Vascular Biology

Reactive Oxygen Species Regulate Heat-Shock Protein 70 via the JAK/STAT Pathway

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Abstract—Reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) activate intracellular signal transduction pathways implicated in the pathogenesis of cardiovascular disease. H₂O₂ is a mitogen for rat vascular smooth muscle cells (VSMCs), and protein tyrosine phosphorylation is a critical event in VSMC mitogenesis. Therefore, we investigated whether the mitogenic effects of H₂O₂, such as stimulation of extracellular signal–regulated kinase (ERK)2, are mediated via activation of cytoplasmic Janus tyrosine kinases (JAKs). JAK2 was activated rapidly in VSMCs treated with H₂O₂, and signal transducers and activators of transcription (STAT) STAT1 and STAT3 were tyrosine-phosphorylated and translocated to the nucleus in a JAK2-dependent manner. Inhibition of JAK2 activity with AG-490 partially inhibited H₂O₂-induced ERK2 activity, suggesting that JAK2 is upstream of the Ras/Raf/mitogen-activated protein kinase–ERK/ERK mitogenic pathway. Because heat-shock proteins (HSPs) can protect cells from ROS, we investigated the effect of H₂O₂ on HSP expression. H₂O₂ stimulated HSP70 expression in a time-dependent manner, and AG-490 abolished H₂O₂-induced HSP70 expression. H₂O₂ activated the HSP70 promoter via enhanced binding of STATs to cognate binding sites in the promoter. Regulation of chaperones such as HSP70 via activation of the JAK/STAT pathway suggests that in addition to its growth-promoting effects, this pathway may help VSMCs adapt to oxidative stress. (Arterioscler Thromb Vasc Biol. 2001;21:321-326.)

Key Words: vascular smooth muscle cells JAK2 reactive oxygen species STAT AG-490

Accumulating evidence supports a critical role for oxidative stress in the pathogenesis of atherosclerosis, cancer, and other human diseases. High levels of reactive oxygen species (ROS) damage DNA and inactivate proteins, resulting in chronic cellular dysfunction. Many cell types have also harnessed ROS, albeit in lower concentrations, as intracellular signaling molecules to mediate growth factor and cytokine responses. Modulation of growth responses by ROS has been demonstrated in a number of cell types, including vascular smooth muscle cells (VSMCs). Stimulation of VSMC proliferation by ROS is thought to be a critical step in atherosclerotic lesion formation.

Tyrosine phosphorylation of cellular proteins and the consequent induction of transcription of early-response genes are key determinants of cell growth and differentiation in response to mitogenic signaling. VSMC mitogens such as platelet-derived growth factor and epidermal growth factor activate receptor protein tyrosine kinases on binding, which stimulate intracellular signaling pathways that result in mitogen-activated protein kinase activation. Other mitogens, such as thrombin and angiotensin II, activate G protein–coupled receptors that do not possess intrinsic tyrosine kinase activity but require tyrosine phosphorylation events to induce mitogenesis.

The necessary role for protein tyrosine phosphorylation in mitogenesis elicited by thrombin and ROS indicates that these mitogens may utilize cytoplasmic protein tyrosine kinases in their signaling cascade. Forming I such group of tyrosine kinases are Janus kinases (JAKs), which along with their substrates, signal transducers and activators of transcription (STATs), have hitherto been characterized as essential mediators of cytokine and polypeptide hormone-induced signaling. Members of the JAK/STAT pathway mediate at least some biological effects of angiotensin II, platelet-derived growth factor-B, and endothelial growth factor. Activation of the JAK/STAT pathway has also been observed in response to generation of intracellular ROS and exogenous hydrogen peroxide (H₂O₃). On phosphorylation by JAKs of tyrosine residues, activated STAT dimers translocate to the nucleus to transactivate target gene expression.

