INACTIVATION OF SRC FAMILY TYROSINE KINASES BY REACTIVE OXYGEN SPECIES IN VIVO

Hua Tang¶, Qin Hao, Stacey A. Rutherford, Brad Low, and Z. Joe Zhao§

From the Department of Biochemistry, The University of Texas Health Center at Tyler, Tyler, TX 75708. §Division of Hematology/Oncology, Department of Medicine, Vanderbilt University, Nashville, TN 37232

Running Title: Inactivation of SFKs by ROS in Vivo

¶Address correspondence to Hua Tang, Department of Biochemistry, The University of Texas Health Center at Tyler, 11937 US Highway 271, Tyler, Texas 75708, Tel: 903-877-7938; Fax: 903-877-2881; E-mail: hua.tang@uthct.edu

Reactive oxygen species including H₂O₂, O₂⁻ and OH· are constantly produced in the human body and are involved in the development of cardiovascular diseases. Emerging evidence suggests that reactive oxygen species, besides their deleterious effects at high concentrations, may be protective. However, the mechanism underlying the protective effects of reactive oxygen species is not clear. Here, we reported a novel finding that H₂O₂ at low to moderate concentrations (50-250 µM) markedly inactivated Src family tyrosine kinases temporally and spatially in vivo but not in vitro. We further showed that Src family kinases localized to focal adhesions and the plasma membrane were rapidly and permanently inactivated by H₂O₂, which resulted from a profound reduction in phosphorylation of the conserved tyrosine residue at the activation loop. Interestingly, the cytoplasmic Src family kinases were activated gradually by H₂O₂, which partially compensated for the loss of total activities of Src family kinases but not their functions. Finally, H₂O₂ rendered endothelial cells resistant to growth factors and cytokines and protected the cells from inflammatory activation. Since Src family kinases play key roles in cell signaling, the rapid inactivation of Src family kinases by H₂O₂ may represent a novel mechanism for the protective effects of reactive oxygen species.

Src family tyrosine kinases (SFKs)¹ play initiating and key roles in the control of cell proliferation, differentiation, survival, adhesion, cytoskeletal rearrangement, and specialized cell signals by a diverse set of cell surface receptors such as growth factor receptors, G protein-coupled receptors (GPCR), antigen receptors, cytokine receptors and integrins (1). SFKs comprise nine family members, Src, Fyn, Yes, Lck, Hck, Blk, Fgr, Lyn and Yrk, and share a conserved domain structure, including a myristoylated N-terminal unique sequence that mediates association with the inner face of the plasma membrane, followed by Src homology (SH)3, SH2 and kinase domains, and a short C-terminal tail with regulatory function (2). The ability of SFKs to initiate and mediate signaling from cell surface receptors is dependent on their catalytic activities, locations and binding partners (3,4).

The enzymatic activities of SFKs are positively and negatively regulated by tyrosine phosphorylation. Phosphorylation of a conserved tyrosine (Tyr-418, using human Src numbering throughout) in the activation loop enhances kinase activity (5-7). Phosphorylation of Src Tyr-418, the major site of autophosphorylation in vitro, has been traditionally thought to occur as an intramolecular event carried out by the kinase itself (8,9). However, a set of evidence suggests that phosphorylation of the activation loop tyrosine can be achieved by a kinase other than SFKs (7,10). On the other hand, phosphorylation of a conserved tyrosine (Tyr-529 in human Src) in the C-terminal tail by tyrosine kinase Csk or its family member Chk inhibits the activity of Src (11,12). Phosphorylation of Tyr-529 causes an intramolecular binding of the SH2 domain to the phosphorylated C-terminus, followed by binding of the SH3 domain to the linker between the SH2 and the catalytic domains, thus resulting in formation of an inactive conformation of Src (13,14). Dephosphorylation of this site is mediated by receptor tyrosine phosphatases (RPTPα and CD45) (15), which facilitates SFKs activation.

Reactive oxygen species (ROS), such as H₂O₂, superoxide (O₂⁻), and hydroxyl radical (OH·), are constantly produced in the human body and are involved in the pathogenesis of cardiovascular...
diseases, cancer and Alzheimer’s diseases (16-18). ROS can be generated in activated phagocytes as one of the defense mechanisms and in non-phagocytes stimulated with cytokines, growth factors, and agonists for GPCRs (19-21). Moreover, tissue injuries such as ischemia/reperfusion and surgical angioplasty, hypercholesterolemia, normal body metabolism and exercise all promote ROS production (22-24). ROS have major impacts on vascular cells including endothelial cells (ECs) that are at the center of most common clinical pathologies. The conventional thought has generally regarded ROS as being harmful to the vasculature, leading to such pathological processes as atherosclerosis, coronary ischemia, angiogenesis, retinopathy, restenosis and hypertension (16,24,25). However, controlled clinical trials have failed to show a consistent benefit of antioxidants on these cardiovascular diseases (26-28). Although a number of factors may contribute to this lack of efficacy of antioxidants, one intriguing possibility is that ROS may play both a physiological and pathophysiological role in vascular homeostasis: i.e. ROS may be both protective and deleterious. It has been shown that endothelial preconditioning by ROS reduces EC inflammatory responses to tumor necrosis factor-α (TNF-α) (29). Preconditioning by ROS also protects reperfusion injury of cardiomyocytes (24). However, the mechanism underlying the protective effect of ROS has not been elucidated yet.

