Src and Cas Mediate JNK Activation but Not ERK1/2 and p38 Kinases by Reactive Oxygen Species*

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Reactive oxygen species (ROS), including H$_2$O$_2$, O$_2^-$, and OH$,^-$, have been identified as important chemical mediators that regulate signal transduction, and the importance of ROS in pathological events is becoming increasingly apparent. Because increased ROS may be a risk factor for cardiovascular events such as unstable angina, myocardial infarction, and sudden death, understanding the biological processes that generate ROS and the intracellular signals elicited by ROS will be useful for gaining insights into the pathogenesis of these diseases (1, 2). Recently, it has been shown that ROS stimulate intracellular signal events similar to those activated by growth factors including members of the mitogen-activated protein kinases, namely extracellular signal regulated kinases (ERK1/2), c-Jun NH$_2$-terminal kinases (JNKs), and p38 kinase (3, 4). The ERKs are involved in anabolic processes (cell growth, division, and differentiation). In contrast, the JNKs and p38 appear to be involved primarily in the responses of cells to cytotoxic agents and interventions. It has been reported by several investigators that ROS also increase JNK activation (5–7), but the proximal mechanisms for ROS-mediated JNK activation remain unknown.

Previous studies have shown that c-Src is involved in signal events stimulated by ROS (3, 8). Three family members (c-Src, Fyn, and Yes) are expressed ubiquitously and appear to have partially overlapping functions on the basis of studies with transgenic mice (9). Functional domains shared by Src family kinases include an amino-terminal myristoylation sequence for membrane targeting, a unique domain, SH2 and SH3 domains, a kinase domain, and a carboxyl-terminal noncatalytic domain. These regions participate in a complex tonic inhibition of Src family kinases which can be overcome when cells are exposed to mitogens. One of the residues that appears to be critical for regulation of c-Src is Tyr$^{527}$, which is not present in v-Src. Phosphorylation of Tyr$^{527}$ by COOH-terminal Src kinase inhibits c-Src activity (10), whereas dephosphorylation of this residue appears to be an activating mechanism. Autophosphorylation of Tyr$^{416}$ in the catalytic domain may be another activating signal. Src activity is also inhibited via intramolecular interactions of the carboxyl-terminal catalytic domains with both the SH2 and SH3 domains (11). These SH2 and SH3 domains probably also stimulate c-Src activity through interactions with regulators and downstream/kinase substrates.

Because ROS activate both Src and JNK, and Cas overexpression stimulates JNK, we hypothesized that H$_2$O$_2$-mediated JNK activation would require Src activity and formation of a Cas-Crk complex. To determine the specific role of Src family kinases, we utilized specific Src family kinase inhibitors and cells derived from animals deficient in Src and Fyn. We show here that H$_2$O$_2$-mediated activation of JNK, but not ERK1/2 or p38, is regulated by Src but not by Fyn. Furthermore, we demonstrate that Src is required for H$_2$O$_2$-mediated tyrosine phosphorylation of Tyr$^{527}$ by COOH-terminal Src kinase inhibits c-Src activity (10), whereas dephosphorylation of this residue appears to be an activating mechanism. Autophosphorylation of Tyr$^{416}$ in the catalytic domain may be another activating signal. Src activity is also inhibited via intramolecular interactions of the carboxyl-terminal catalytic domains with both the SH2 and SH3 domains (11). These SH2 and SH3 domains probably also stimulate c-Src activity through interactions with regulators and downstream/kinase substrates.
phosphorylation of Cas and Cas-Crk complex formation. Thus, the Src-Cas-Crk-JNK signaling pathway described here may represent a new redox-sensitive mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Culture, and Transfection—**Vascular smooth muscle cells (VSMC) were isolated from 200–250-g male Harlan Sprague-Dawley rats and maintained in 10% calf serum and Dulbecco’s modified Eagle’s medium as described previously (23). Passage 5–15 VSMC at 70–80% confluence in 100-mm dishes were growth arrested by incubation in 0.4% calf serum and Dulbecco’s modified Eagle’s medium for 48 h to use. Fibroblasts deficient in c-Src (Src<sup>−/−</sup>) or Fyn (Fyn<sup>−/−</sup>) were isolated from mouse embryo fibroblasts homozygous for disruption of the Src and Fyn genes, immortalized with large T antigen (24). Cells were kindly provided by Sheila M. Thomas, Fred Hutchinson Cancer Center, Seattle, WA. Fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum as described previously (24). Cells at 70–80% confluence in 100-mm dishes were growth arrested by incubation in RPMI 1640 for 24 h prior to use. Chinese hamster ovary cells were maintained in F-12/Ham’s medium supplemented with 10% fetal calf serum. pSSR<sub>a</sub>mammalian cell expression vectors encoding p130Cas or Cas<sub>D</sub>SD were kindly provided by Dr. Vuori, La Jolla Cancer Research Center. For transient expression experiments, cells were transfected 1 day after replating by LipofectAMINE as described previously (25). After 48 h of incubation, cells were harvested for experiments.