The 70- and 90-kDa heat-shock proteins (HSPs) are among the subset of stress-responsive proteins known to be transactivated by STATs. Elevated levels of HSPs are rapidly synthesized within the cell in response to environmental stresses. Under physiological conditions, constitutively expressed HSPs function as molecular chaperones, whereas under stress conditions, HSPs prevent protein aggregation.

In addition, HSPs may directly regulate specific stress-
responsive signaling pathways and may antagonize signaling cascades that result in apoptosis.25

Because ROS do not directly activate receptor protein tyrosine kinases to exert their mitogenic effects on VSMCs, we investigated the effect of H2O2 on the activation of cytoplasmic tyrosine kinases, JAKs, and their substrates, STATs. We show here that H2O2 causes activation of JAK2 and subsequent tyrosine phosphorylation and nuclear translocation of STAT1 and STAT3. Our results also indicate that JAK2 partially regulates extracellular signal–regulated kinase (ERK)2 activity and thus, contributes to VSMC proliferation. Furthermore, we demonstrate that the JAK/STAT pathway mediates H2O2-induced HSP70 expression, which may help cells adapt to oxidative stress.

Methods

Cell Culture and Reagents

VSMCs were isolated from the thoracic aortas of 200- to 250-g male Sprague-Dawley rats. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) calf serum as described previously.12 VSMCs were growth-arrested by incubation in Dulbecco’s modified Eagle’s medium containing 0.1% calf serum for 72 hours. Antibodies used were as follows: anti-phosphotyrosine (4G10), anti-JAK1, anti-JAK2, anti-TYK2, anti-STAT1, anti-STAT3, and anti-ERK2 (UBI and Santa Cruz Biotechnology); anti-phosphospecific JAK2 (Biosource International); anti-phosphospecific and anti-nonphosphospecific ERK1/2 (New England Biolabs); and anti-HSP70 (Affinity BioReagents). AG-490 was obtained from Calbiochem.

Immunoprecipitation, ERK2 Activity Assay, and Western Blotting

Growth-arrested VSMCs were treated with 200 μmol/L H2O2 in the presence or absence of inhibitors for the specified time periods at 37°C. Cell lysates were prepared, and immunoprecipitation, ERK2 activity assay, and Western blotting were performed as described previously.26

Electrophoretic Mobility Shift Assay

Nuclear extracts were prepared from growth-arrested VSMCs that were either left untreated or treated with 200 μmol/L H2O2.27 DNA binding was performed by incubating 5 μg of nuclear protein with 100,000 counts per minute of 32P-labeled, double-stranded HSP70 oligonucleotide (5′-GATCCGGCGAAACCCCTGGAATATTC-CCCCAGGCT-3′) for 20 minutes at room temperature. Canonical double-stranded oligonucleotides for SP1 (5′-ATTCGATC GG-CCGGGGCCGAGC-3′) and STAT3 (5′-TGGATTACGGGAATGTGCACT-3′) and a high-affinity, double-stranded STAT1 binding sequence SIEEm67 (5′-GATCTGATTACGGGAATGTGCACT-3′) were used in competition studies. For identifying the STAT1-specific band, samples were incubated with STAT1 or STAT3 antibody for 30 minutes before the DNA binding reaction was performed. Protein-DNA complexes were resolved on a 4% polyacrylamide gel.

Transient Transfection and CAT Assay

The HSP70 chloramphenicol acetyltransferase (CAT) reporter construct LSN (-188 to +1) and LSNP (-100 to +1) were kindly provided by Richard Morimoto (Northwestern University, Evanston, Ill.). VSMCs at 70% to 80% confluence were transfected with 10 μg of reporter plasmid DNA by using the calcium precipitate method.26 Cells were cotransfected with β-galactosidase expression vector to normalize for transfection efficiency and were quiesced for 16 hours after transfection. After quiescence was maintained for 36 hours, cells were either left untreated or treated with 200 μmol/L H2O2 for 6 hours. In experiments with AG-490, cells were treated with the inhibitor for 16 hours before H2O2 treatment. Cell lysates were prepared,26 and CAT activity was measured by the method of Gorman et al.29 The β-galactosidase assay was performed by following the manufacturer’s protocol (Promega).