In the present study, we report a novel finding that H2O2 at a low to moderate concentration (50-250 µM) markedly inactivates SFKs temporally and spatially in vivo but not in vitro and renders ECs resistant to growth factors and cytokines thereby protecting the cells from inflammatory activation. Furthermore, we show that a reduced phosphorylation of the tyrosyl residue in the activation loop (Tyr-418 in human Src) is responsible for the inactivation of SFKs by H2O2 in vivo.

EXPERIMENTAL PROCEDURES

Reagents — H2O2 was from Sigma and Fisher, respectively. Recombinant human full-length Src and Fyn, Src substrate peptide (KVEKIGEGTVYVYK) and P81 phosphocellulose paper were from Upstate. Reagents for chemiluminescence detection were from Cell Signaling.

Antibodies—Antibodies against phospho-Src (Tyr-418) and phospho-Src (Tyr-529) were from Biosource. Antibodies against phospho-p130Cas (Tyr-410), phospho-paxillin (Tyr-118), phospho-ERK (Thr202/Tyr204), phospho-Src (Tyr-416) and phospho-Src (Tyr-527) were from Cell Signaling. A phosho-specific antibody against Fyn (Tyr-528) or Src (Tyr-530) was from BD Pharmingen. Antibodies against SFKs (SRC-2) and VCAM-1 were from Santa Cruz Biotechnology. Monoclonal antibodies against Src (GD11) and phosphotyrosine (4G10) were from Upstate. CyTM3-conjugated donkey anti-rabbit antibody was from Jackson ImmunoResearch.

Cell Culture—HAEC and HUVEC were from Cambrex Bio Science and cultured in EGM-2 medium and used for experiments within 10 passages. 293 human embryonic kidney cells and CHO-K1 were from American Type Culture Collection and cultured respectively in Dulbecco’s modified Eagle’s medium (DMEM) or Kaighn’s modification of Ham’s F12 medium supplemented with 10% fetal bovine serum. Murine embryonic E6 fibroblasts (30) were kindly provided by Dr. Jan Sap (New York University, New York) and cultured in DMEM supplemented with 10% fetal bovine serum.

Immunoprecipitation and Immunoblotting—Immunoblotting and immunoprecipitation were performed essentially as we described previously (31). Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then lysed on ice in Nonidet P-40 lysis buffer (25 mM Tris-HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 10 mM NaF, 1mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml each of leupeptin and aprotinin). The extract was clarified by centrifugation and incubated sequentially with primary antibodies and protein A- or G-Agarose. The immunoprecipitates were collected and washed three times with the lysis buffer. For immunoblotting, whole cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membrane. The membrane was probed with various primary antibodies as indicated and...
detected using the ECL system with horseradish peroxidase-conjugated secondary antibodies according to the manufacturer’s protocol.

In Vitro SFKs Assay—The activities of SFKs were measured essentially according to the manufacturer’s protocol. To determine the effect of H2O2 on SFKs in vitro, purified recombinant human full-length Src (6 Units) or Fyn (25 ng) was incubated with various concentrations of H2O2 (0-1000 μM) for 20 min at 30 °C, then catalase was added at final concentration of 100 ng/μl for 10 min at room temperature to remove the excess H2O2. Kinase reactions were initiated by the addition of ATP, MgCl2, MnCl2, and kinase reaction buffer containing 100 mM Tris-HCl (pH 7.4), 0.1% Nonidet P-40 lysis buffer and incubated in 40 μl of reaction mixture containing 0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH2PO4, and 4% (w/v) Norit A. After centrifugation in a microcentrifuge, the amount of radioactivity present in the supernatant was determined by scintillation counting. The phosphatase activity was evaluated by the extent of Tyr-Raytide dephosphorylation in vitro. To determine the effect of H2O2 on SHP-2 in vitro, purified full-length SHP-2 (0.5 μg/each) was incubated with various concentrations of H2O2 (0-1000 μM) for 30 min at 25 °C, then catalase was added at final concentration of 100 ng/μl for 10 min at room temperature to remove the excess H2O2. The activity of SHP-2 was measured using the 32P-labeled Tyr-Raytide or substrate p-nitrophenyl phosphatase (10 mM) as described previously (32).

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed essentially as we described recently (34). Cells grown on glass coverslips in a 6-well plate were treated with H2O2 for indicated time periods at 37°C, then washed with PBS and fixed in 3.7% formaldehyde solution in PBS for 10 min at 25°C. The fixed cells were extracted in ice-cold acetone at –20°C for 5 min, washed, and preincubated in PBS containing 1% BSA. Control stainings were performed without either primary or secondary antibodies. After washing with PBS, coverslips were mounted on slides with cell-side down with CytosealTM and examined and photographed using a PerkinElmer Ultra VIEW LCI confocal imaging system configured with a Nikon TE2000-S fluorescence microscope fitted with PlanApo 60 and 100 X oil objectives. Adobe Photoshop 6.0 software was used for image processing.
RESULTS

