fluor 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 H₂O₂ 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 H₂O₂ 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 \pm S.E.M.

3. Results

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

We have previously shown that H₂O₂ activates CaMKII in endothelial cells under identical experimental protocols [5]. In this earlier study, we demonstrated that H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ (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 H₂O₂ (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 H₂O₂ (100, 200, and 400 µmol/L) or at different time points post H₂O₂ exposure (5, 20, and 40 min) was examined. As is evident, H₂O₂ 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 H₂O₂ 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, H₂O₂ 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 H₂O₂ 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 H₂O₂ in human endothelial cells [9]. Consistent with this previous observation, H₂O₂ phosphorylation of HSP27 was found attenuated by SB202190 (10 µmol/L), a selective inhibitor of p38 MAPK (Fig. 2B).

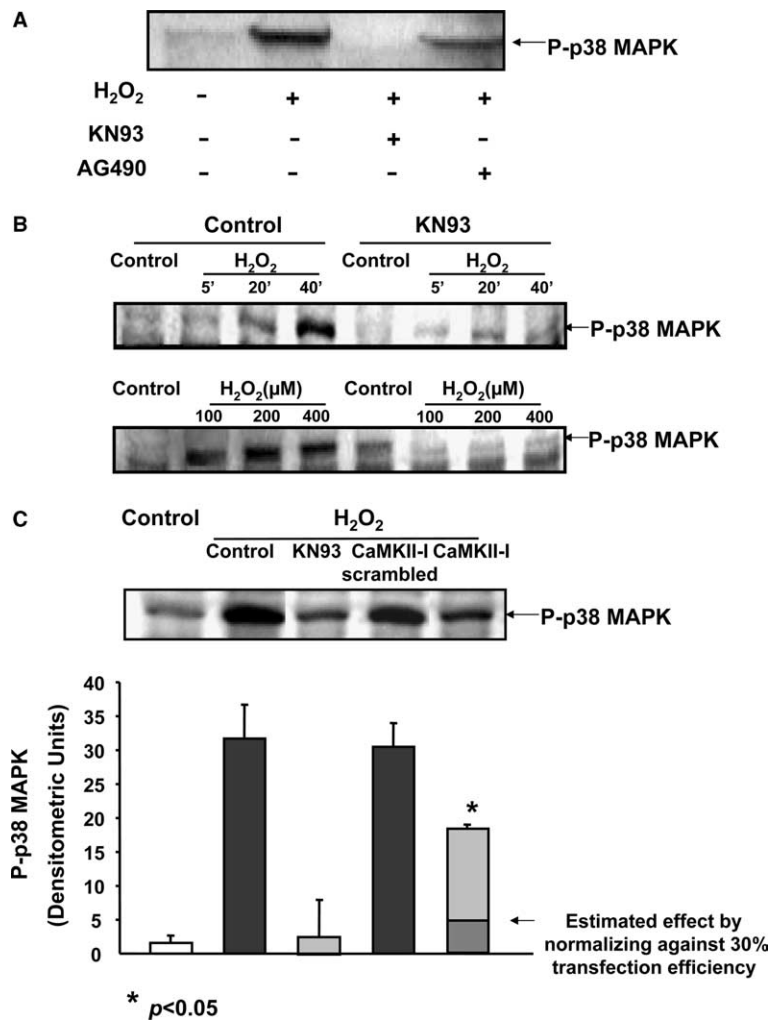


Fig. 1. H₂O₂ activation of p38 MAPK requires CaMKII. (A) Post-confluent endothelial cells were pretreated with a selective CaMKII inhibitor KN93 (10 μmol/L) or AG490, a specific inhibitor for janus kinase 2 (JAK2) for 1 h prior to stimulation by H₂O₂ (100 μmol/L) for 40 min. Cellular proteins were subjected to Western blotting for p38 MAPK phosphorylation with a phospho-specific antibody (Cell Signaling Technology). (B) Endothelial cells were treated with H₂O₂ (100 μmol/L) for 5, 20 and 40 min or different concentrations of H₂O₂ (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 H₂O₂ (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.).

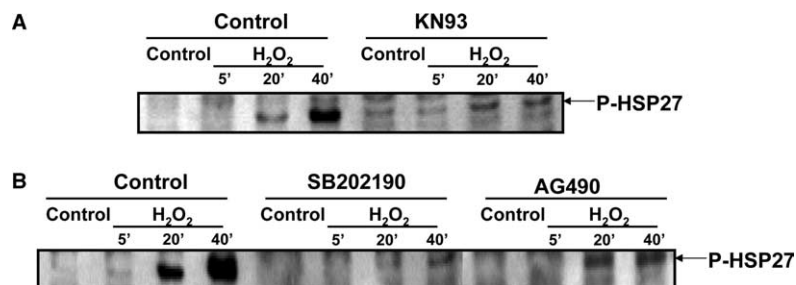


Fig. 2. Role of CaMKII/JAK2 in H₂O₂ activation of heat shock protein 27 (HSP27). (A) Endothelial cells were exposed to H₂O₂ (100 μmol/L) for 5, 20, and 40 min in the presence or absence of one-hour KN93 (10 μmol/L) pretreatment. HSP27 phosphorylation was analyzed by Western blotting using a phospho-specific antibody (Cell Signaling Technology). (B) Endothelial cells were exposed to H₂O₂ (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.