Statistical Analysis

Differences were analyzed with 1-way ANOVA, and post hoc analysis was performed with the Student-Newman-Keuls method. Values of P<0.05 were considered statistically significant.

Results

H2O2 Causes Activation of Kinases JAK2 and TYK2 in Rat VSMCs

To test the hypothesis that JAK/STAT pathway activation contributes to the mitogenic effects of ROS on VSMCs, we treated growth-arrested VSMCs with 200 μmol/L H2O2, a physiological concentration of this representative ROS that elicits VSMC proliferation.6 H2O2 caused rapid tyrosine phosphorylation of JAK2, which was sustained for 60 minutes (Figure 1A), but it had no effect on JAK1 phosphorylation (data not shown). JAK2 phosphorylation was biphasic, with peaks at 5 minutes (9.10±2.97-fold increase) and 30 minutes (3.0±1.52-fold increase; Figures 1A, 1B [top], and 1C). A similar biphasic stimulation of various kinases by ROS has been reported, and the second peak has been attributed to the secretion of autocrine factors.30 Tyrosine phosphorylation of JAK2 by H2O2 was concentration dependent, and maximum phosphorylation was observed with 200 μmol/L H2O2 at 5 minutes (please see http://www.atvb.ahajournals.org). In contrast to JAK2, peak TYK2 tyrosine phosphorylation was observed after a 15-minute exposure to H2O2, and TYK2 phosphorylation (3.86±0.64-fold...

Figure 1. H2O2 causes rapid tyrosine phosphorylation of JAK2. Growth-arrested VSMCs were treated with 200 μmol/L H2O2 and harvested in lysis buffer. A, Cell lysates containing equal...
increase) was not as pronounced as that of JAK2 (please see http://atvb.ahajournals.org). H\(_2\)O\(_2\) altered tyrosine phosphorylation without affecting steady-state protein levels of JAK2 (Figure 1B, bottom) and TYK2 (please see http://atvb.ahajournals.org).

**H\(_2\)O\(_2\) Induces Tyrosine Phosphorylation and Nuclear Translocation of STAT1 and STAT3 in Rat VSMCs**

To determine whether activation of JAK2 and TYK2 by H\(_2\)O\(_2\) leads to activation of STAT proteins, H\(_2\)O\(_2\)-treated VSMC lysates were immunoprecipitated with an anti-phosphotyrosine antibody, and immunoprecipitates were immunoblotted with antibodies against STAT1\(\alpha/\beta\) or STAT3. Constitutive phosphorylation was lower for STAT1\(\alpha\) than for STAT1\(\beta\) in untreated VSMCs (Figure 2A), but STAT1\(\alpha\) phosphorylation was greater than that for STAT1\(\beta\) after 10 minutes of H\(_2\)O\(_2\) treatment. Tyrosine-phosphorylated STAT1\(\alpha/\beta\) were not detectable 15 and 30 minutes after H\(_2\)O\(_2\) treatment, and phosphorylated STAT1\(\alpha/\beta\) levels at 60 minutes were much lower than in untreated cells. In broad terms, the biphasic STAT1 phosphorylation corresponded to the biphasic JAK2 phosphorylation, suggesting that the former is dependent on JAK2 activation. STAT1 was rapidly translocated to the nucleus within 5 minutes after H\(_2\)O\(_2\) treatment (Figure 2B, top), whereas no detectable change was observed in cytosolic STAT1 protein levels (Figure 2B, bottom). Peak nuclear translocation of STAT1 was observed at 10 minutes (4.03±0.32-fold increase), and in contrast to the tyrosine phosphorylation in immunoprecipitation studies (Figure 2A), was sustained for 60 minutes (Figure 2C). The reason for this discrepancy could be that nuclear fractions may contain some dephosphorylated STAT1. Constitutive tyrosine phosphorylation of STAT3 was observed in growth-arrested VSMCs, and phosphorylation increased within 5 minutes of H\(_2\)O\(_2\) treatment and was sustained for 60 minutes (Figure 3A). As with STAT1, rapid nuclear translocation of STAT3 was observed in H\(_2\)O\(_2\)-treated VSMCs (Figure 3B, top), with no detectable change in protein levels in the cytosolic fractions (Figure 3B, bottom). Peak nuclear STAT3 protein levels were observed at 15 minutes (2.90±0.49-fold increase; Figure 3C) and were sustained for 60 minutes. These experiments demonstrate the phosphorylation and nuclear translocation of STATs in H\(_2\)O\(_2\)-stimulated VSMCs.