H2O2 Suppresses the Tyrosine Phosphorylation of Focal Adhesion Proteins in Vivo—H2O2, the most stable form of ROS, can easily diffuse across the membrane and has been widely used to study the role of ROS in cells (16). To determine H2O2 signaling, primary human aortic ECs (HAECs) and murine embryonic E6 fibroblasts (30) were serum-starved for 2 h and treated with a subcytolytic concentration of H2O2 (300 µM) for the indicated time periods, and lysates were immunoblotted with a phosphotyrosine antibody. As shown in Fig. 1 (A and B), H2O2 markedly suppressed the tyrosine phosphorylation of two major protein bands (∼130 kDa and 70 kDa) in a time-dependent manner in HAECs and E6 fibroblasts. Furthermore, the inhibitory effect of H2O2 on tyrosine phosphorylation of the 130 kDa protein band but not the 70 kDa protein was reversible. In addition, we found that H2O2 enhanced the tyrosine phosphorylation of a 190-kDa protein in HAECs. To determine whether the 130 kDa and the 70 kDa protein bands represent, at least, the focal adhesion proteins p130Crk-associated substrate (p130Cas) and paxillin (68 kDa) that have similar molecular sizes and are known to be heavily tyrosine-phosphorylated (35), we determined the effects of H2O2 on the tyrosine phosphorylation of p130Cas and paxillin using phospho-specific antibodies in E6 fibroblasts. As shown in Fig. 1 C, the phosphorylation of p130Cas on Tyr-410 was profoundly suppressed by H2O2 at 5 min but was fully recovered at 30 min. However, the H2O2-induced reduction in paxillin phosphorylation on Tyr-118 could not be reversed at 30 min. Since p130Cas and paxillin are native substrates of SFKs (35), these data suggest that H2O2 may inactivate SFKs in vivo.

H2O2 Inactivates SFKs in Vivo—SFKs are activated when the conserved tyrosine (Tyr-418 in human Src) in the activation loop is phosphorylated (5-7). Thus, the activated form of SFKs can be detected with a phospho-specific antibody against the conserved tyrosine. Fig. 2 shows a time-dependent effect of H2O2 (250 µM) on phosphorylation of the conserved tyrosine in various types of cells using an antibody against Tyr-418-phosphorylated Src (Biosource). In agreement with previous reports (36,37), several bands around 60 kDa were detected with the phospho-Src (Tyr-418) antibody (Fig. 2), indicating that the phospho-specific antibody also recognizes other SFK members when phosphorylated at the equivalent sites. We found that the phosphorylation of Src Tyr-418 and the equivalent sites of other members was suppressed more than 80% by H2O2 within 10 min and gradually returned to a near basal level by 60 min in primary human umbilical vein ECs (HUVECs), HAECs and E6 fibroblasts (Fig. 2, A-C). Similar time-dependent effect of H2O2 was also observed in 293 human embryonic kidney cells (Fig. 2D) and CHO-K1 cells (data not shown). These findings indicate that SFKs can be inactivated by H2O2 in vivo. We next determined the dose-dependent inactivation of SFKs by H2O2 using the phospho-Src (Tyr-418) antibody. As shown in Fig. 3 A, the phosphorylation of Src Tyr-418 was suppressed 51% by 5 min treatment of HUVECs with as low as 50 µM H2O2, 54% by 100 µM H2O2, and ~85% by 250-500 µM H2O2, respectively. Similar dose-dependent inactivation of SFKs by H2O2 was observed in HAECs (Fig. 3B). In addition, H2O2 over 100 µM was required to inactivate SFKs in E6 fibroblasts (Fig. 3C) and 293 cells (data not shown), and the maximal inactivation of SFKs can be achieved by 250 µM H2O2 in both cells. Thus, SFKs can be inactivated maximally by H2O2 at 250 µM in all the cells we examined. It seems that the primary human ECs are more sensitive to H2O2 than fibroblasts and 293 cells on the inactivation of SFKs. Similar results were obtained by using an antibody against Tyr-416-phosphorylated Src (Cell Signaling) (data not shown).

The effect of H2O2 on the catalytic activities of SFKs was then determined in ECs. SFKs were immunoprecipitated with an antibody (SRC-2) that recognizes the C-terminal sequence of SFKs, and kinase activities of the immune complexes towards a SFK-specific peptide substrate were measured. As shown in Fig. 4 A, the activities of SFKs were profoundly inhibited by H2O2 in a time-dependent manner in ECs. At 1 min, the activities of SFKs were inhibited 90% or 80% by H2O2 in HUVECs and HAECs, respectively. The pattern of the inhibition in kinase activity correlated well with the H2O2-induced suppression in the phosphorylation of Tyr-418 shown in Fig. 2. Since
SFKs interact with substrates to transduce signal, inactivation of SFKs by H_2O_2 should suppress the tyrosine phosphorylation of SFK-bound substrates. As shown in Fig. 4B, the tyrosine phosphorylation of all the SFK-bound substrates was markedly inhibited by H_2O_2 (250 μM) at 5 min in HUVECs. Excluding the protein bands indicated with arrows, the tyrosine phosphorylation of several protein bands including the 130 kDa protein was recovered to basal levels by 1 h after H_2O_2 treatment. Taken together, these findings from different approaches demonstrate that H_2O_2 strongly inactivates SFKs in vivo.