**Immunoprecipitation and Western Blot Analysis—**After treatment, the cells were washed with phosphate-buffered saline, harvested in 0.5 ml of lysis buffer (50 mM sodium pyrophosphate, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM Na<sub>3</sub>VO<sub>4</sub>, 10 mM HEPES, pH 7.4, 0.1% Triton X-100, 500 μM phenylmethanesulfonyl fluoride, and 10 μg/ml leupeptin), and flash-frozen on a dry ice/ethanol bath. After allowing the cells to thaw, cells were scraped off the dish and centrifuged at 14,000 × g (4 °C for 30 min), and protein concentration was determined using the Bradford protein assay (Bio-Rad). For immunoprecipitation, cell lysates were incubated with rabbit anti-Cas antibody

**Fig. 1.** ERK1/2, JNK, and p38 are activated by H<sub>2</sub>O<sub>2</sub> in VSMC and fibroblasts. VSMC (panels A and B) and mouse fibroblasts (panels C and D) were growth arrested for 24 h and stimulated with 1 mM H<sub>2</sub>O<sub>2</sub> for the indicated times. Activity of ERK1/2, JNK, and p38 was measured as described under “Experimental Procedures.” No difference in the amount of ERK1/2, JNK, and p38 was observed in samples by Western blot analysis with anti-JNK, ERK1/2, and p38 (data not shown). Panels B and D, densitometric analysis of JNK, ERK1/2, and p38 activation. Results were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0 (shown is the mean ± S.D., n = 3).

**Fig. 2.** H<sub>2</sub>O<sub>2</sub> stimulates a concentration-dependent increase in ERK1/2, JNK, and p38 activity. VSMC (panels A and B) and mouse fibroblasts (panels C and D) were growth arrested for 24 h and stimulated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for the following times: ERK1/2, 20 min; JNK, 40 min; and p38, 60 min for VSMC and 40 min for fibroblasts. The activity of ERK1/2, JNK, and p38 was measured as described under “Experimental Procedures.” No difference in the amount of ERK1/2, JNK, and p38 was observed in samples by Western blot analysis with anti-JNK, ERK1/2, and p38 (data not shown). Panels B and D, densitometric analysis of JNK, ERK1/2, and p38 activation. Results were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0 (shown is the mean ± S.D., n = 3).
nase inhibitor, inhibits H2O2-induced JNK activation in a concentration-dependent manner, but not ERK1/2 and p38 in VSMC and fibroblasts. Growth-arrested VSMC (panels A and B) and fibroblasts (panels C and D) were pre-treated with dimethyl sulfoxide or the indicated concentrations of PP2 for 15 min. ERK1/2, JNK, and p38 activation assay was performed as described under “Experimental Procedures.” Western blots were performed with an anti-phosphospecific c-Jun antibody for JNK activity assay, and ERK1/2 and p38 activity was measured by Western blot analysis with a phosphospecific ERK1/2 and p38 antibody (panels A and C, top and bottom). No difference in the amount of ERK1/2, JNK, and p38 was observed in samples by Western blot analysis with anti-JNK, ERK1/2, and p38 (data not shown). Panels B and D, densitometric analysis of JNK, ERK1/2, and p38 activation. Results were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0 (shown is the mean ± S.D., n = 3, *p < 0.05, **p < 0.01).