**JAK2 Contributes to ERK2 Activation in Response to H\(_2\)O\(_2\) in VSMCs**

Because ERK1/2 activation is associated with H\(_2\)O\(_2\)-induced mitogenesis,\(^{31,32}\) we investigated the relationship between JAK2 stimulation by H\(_2\)O\(_2\) and ERK2 activity. Pretreatment of VSMCs with 50 \(\mu\)mol/L AG490, a specific JAK2 inhibitor, inhibited H\(_2\)O\(_2\)-induced JAK2 tyrosine phosphorylation and the consequent phosphorylation of STAT1 and STAT3, whereas it had no effect on c-Src, a non-JAK cytosolic tyrosine kinase (data not shown). Peak ERK2 activity, as measured by an immunocomplex kinase assay, was observed 15 minutes after treatment with H\(_2\)O\(_2\) (Figure 4). This increase in ERK2 activation by H\(_2\)O\(_2\) is consistent with 1 previous report\(^{32}\) but is contradictory to another\(^{33}\); the reasons for this discrepancy are not clear.
Activation of the JAK/STAT Pathway

Pretreatment of VSMCs with 50 μM H₂O₂ causes accumulation of these proteins in rat VSMCs. Binding sites for STATs have been reported during conditions known to produce ROS. The 5'-flanking sequences of the HSP70 and HSP90 genes contain functional binding sites for STATs. Hence, we investigated whether ERK1/2 activation by a JAK2 antagonist indicates that, along with JAK2-induced ERK2 activation. Partial inhibition of ERK2 activity blocked partially by AG-490 (4.5±1.4-fold increase with H₂O₂, at 15 minutes vs 2.7±0.7 and 1.9±0.4-fold for H₂O₂ in the presence of 10 and 50 μmol/L AG490, respectively), suggesting that JAK2 activation is necessary for H₂O₂-induced ERK2 activation. Partial inhibition of ERK2 activity by a JAK2 antagonist indicates that, along with JAK2-mediated stimulation, there may be an alternative pathway for ERK1/2 activation.