**H_2O_2 Inactivates Predominantly the SFKs Localized to Focal Adhesions and the Plasma Membrane**—Although the H_2O_2-induced inhibition in the total SFKs’ activities was reversible (Fig. 4A), the recovery of tyrosine phosphorylation of SFK-bound substrates was only observed in certain protein bands (Fig. 4B). These data suggest that SFKs localized at different cellular compartments may be regulated differently by H_2O_2. We performed immunofluorescence microscopy using a confocal microscope to access the localization of activated SFKs in HUVECs. In agreement with early reports (38-40), we found that the activated SFKs detected with the phospho-Src (Tyr-418) antibody were mainly localized to cell peripheral focal adhesions (Fig. 5A, solid arrow). The signal was also detected at plasma membrane (Fig. 5A, broken arrow). Remarkably, the staining signals of the activated SFKs localized at cell periphery were virtually abolished by 5 min treatment of HUVECs with H_2O_2 (Fig. 5B), which correlated well with a profound reduction in the tyrosine phosphorylation of focal adhesion proteins p130Cas and paxillin (Fig. 1C). At this moment, the activated SFKs were mainly detected as a granular cytoplasmic staining, likely associated with endosomes as shown previously (41). By 1 h after H_2O_2 treatment, the activated SFKs were mainly detected as big clusters in cytoplasm (Fig. 5C). These data were highly reproducible in HAECs (data not shown). These findings indicate that H_2O_2 rapidly and sustainedly inactivates SFKs localized to cell peripheral focal adhesion sites and the plasma membrane in HUVECs.

**H_2O_2 Does Not Affect Directly the Catalytic Activities of SFKs in Vitro**—To determine whether H_2O_2 directly inactivates SFKs in vitro, purified recombinant full-length human Src or Fyn was incubated with various concentrations of H_2O_2 and the kinase activity was measured with a specific substrate peptide. As shown in Fig. 6, H_2O_2 (0-1 mM) did not significantly affect the catalytic activity of Src or Fyn in vitro.

**Inhibition of PTPs Is Involved in the H_2O_2-induced Inactivation of SFKs in Vivo**—Since PTPs contain a catalytically essential cysteine residue in the signature active site motif, HCXXGXXR(S/T), which can be reversibly oxidized by ROS to inactivate PTPs (42), it has been proposed that the inhibition of PTPs by ROS could regulate their counterpart protein tyrosine kinases and cellular signaling. As shown in Fig. 7A, SHP-2, a non-receptor PTP, was directly inactivated by H_2O_2 in a dose-dependent manner with an IC_{50} of 75 μM. SHP-2 was also directly inhibited by other PTP inhibitors such as Na_{3}VO_{4} (43) and phenylarsine oxide (PAO) (44) but not by the serine/threonine phosphatase inhibitor NaF (45) (data not shown). Fig. 7B shows the effect of H_2O_2 on total activities of PTPs in HUVECs. The total activities of PTPs in HUVECs were inhibited ~50% by H_2O_2 at 5 min, then gradually recovered to basal level at 20 min and increased to a higher level (1.62-fold) at 60 min. We next determined whether SFKs can be inactivated by other PTPs inhibitors. As shown in Fig. 7 (C and D), SFKs were markedly inactivated by H_2O_2, Na_{3}VO_{4}, and PAO but not by NaF in HUVECs. In addition, we found that protein kinase C and calcium did not play a role in the inactivation of SFKs by H_2O_2 (data not shown). These data indicate that PTPs can be reversibly inhibited by H_2O_2 in HUVECs, and the inhibition of PTPs may be involved in the H_2O_2-induced inactivation of SFKs.

It has been shown that phosphorylation of a conserved tyrosine (Tyr-529 in human Src) in the C-terminal tail causes formation of an inactive conformation of SFKs, thus inhibiting SFKs (13,14). We determined effect of H_2O_2 on the phosphorylation of the conserved tyrosine using a phospho-Src (Tyr-529) antibody (Biosource) that recognizes SFKs when phosphorylated on Tyr-529. As shown in Fig. 8A, the phosphorylation of Src Tyr-529 was slightly increased by H_2O_2 at 5 and 10 min (1.3-1.4 fold), then gradually returned to basal level at 30 min and even to a level less than control (85%) at 60 min in HUVECs. However, H_2O_2 did not affect
the phosphorylation of Tyr-529 in E6 fibroblasts and 293 cells (Fig. 8, B and C). Similar findings were observed in HUVECs, E6 fibroblasts and 293 cells when another phospho-specific antibody against Tyr-528-phosphorylated Fyn or Tyr-530-phosphorylated Src (BD Pharmingen) was employed (data not shown). Since SFKs were inactivated with a similar extent in ECs, fibroblasts and 293 cells, the slight increase in the phosphorylation of Tyr-529 observed only in HUVECs may not be the key mechanism for the H₂O₂-induced inactivation of SFKs.

**H₂O₂ Suppresses the Responses of ECs to Growth Factors and Cytokines**—Since SFKs play a key roles in the signal transduction of growth factors (3), we determined effect of H₂O₂ on the mitogenic responses of ECs to growth factors. As shown in Fig. 9A (top panel), pretreatment of HUVECs with H₂O₂ (250 µM) for 3 min virtually abolished the activation of extracellular signal-regulated kinase (ERK1/2) by platelet-derived growth factor (PDGF). Furthermore, PDGF alone slightly, if any, activated SFKs by enhancing the phosphorylation of Tyr-418 in HUVECs. However, PDGF was unable to rescue the H₂O₂-induced inactivation of SFKs (Fig. 9A, middle panel), suggesting that different mechanisms may be involved in the regulation of SFKs by PDGF and H₂O₂. In addition, we found that the responses of HUVECs to epidermal growth factor (EGF) were also blunted by H₂O₂ pretreatment (data not shown).