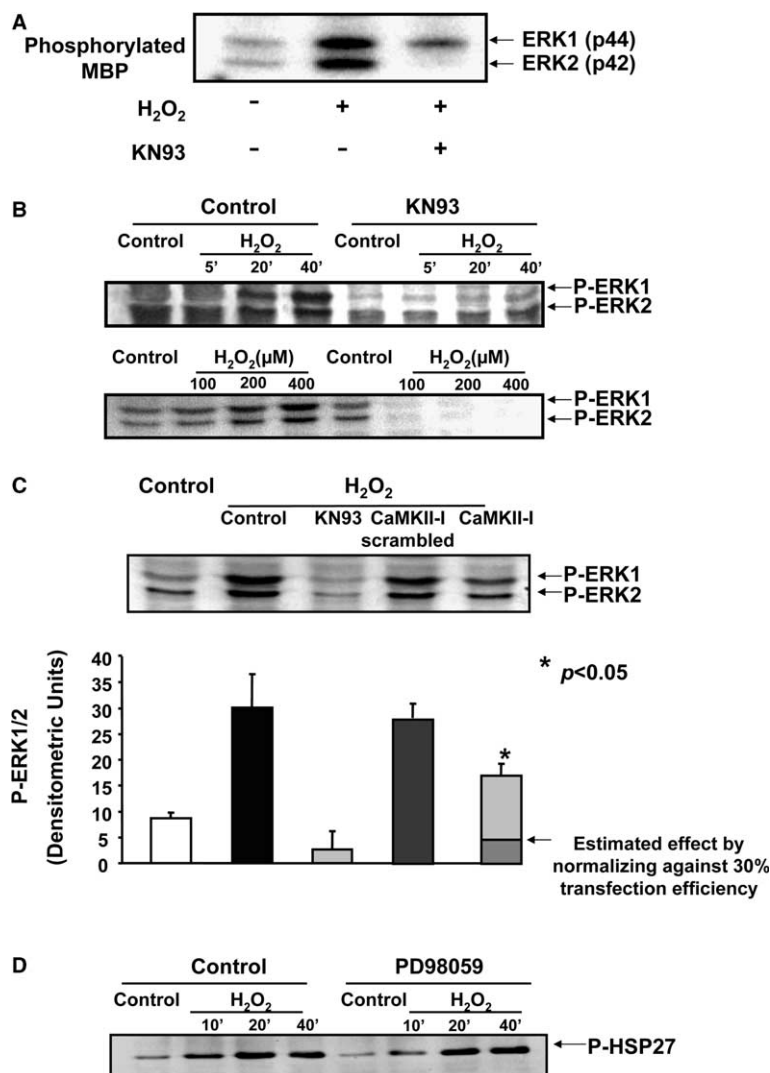


Fig. 3. H₂O₂ activation of ERK1/2 requires CaMKII. (A) Post-confluent endothelial cells were pretreated with a selective CaMKII inhibitor KN93 (10 μmol/L) for 1 h prior to stimulation by H₂O₂ (100 μmol/L) for 40 min. In-gel kinase assay was performed for analysis of ERK1/2 activity. (B) Endothelial cells were treated with H₂O₂ (100 μmol/L) for 5, 20 and 40 min or different concentrations of H₂O₂ (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 H₂O₂ (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 H₂O₂ (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 H₂O₂ 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 H₂O₂ activation of ERK1/2, endothelial cells were pretreated with KN93 (10 μmol/L) for 1 h prior to H₂O₂ 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 H₂O₂ for 60 min and this response was completely prevented by KN93 (Fig. 3A). Likewise, ERK1/2 phosphorylation in responses to different concentrations of H₂O₂ (100, 200, and 400 μmol/L) or at different time points post H₂O₂ exposure (5, 20, and 40 min) was determined by Western blot

analysis. Of note, H₂O₂ 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 H₂O₂ 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 $\mu\text{mol/L}$) 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.