H₂O₂ Induces HSP70 Expression Through Activation of the JAK/STAT Pathway

Accumulation of RNA for HSPs has been reported during conditions known to produce ROS. The 5'-flanking sequences of the HSP70 and HSP90β genes contain functional binding sites for STATs. Hence, we investigated whether H₂O₂ causes accumulation of these proteins in rat VSMCs. H₂O₂ stimulated expression of both HSP70 (Figure 5) and HSP90 (data not shown). Because of more pronounced upregulation, we further investigated the expression of HSP70. HSP70 protein levels increased in VSMCs in a time-dependent manner, with a 5.90±0.97-fold increase at 24 hours after 200 μmol/L H₂O₂ treatment (Figures 5A and 5B). Pretreatment of VSMCs with 50 μmol/L AG-490 abolished the increase in HSP70 protein levels induced by H₂O₂ at 24 hours (Figures 5C and 5D; 5.70±0.85 vs 1.40±0.50, P<0.05). To investigate whether HSP70 expression in VSMCs exposed to H₂O₂ is mediated by the binding of STATs to their responsive element (−122 to −90 bp of the HSP70 promoter), we measured the ability of nuclear proteins from H₂O₂-treated cells to bind an HSP70-STAT oligonucleotide (please see http://www.atvb.ahajournals.org). Nuclear extracts from H₂O₂-treated VSMCs produced 2 shifted bands that were competed with an excess of unlabeled specific oligonucleotide but not with a nonspecific sequence. The faster migrating band was competed with an unlabeled, high-affinity STAT1 binding site (SIEm67 oligonucleotide) from the c-fos promoter, but not with an unlabeled STAT3 consensus sequence, and was abolished by preincubation of the complexes with an anti-STAT1 antibody (but not by an anti-STAT3 antibody), demonstrating the presence of STAT1 protein in this complex. The slower migrating band was partially competed by STAT1 and STAT3 oligonucleotides and partially abolished by anti-STAT1 and anti-STAT3 antibodies, suggesting that it contains a STAT1/STAT3 heterodimer.

H₂O₂ activates the HSP70 promoter in a JAK2-dependent manner. To investigate whether H₂O₂-induced HSP70 expression was mediated via a direct effect on its promoter, VSMCs were transfected with HSP70 promoter-reporter constructs either containing (LSN, −188 to +1) or lacking (LSNP, −100 to +1) a functional STAT1 binding site. The reporter construct LSN was activated 2.5-fold by 200 μmol/L H₂O₂, whereas transactivation in response to H₂O₂ was lost by deletion of the STAT binding site (Figure 6A). AG-490 inhibited the H₂O₂-induced increase in HSP70 promoter activity, suggesting that STATs require JAK2 activation for activity (Figure 6B). Taken together with the results shown in Figure 5, these findings indicate that H₂O₂ activates the JAK/STAT pathway in VSMCs, which has functional consequences on the expression of physiologically relevant proteins such as HSP70.

Discussion

We have shown that H₂O₂ stimulates specific members of the JAK/STAT pathway, an effect that participates in stress-related processes.
HSP70 expression in VSMCs treated with H_2O_2 via the JAK/STAT pathway. Indeed, significant heat-shock factor-1 activation has been observed in heart tissue exposed to H_2O_2, confirming that HSPs are induced under oxidative stress.

Our results indicate that the HSP70 promoter construct containing a functional STAT-binding site is activated by H_2O_2, which is blocked by pretreatment with AG-490. JAK2-mediated phosphorylation of STATs is therefore required for HSP70 promoter activity via H_2O_2. STAT1 is the major promoter binding element in VSMCs, and STAT3 also participates, as indicated by anti-STAT3 antibody inhibition of H_2O_2-induced binding activity. Further studies will determine the role of HSP70 in VSMC growth.

Increased HSP70 protein synthesis is seen in cardiovascular tissues exposed to various stresses and in VSMCs surrounding the necrotic zones of atherosclerotic plaques. Enhanced HSP70 protein levels protect against lethal heat stress and ischemia. ROS such as H_2O_2 have pleiotropic effects on cells, eliciting apoptosis in some cell types while stimulating early growth–response gene expression and proliferation in others. The cell types that exhibit proliferative responses likely have adaptive mechanisms to overcome the adverse effects of oxidants. Because HSPs modulate the stress response, we hypothesized that they might be induced in VSMCs exposed to H_2O_2, especially because HSP promoters contain functional STAT binding sites. Our results demonstrate a time-dependent increase in HSP70 protein levels in VSMCs treated with H_2O_2, which is blocked by pretreatment with AG-490, indicating that this protein is indeed regulated via activation of the JAK/STAT pathway. HSP70 has been shown to promote cell proliferation.

Oxidative stress–induced HSP90α and cyclophilin may promote VSMC growth. These observations suggest that regulation of HSP70 could be 1 of the mechanisms by which the H_2O_2-induced JAK/STAT pathway promotes VSMC growth. Further studies will determine the growth-promoting versus protective effects of HSP70 on H_2O_2-induced VSMC growth.