Associated with the induction of cell adhesion molecules, EC inflammatory activation by cytokines is a major feature during the development of EC-related cardiovascular diseases (48). It has been shown that the expression of vascular cell adhesion molecule-1 (VCAM-1) by thrombin and TNF-α is dependent on nuclear factor-κB (NF-κB) in ECs (49,50). Since SFKs are essential for the activation of NF-κB (51,52), we assessed the effect of H₂O₂ on the expression of VCAM-1 by thrombin and TNF-α in HUVECs. As shown in Fig. 9 (B and C), the expression of VCAM-1 induced by thrombin and TNF-α was suppressed by H₂O₂ in a dose-dependent manner. In particular, the expression of VCAM-1 by thrombin or TNF-α was almost completely suppressed by 250 µM or 350 µM H₂O₂, respectively. Moreover, PAO (44), which inactivated SFKs (Fig. 7D), also markedly inhibited the expression of VCAM-1 by thrombin and TNF-α in HUVECs (Fig. 9, B and C). These findings suggest that ROS (H₂O₂) may be protective through suppression of EC responses to cytokines via inactivation of SFKs, thus preventing endothelial inflammation.

**DISCUSSION**

In the present study, we report a novel finding that H₂O₂ at a low to moderate level (50-250 µM) markedly inactivates SFKs temporally and spatially in vivo but not in vitro using multiple approaches. We further show that SFKs localized to EC peripheral focal adhesion sites and the plasma membrane are rapidly and permanently inactivated by H₂O₂, which results from a profound reduction in phosphorylation of the activation loop conserved tyrosine (Tyr-418 in human Src). The inactivation of SFKs correlates well with the H₂O₂-induced resistance of ECs to growth factors and cytokines. Since SFKs play initiating and key roles in cell signaling by a diverse set of cell surface receptors (1), our findings that the rapid inactivation of SFKs by H₂O₂, a stable form of ROS, may provide new insights into the mechanism of the protective effects of ROS.

ROS including H₂O₂, O₂⁻ and OH are constantly produced in the human body in physiological conditions such as metabolism and activation of cell surface receptors to maintain cell survival (19-21), and in pathophysiological settings like inflammation, infection, tissue injury and hypercholesterolemia (22-24). Although the exact concentrations of ROS in these settings are not clear, it has been showed that the oxidant generation induced by TNF-α (100 U/ml), detected with an oxidant-sensitive dye, is comparable to that by 500 µM H₂O₂ in cultured human dermal microvascular endothelial cells (19). The first finding obtained from our study is that SFKs can be rapidly inactivated by H₂O₂ in HAECs, HUVECs, 293 cells, E6 fibroblasts as well as CHO-k1 cells. The enzymatic activities of SFKs are positively regulated by phosphorylation of a conserved tyrosine (Tyr-418 in human Src) in the activation loop (5-7). We found that the phosphorylation of Tyr-418 was suppressed by H₂O₂ in a time- and dose-dependent manner in all the cells we examined. In HAECs and HUVECs,
H$_2$O$_2$ over 50 µM was required to induce a reduction in the phosphorylation of Tyr-418, while H$_2$O$_2$ over 100 µM was required in E6 fibroblasts and 293 cells. These data suggest that the primary human ECs are more sensitive to H$_2$O$_2$ than fibroblasts and 293 cells on the inactivation of SFKs. Furthermore, the phosphorylation of Src Tyr-418 was almost abolished by 250 µM H$_2$O$_2$ in all cells examined, which correlated with a profound inhibition in the catalytic activities of SFKs. Thus, it is likely that the reduction in phosphorylation of the activation loop conserved tyrosine may be involved in the H$_2$O$_2$-induced inactivation of SFKs in vivo. Immunofluorescence study revealed that, consistent with early reports (38-40), the activated SFKs were predominantly localized to cell peripheral focal adhesion sites and the plasma membrane at resting HUVECs. Remarkably, we found that the activated SFKs localized at these sites were completely inactivated by H$_2$O$_2$ at 5 min in HUVECs, and the inactivation can not be recovered even by a prolonged (1 h) H$_2$O$_2$ treatment. These findings were further supported by the notion that H$_2$O$_2$ induced a great reduction in the tyrosine phosphorylation of focal adhesion proteins p130Cas and paxillin. Interestingly, along with a complete inhibition of SFKs localized at cell periphery, the SFKs associated with endosomes (41) in cytoplasm were activated by H$_2$O$_2$ in a time-dependent manner through an enhanced phosphorylation of Src Tyr-418. By 1 h after H$_2$O$_2$ treatment, the activated SFKs were mainly detected as big and bright clusters in the cytoplasm of HUVECs. Thus, the activation of cytoplasmic SFKs may compensate for the lost activities of SFKs localized to focal adhesions and the plasma membrane and account for the H$_2$O$_2$-induced reversible inhibition in total phosphorylation of Src Tyr-418 and total catalytic activities of SFKs. However, this compensation did not fully restore the function of SFKs since the recovery of tyrosine phosphorylation of SFK-bound substrates was only observed in certain protein bands by 1 h after H$_2$O$_2$ treatment in HUVECs. Indeed, we found that the activation of ERK by PDGF and EGF and the induction of VCAM-1 by thrombin and TNF-α, which require a proper localization of the activated SFKs and an intact focal adhesion complexes (3, 4,53), were greatly inhibited by H$_2$O$_2$ in HUVECs.