RESULTS

H2O2 Stimulates ERK1/2, JNK, and p38 Kinase Activity in VSMC and Fibroblasts in a Concentration-dependent Manner—To evaluate the relative magnitude of mitogen-activated protein kinase activation by H2O2, growth-arrested VSMC and fibroblasts were stimulated for the indicated times with 1 mM H2O2, cell lysates were prepared, and Western blot analysis was performed on whole cell lysates using anti-c-Jun antibody (Santa Cruz) or mouse anti-Crk antibody (Transduction Laboratories) for 3 h at 4°C and then incubated with 20 μl of protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) for 1 h on a roller system at 4°C. The beads were washed twice with 1 ml of lysis buffer, twice with 1 ml of LiCl wash buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, 0.1% Triton X-100, 1 mM dithiothreitol), and twice in 1 ml of washing buffer (20 mM HEPES, pH 7.2, 2 mM EGTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100). For Western blot analysis, cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to nitrocellulose membranes (Hybond™, ECL, Amersham Pharmacia Biotech) as described previously (26). The membrane was blocked for 1 h at room temperature with a commercial blocking buffer from Life Technologies, Inc. The blots were then incubated for 1 h at room temperature with the anti-phosphotyrosine 4G10, Upstate Biotechnology) or anti-Cas antibody (Santa Cruz), followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated). For ERK1/2 activation, the blots were incubated for 12 h with anti-phosphospecific ERK1/2 or p38 (New England Biolabs) or antibodies that recognize ERK1 and ERK2 or p38 (Santa Cruz). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

JNK Activation Assay—JNK activity was measured as a commercially available kit based on phosphorylation of recombinant c-Jun (New England Biolabs). After treatment, cells were rinsed twice with ice-cold phosphate-buffered saline, scraped off the plates into lysis buffer (included in the kit), and sonicated three times on ice. After removing the cell debris (20,000 × g, 10 min, 4°C), protein content was measured in the supernatant using Bradford protein assay reagent. Equal amounts of protein were then immunoprecipitated with c-Jun (1–89) fusion protein beads overnight. After washing the beads, kinase assays were performed according to the instructions of the manufacturer. Beads were loaded on a 10% SDS-polyacrylamide gel, and Western blots were performed with an antibody against phosphospecific c-Jun.

Materials—All materials were from Sigma except where indicated. H2O2 was from Fisher Scientific, and PP2 was from Calbiochem.

Statistical Analysis—Data are reported as the mean ± S.D. Statistical analysis was performed with the StatView 4.0 package (ABACUS Concepts, Berkeley, CA). Differences were analyzed with an unpaired two-tailed Student's t test or Welch's t test as appropriate.

H2O2-mediated JNK Activation via Src and Cas
fibroblasts were exposed to 1 mM H$_2$O$_2$. Cell lysates were then immunoprecipitated with c-Jun (1–89) fusion protein, and Western blotting was performed with anti-phosphosphpecific c-Jun antibody for JNK activity assay (panel A), and ERK1/2 and p38 activities were determined by immunoblotting for dually phosphorylated ERK1/2 and p38 in whole cell lysates. In both VSMC and fibroblasts, ERK1/2 was activated most rapidly (peak at 5–20 min) and to the greatest extent (~10 fold) (Fig. 1, A and C, top). JNK was activated within 20 min after the stimulation by H$_2$O$_2$ in VSMC and fibroblasts (Fig. 1, A and C, middle). Peak JNK activation (5.3 ± 0.8-fold increase in VSMC and 5.4 ± 0.7 fold in fibroblasts) occurred at 40 min and was sustained for 120 min in fibroblasts (Fig. 1, B and D). p38 activation was of greater extent in VSMC (~8 fold) than in fibroblasts (~5 fold) and was more rapid in fibroblasts (peak at 40 min) than in VSMC (peak at 60 min) (Fig. 1, A and C, bottom). These results are summarized in Fig. 1, B and D.