Our results also indicate that the HSP70 promoter construct containing a functional STAT-binding site is activated by H_2O_2, which is blocked by pretreatment with AG-490. JAK2-mediated phosphorylation of STATs is therefore required for HSP70 promoter activity via H_2O_2. STAT1 is the major promoter binding element in VSMCs, although it is possible that STAT3 also participates, as indicated by anti-STAT3 antibody inhibition of H_2O_2-induced binding activity. In addition to STAT binding sites, the HSP70 promoter (LSN, −188 to +1) has overlapping binding sites for two transcription factors, heat-shock factor-1. Although we do not specifically address this point, it is possible that heat-shock factor-1 may also be involved in H_2O_2-mediated HSP70 expression in VSMCs. Indeed, significant heat-shock factor-1 activation has been observed in heart tissue exposed to H_2O_2, confirming that HSPs are induced under oxidative stress.

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References


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FIGURE LEGENDS

Fig. I. **H₂O₂ causes concentration-dependent tyrosine phosphorylation of JAK2.**

Growth-arrested VSMC were treated with the indicated concentrations of H₂O₂ for 5 min and harvested in lysis buffer. **A.** Cell lysates containing equal amounts of protein were immunoblotted with phosphotyrosine-specific anti-JAK2 antibody (Top). Densitometric analysis of 3 immunoblots, from 3 independent experiments, probed with phosphotyrosine-specific anti-JAK2 antibody (Bottom) (mean ± S.D.). The asterisks represent significant differences compared with control (P < 0.05).

**B.** Western blots probed with phosphotyrosine-specific anti-JAK2 antibody were reprobed with nonphospho-specific anti-JAK2 antibody (Bottom).

Fig. II. **H₂O₂ causes rapid tyrosine phosphorylation of TYK2.** Growth-arrested VSMC were treated with 200 µM H₂O₂ and harvested in lysis buffer. **A.** Cell lysates immunoprecipitated with anti-phosphotyrosine antibody were immunoblotted with anti-TYK2 antibody. **B.** Cell lysates were immunoprecipitated with anti-TYK2 antibody and immunoblotted with anti-phosphotyrosine antibody (Top) or anti-TYK2 antibody (Bottom). **C.** Densitometric analysis of 3 immunoblots, from 3 independent experiments, probed with phosphotyrosine-
specific anti-TYK2 antibody (mean ± S.D.). The asterisks represent significant
differences compared with control (P < 0.05).

Fig. III. **H₂O₂ induces DNA binding of the Hsp70 STAT sequence.** Nuclear extracts
from VSMC, either untreated (*lanes 1*) or treated with 200 µM H₂O₂ for 10 min,
(*lanes 2-9*) were subjected to an electrophoretic mobility shift assay using a labeled
Hsp-70 STAT probe. For determining the specificity of the HSP-70 STAT-binding
complex, nuclear extracts were preincubated with unlabeled specific or nonspecific
competitors. The specific competitors used were Hsp-70 STAT (*lanes 3 and 4, 50
and 100-fold molar excess of Hsp-70 STAT, respectively), 100-fold molar excess of
SIE containing a consensus-binding site for STAT1 (*lane 5*), 100-fold molar excess
of STAT3 consensus oligo (*lane 6*) and the nonspecific competitor was 100-fold
molar excess of SP1 (*lane 7*). To characterize the protein components of the H₂O₂-
induced binding complex, nuclear extracts were preincubated with anti-STAT1α
(*lane 8*) or STAT3 (*lane 9*) antibody. Arrow on the left indicates STAT1 specific
protein-DNA complex and the arrow on the right may denote protein-DNA
complex with STAT1 and STAT3 heterodimer. Autoradiogram shown represents an
experiment that was repeated at least twice with similar results.