Previous studies on the regulation of SFKs by H$_2$O$_2$ have yielded contradictory results. Cunnick et al (54) reported that, consistent with our findings, Src kinase activity was inhibited by H$_2$O$_2$ (500-2000 µM) in HeLa cells. In contrast, several other studies showed that treatment of cells with an extreme high dose of H$_2$O$_2$ (5 mM) augmented the phosphorylation of Lck on Tyr-394 and activated the kinase (7,10). However, these studies did not show what compartment of Lck was activated and the mechanism was not clear. Herein, we demonstrated that SFKs at different cellular compartments were regulated differently by H$_2$O$_2$ when a low to moderate concentration (50-250 µM) of H$_2$O$_2$ was employed. We showed that SFKs localized to cell peripheral focal adhesion sites and the plasma membrane were rapidly and permanently inactivated by H$_2$O$_2$ in HUVECs, while the cytoplasmic SFKs associated with endosomes were gradually activated by H$_2$O$_2$. Based on our findings, the discrepancy between these previous studies and our results may be reconciled with following interpretation. First, H$_2$O$_2$ at an extreme high level (5 mM) may override the normal regulation of Lck and activate the kinases. Second, it is possible that Lck localized at cell periphery is inactivated by 5 mM H$_2$O$_2$, but the cytoplasmic Lck is hyper-phosphorylated on Tyr-394 and over activated, leading to an increase in total phosphorylation of Lck on Tyr-394 and an elevated kinase activity.

We found that the H$_2$O$_2$-induced rapid inactivation of SFKs was mimicked by two other PTP inhibitors such as Na$_3$VO$_4$ (43) and PAO (44). These findings suggest that inhibition of PTPs may be involved in the inactivation of SFKs by H$_2$O$_2$ in vivo. PTPs contain a catalytically essential cysteine residue in the signature active site motif, HCXXGXXR(S/T), which can be reversibly oxidized by ROS to inactivate PTPs (42). Several lines of evidence including our results (Fig. 7) have demonstrated that PTPs can be directly inhibited by H$_2$O$_2$ in vitro and reversibly inhibited by H$_2$O$_2$ in intact cells (55,56). Our data also suggest that inhibition of PTPs does not always lead to an increase in the tyrosine phosphorylation of cellular proteins because we found that H$_2$O$_2$ inactivated SFKs and suppressed the tyrosine phosphorylation of two major protein bands of 130 kDa and 70 kDa in HAECs and E6 fibroblasts, which may at least represent p130Cas.
and paxillin. SFKs are negatively regulated by phosphorylation of a conserved tyrosine (Tyr-529 in human Src) in the C-terminal tail, which causes formation of an inactive conformation of SFKs (13,14). Dephosphorylation of this site has been shown to be mediated by RPTPα and CD45, resulting in the activation of SFKs (15). It is conceivable that inhibition of PTPs may augment phosphorylation of the C-terminal tyrosine and render SFKs to an inactive form. However, the hypothesis seems not a key mechanism for the H2O2-induced inactivation of SFKs localized at cell periphery. We found that the phosphorylation of Src Tyr-529 was only slightly increased (∼1.35 fold) by H2O2 at 5 and 10 min in HUVECs, which was not correlated well with a profound inactivation of SFKs by H2O2. Furthermore, the phosphorylation of Tyr-529 was not altered by H2O2 in E6 fibroblasts and 293 cells, in which SFKs were equally well inactivated by H2O2 compared with that in HUVECs. It appears likely other mechanisms may be involved. SFKs are activated through phosphorylation of the activation loop conserved tyrosine (Tyr-418 in human Src) (5-7). This phosphorylation has been traditionally thought to occur as an intermolecular event carried out by the kinase itself (8,9). Since H2O2 does not directly inhibit SFKs in vitro (Fig. 6), the fact that a rapid and profound reduction in the phosphorylation of Src Tyr-418 by H2O2 in vivo argues strongly against that the phosphorylation of Src Tyr-418 is merely catalyzed by the kinase itself in cells. Indeed, a set of evidence has indicated that phosphorylation of the activation loop tyrosine can be achieved by a kinase other than SFKs in several types of cells (7,10). Therefore, the simplest interpretation of our results is that H2O2 may inhibit the novel kinase through oxidation and inhibition of PTPs and suppress phosphorylation of the activation loop tyrosine in SFKs, leading to inactivation of SFKs in H2O2-treated cells. The hypothesis merits further investigation.

Another interesting finding from this study is that H2O2 induces insensitivity of ECs to growth factors and cytokines and protects endothelial inflammatory activation by cytokines. Conventional thought has generally regarded ROS as harmful to the vasculature, leading to such pathological processes as atherosclerosis, coronary ischemia, retinopathy, restenosis and hypertension (16, 24, 25). However, controlled clinical trials have failed to show a consistent benefit of antioxidants on these cardiovascular diseases (26-28). If oxidants are toxic to human body, why clinical trials of antioxidants have produced such mixed and mostly negative outcomes? Although the mechanism is not yet clear, a growing body of evidence suggests that ROS may be both protective and deleterious depending on concentration (24, 29). In the present study, we found that acute (3 min) pretreatment of HUVECs with a subtoxic concentration of H2O2 (250 µM) abolished the cell responses to PDGF and EGF. Moreover, H2O2 at 100-350 µM markedly suppressed the expression of VCAM-1 by thrombin and TNFα in HUVECs. These data indicate that ROS could blunt signal transduction of the activated receptors in ECs. The hypothesis is supported by a finding that HepG2 cells overexpressed mitochondrial catalase are more resistant to H2O2 but develop increased sensitivity to TNF-α (57). Since SFKs play initiating and key roles in cell signaling by a diverse set of cell surface receptors (1), our data suggest that the rapid and profound inactivation of SFKs by H2O2 may be at least one of the mechanisms for the protective effect of H2O2 on vascular cells. The production of cytokines and ROS is always accompanied with inflammation. At the sites of inflammation caused by infection or injury, high concentration of ROS may harm the vasculature and cause endothelial damage. On the other hand, ROS can be easily diffused and diluted to joint areas or tissues. Before destroyed by antioxidant system, the diluted ROS, on the basis of present findings, will inactivate SFKs and render the ECs resistant to cytokines in these adjoining areas, thus protecting development of a spread inflammation caused by cytokines. It is possible that an elevated antioxidant system may protect cells at the inflammation sites, but it may sacrifice the vascular protective effect of ROS for nearby vascular cells. The imbalance between these two opposite effects of ROS may impact outcomes in the antioxidant trials.