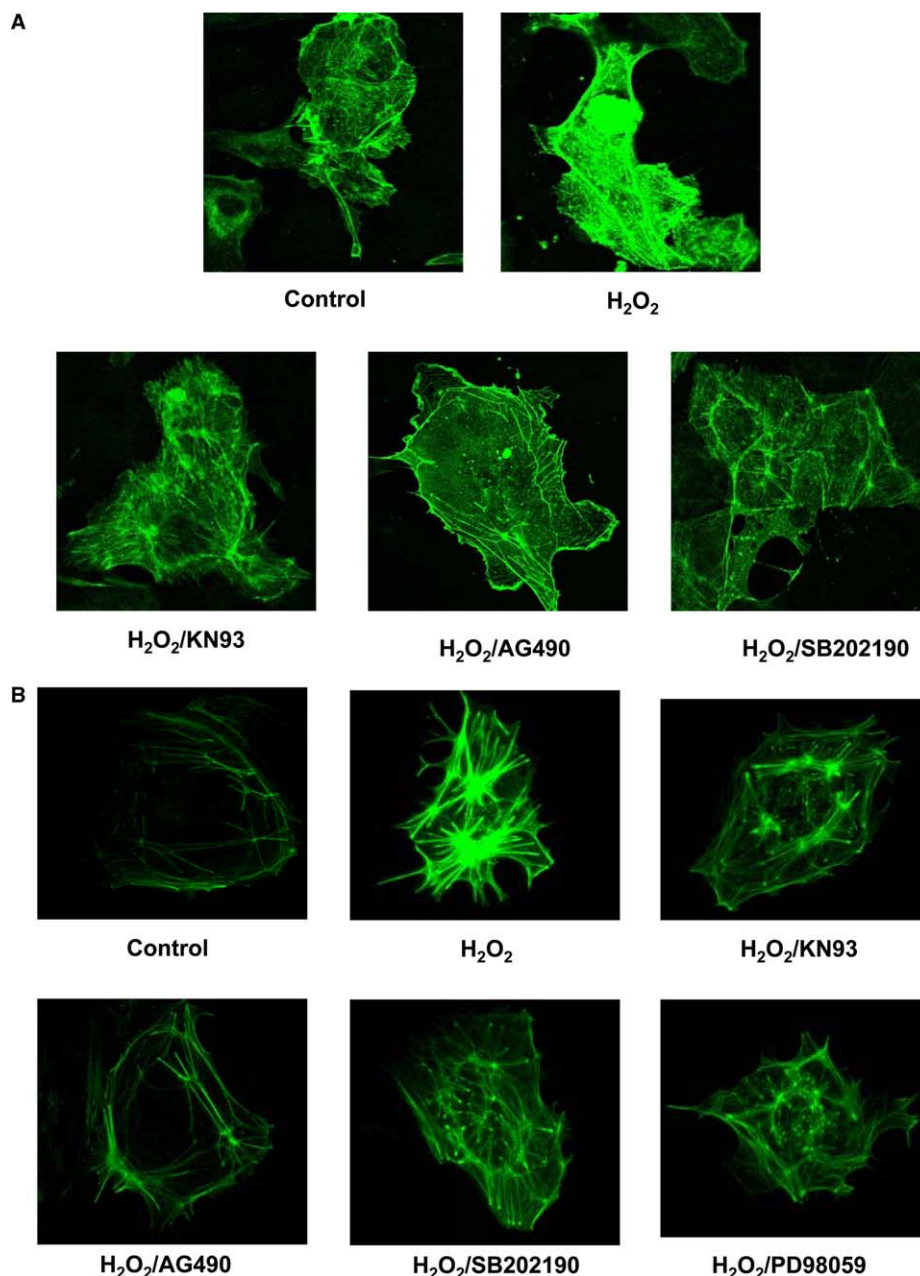


Fig. 4. (A) Role of CaMKII and JAK2 in H_2O_2 modulation of actin cytoskeleton. Endothelial cells were transfected with a DNA construct of pEYFP-actin for 48 h. Cells were stimulated with H_2O_2 (100 $\mu\text{mol/L}$) in the presence or absence of one-hour pretreatment with KN93, AG490, or SB202190, 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 Fluor 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 H₂O₂ induced reorganization of actin cytoskeleton

To examine the effect on H₂O₂-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 H₂O₂ 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 H₂O₂ 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 H₂O₂ up-regulation of eNOS mRNA.

In separate experiments, polymeric actin filaments were stained using Alexa Fluoro 488–Phalloidin. Consistent to findings using pEYFP-actin transfection, three-hour treatment with H₂O₂ 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 H₂O₂ 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 H₂O₂ activation of p38 MAPK/HSP27 pathway and may subsequently be responsible for H₂O₂ induction of actin stress fibers. Though ERK1/2 is only transiently involved in HSP27 phosphorylation, it appears partially involved in actin reorganization induced by H₂O₂.

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 H₂O₂ upregulation of eNOS [5]. Recent studies demonstrate that CaMKII is also involved in acute production of NO[•] 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 H₂O₂ 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 H₂O₂

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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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].

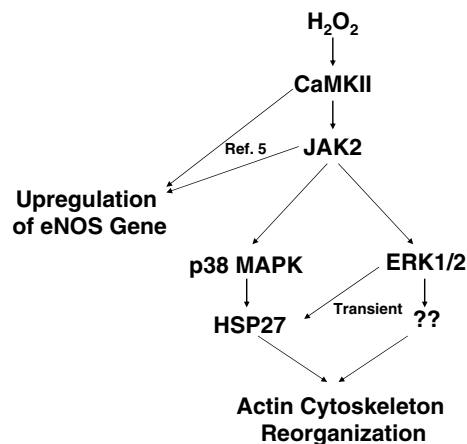


Fig. 5. Proposed signaling cascades mediating H₂O₂ induced reorganization of actin cytoskeleton.

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