In summary, we have obtained substantial evidence indicating that ROS inactivate SFKs temporally and spatially in vivo but not in vitro, which may offer new insights into the mechanism of the protective effect of ROS in vivo.

FOOTNOTES

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†The abbreviations used are: SFKs, Src family tyrosine kinases; ROS, reactive oxygen species; PTPs, protein tyrosine phosphatases; EC, endothelial cells; HAECs, human aortic endothelial cells; HUVECs, human umbilical vein endothelial cells; PAO, phenylarsine oxide; GPCR, G protein-coupled receptor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; VCAM-1, vascular cell adhesion molecule-1.

FIGURE LEGENDS

**Fig. 1.** *H₂O₂ suppresses the tyrosine phosphorylation of focal adhesion proteins in vivo.* HAECs (A) or E6 fibroblasts (B) were serum-starved for 2 h, treated with *H₂O₂* (300 µM) for the indicated time periods, and cell lysates were immunoblotted with a phosphotyrosine antibody (*pTyr*). C, lysates from E6 fibroblasts treated with *H₂O₂* (250 µM) were subjected to immunoblotting (IB) with phospho-specific antibodies against Tyr-410-phosphorylated p130Cas or Tyr-118-phosphorylated paxillin. Results shown are representative immunoblots of 2 independent experiments.
Fig. 2. Time-dependent inactivation of SFKs by H₂O₂ in vivo. HAECs (A), HUVECs (B), E6 fibroblasts (C) or 293 cells (D) were serum-starved and treated with H₂O₂ (250 µM) for the indicated time periods, and lysates were subjected to immunoblotting (IB) with Src [pY418] antibody that recognizes Tyr-418-phosphorylated Src and other SFK members when phosphorylated at the equivalent sites. The phosphorylation of Tyr-418 was also shown as percentage of untreated control cells determined by densitometric analyses. The same blot was stripped and reprobed with SRC-2 antibody against the C-terminal sequence of SFKs to show the equal loading. Results shown are representative immunoblots of 3 independent experiments.

Fig. 3. Dose-dependent inactivation of SFKs by H₂O₂ in vivo. HUVECs (A), HAECs (B) or E6 fibroblasts (C) were serum-starved and treated with various concentrations of H₂O₂ for 5 min, and lysates were subjected to immunoblotting (IB) with Src [pY418] antibody that recognizes Tyr-418-phosphorylated Src and other SFK members when phosphorylated at the equivalent sites. The phosphorylation of Tyr-418 was also shown as percentage of untreated control cells determined by densitometric analysis. The same blot was stripped and reprobed with SRC-2 antibody against the C-terminal sequence of SFKs to show the equal loading. Results shown are representative immunoblots of 3 independent experiments.

Fig. 4. H₂O₂ inhibits the catalytic activities of SFKs and the tyrosine phosphorylation of SFK-bound substrates in ECs. A, HAECs or HUVECs were treated with H₂O₂ (250 µM) for the indicated time periods. SFKs were immunoprecipitated with SRC-2 antibody that recognizes the C-terminal sequence of SFKs, and kinase activities of the immune complexes towards a SFK-specific peptide substrate were measured. ● indicates HAECs; ○ HUVECs. Error bars indicate standard deviation. B, HUVECs were treated with H₂O₂ (250 µM) for the indicated time periods, and lysates were immunoprecipitated (IP) with SRC-2 antibody and subjected to immunoblotting (IB) with a phosphotyrosine antibody (pTyr). Arrows indicate protein bands in which the tyrosine phosphorylation was not recovered to basal levels by 1 h after H₂O₂ treatment. Results shown are representative immunoblots of 3 independent experiments.

Fig. 5. H₂O₂ inactivates predominantly the SFKs localized to focal adhesions and the plasma membrane of ECs. HUVECs grown on glass coverslips were left untreated (control) or treated with H₂O₂ (250 µM) for 5 or 60 min, washed twice and fixed. The activated SFKs were viewed (10 X 60) by staining the fixed cells with the phospho-Src (Tyr-418) antibody. Arrows indicate localization of the activated SFKs. Results shown are representative of 3 independent experiments.

Fig. 6. H₂O₂ does not affect directly the catalytic activities of SFKs in vitro. Purified recombinant human full-length Src (6 Units) or Fyn (25 ng) was incubated with various concentrations of H₂O₂ (0-1000 µM) for 20 min at 30 ºC and the kinase activity was measured with a SFK-specific substrate peptide as described in Methods. Results shown are representative of 3 independent experiments.

Fig. 7. Inhibition of PTPs is involved in the H₂O₂-induced inactivation of SFKs in vivo. A, dose-dependent inhibition of SHP-2 phosphatase activity by H₂O₂ in vitro. B, time-dependent effect of H₂O₂ (250 µM) on total activities of PTPs in HUVECs. C and D, inactivation of SFKs by PTP inhibitors in HUVECs. HUVECs were treated with H₂O₂ (250 µM) for 5 min or Na₃VO₄ for 1 or 5 min (C), or for 5 min with H₂O₂ (250 µM), phenylarsine oxide (PAO, 25 µM) or NaF (10 mM) (D). Lysates were immunoblotted (IB) with Src [pY418] antibody that recognizes the activated SFKs. The same blot was stripped and reprobed with SRC-2 antibody against the C-terminal sequence of SFKs to show the equal loading. Results shown are representative immunoblots of 3 independent experiments.

Fig. 8. Effect of H₂O₂ on phosphorylation of the C-terminal conserved tyrosine (Tyr-529 in Src). HUVECs (A), E6 fibroblasts (B) or 293 cells (C) were serum-starved and treated with H₂O₂ (250 µM) for...
the indicated time periods, and lysates were subjected to immunoblotting (IB) with Src [pY529] antibody that recognizes Tyr-529-phosphorylated Src and other SFK members when phosphorylated at the equivalent sites. The same blot was stripped and reprobed with the SRC-2 antibody to show the equal loading. Results shown are representative immunoblots of 3 independent experiments.

Fig. 9. H$_2$O$_2$ suppresses the responses of ECs to growth factors and cytokines. A, HUVECs were left untreated (-) or pretreated 3 minutes with H$_2$O$_2$ followed with (+/-) or without (+) a wash to remove H$_2$O$_2$, then stimulated with PDGF (50 ng/ml) for 5 min. Lysates were immunoblotted with antibodies against phospho-ERK1/2 (Thr202/Tyr204) or phospho-Src (Tyr-418). The same blot was stripped and reprobed with the ERK1/2 antibody to show the equal loading. B and C, HUVECs were left untreated (-) or pretreated 30 min with various concentrations of H$_2$O$_2$ as indicated or PAO (100 nM), then incubated without (-) or with thrombin (3 U/ml) (B) or TNF-α (10 ng/ml) (C) for 4 h. Lysates were immunoblotted with VCAM-1 antibody. The same blots were stripped and reprobed with tubulin antibody to show the equal loading. Results shown are representative immunoblots of 3 independent experiments.
A: HAECs

B: E6 fibroblasts

C

Figure 1. H. Tang et al
**A: HAECs**

<table>
<thead>
<tr>
<th>H$_2$O$_2$: 0 5 10 20 30 60 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB: Src [pY418] ( % ): 100 19 23 33 46 90</td>
</tr>
<tr>
<td>IB: SRC-2</td>
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</tbody>
</table>

**B: HUVECs**

<table>
<thead>
<tr>
<th>H$_2$O$_2$: 0 5 10 30 60 120 (min)</th>
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</thead>
<tbody>
<tr>
<td>IB: Src [pY418] ( % ): 100 11 12 18 70 50</td>
</tr>
<tr>
<td>IB: SRC-2</td>
</tr>
</tbody>
</table>

**C: E6 fibroblasts**

<table>
<thead>
<tr>
<th>H$_2$O$_2$: 0 5 10 30 60 120 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB: Src [pY418] ( % ): 100 21 25 57 80 88</td>
</tr>
<tr>
<td>IB: SRC-2</td>
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</tbody>
</table>

**D: 293 cells**

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>IB: Src [pY418]</td>
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<tr>
<td>IB: SRC-2</td>
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</tbody>
</table>

Figure 2. H. Tang et al
A: HUVECs

\[
\text{H}_2\text{O}_2: \quad 0 \quad 50 \quad 100 \quad 250 \quad 500 \quad (\mu\text{M})
\]

\[
\text{IB: Src [pY418]} \quad \text{IB: SRC-2}
\]

B: HAECs

\[
\text{H}_2\text{O}_2: \quad 0 \quad 50 \quad 100 \quad 250 \quad 500 \quad (\mu\text{M})
\]

\[
\text{IB: Src [pY418]} \quad \text{IB: SRC-2}
\]

C: E6 fibroblasts

\[
\text{H}_2\text{O}_2: \quad 0 \quad 100 \quad 250 \quad 500 \quad (\mu\text{M})
\]

\[
\text{IB: Src [pY418]} \quad \text{IB: SRC-2}
\]

Figure 3. H.Tang et al
Figure 4. H. Tang et al
Src [pY418] staining

control

H$_2$O$_2$, 5 min

H$_2$O$_2$, 60 min

Figure 5. H. Tang et al
Figure 6. H. Tang et al
Figure 7. H. Tang et al
A: HUVECs

H$_2$O$_2$: 0 5 10 30 60 (min)

IB: Src [pY529]

Fold basal: 1 1.3 1.4 .95 .85

IB: SRC-2

B: E6 fibroblasts

H$_2$O$_2$: 0 5 10 30 60 (min)

IB: Src [pY529]

IB: SRC-2

C: 293 cells

H$_2$O$_2$: 0 5 10 (min)

IB: Src [pY529]

IB: SRC-2

Figure 8. H. Tang et al
Figure 9. H. Tang et al