We also determined the concentration dependence for mitogen-activated protein kinase activation by H$_2$O$_2$ in VSMC and fibroblasts (Fig. 2). In VSMC, ERK1/2 was activated by the lowest [H$_2$O$_2$] ~0.1 mM, was maximal at 1 mM, and showed half-maximal effect at 0.1 mM. H$_2$O$_2$ also stimulated a concentration-dependent increase in JNK and p38 activity, although the magnitude was smaller than ERK1/2 and required higher [H$_2$O$_2$] (Fig. 2, A and B). Similar results were obtained in fibroblasts (Fig. 2, C and D).

H$_2$O$_2$ Stimulates JNK, but Not ERK1/2 and p38, via a Src-dependent, Fyn-independent Mechanism—Previous investigators including our laboratory have suggested that Src family kinases may be upstream mediators of redox-sensitive signal transduction (3, 8). To determine the role of Src family kinases as upstream mediators of H$_2$O$_2$-mitogen-activated protein kinase activation, we studied the effect of a specific inhibitor for Src family kinase, PP2 (Fig. 3). PP2 has been shown to exhibit specific inhibition of Src (27, 28). PP2 inhibited 1 mM H$_2$O$_2$-mediated JNK activation in both VSMC (Fig. 3A) and fibroblasts (Fig. 3C) in a dose-dependent manner with an IC$_{50}$ value of ~981 mM (Fig. 3, B and D). In contrast, PP2 failed to inhibit ERK1/2 and p38 activation by H$_2$O$_2$ (Fig. 3).

Previously we found a significant role for Fyn in H$_2$O$_2$-mediated signal transduction because H$_2$O$_2$-mediated ERK1/2 activation in Fyn$^{-/-}$ cells was inhibited in a time-dependent manner (29). Therefore, we determined the effect of PP2 on the time course of JNK activation. PP2 (10 μM) inhibited JNK activation completely for up to 120 min after stimulation by H$_2$O$_2$ (data not shown). These results suggested that Src family kinases are the main regulator of ROS-mediated JNK activation in VSMC and fibroblasts.

To determine the role of Src family kinases in JNK activation by H$_2$O$_2$ we utilized cells derived from mice deficient in Src or Fyn (24). There was no immunoreactive c-Src in Src$^{-/-}$ cells, whereas immunoreactive Fyn was expressed to the same extent as in wild type cells (Fig. 4A). Likewise, there was no immunoreactive Fyn in Fyn$^{-/-}$ cells, whereas there was no change in expression of c-Src in Fyn$^{-/-}$ cells compared with wild type cells (Fig. 4A). H$_2$O$_2$ stimulated JNK activity in wild type fibroblasts which was maximal at 40 min (5.4 ± 0.7-fold increase) (Figs. 1C and 4B). In Fyn$^{-/-}$ fibroblasts, JNK activation increased with maximum at 60 min (4.3 ± 1.0-fold increase, n = 3) after H$_2$O$_2$ stimulation (Fig. 4, B and C).
contrast, in Src−/− fibroblasts, H$_2$O$_2$ failed to stimulate JNK activity at any time (Fig. 4, B and C). These results indicate that H$_2$O$_2$-mediated activation of JNK is dependent on Src but not Fyn in fibroblasts.

H$_2$O$_2$ Stimulates JNK Activation, but Not ERK1/2 and p38, via a Cas-dependent Mechanism—An important role for Cas in JNK activation was suggested by Dolfi et al. (22), who reported that expression of Cas induced JNK activation. Cas-mediated JNK activation likely requires Crk because a mutant form of Cas (CasΔSD), in which a region known as the substrate domain is deleted, prevents JNK activation (22).

Transfection of wild type Cas had no significant effect on H$_2$O$_2$-induced JNK activation (Fig. 5, A and B). Transfection of CasΔSD inhibited JNK activation by H$_2$O$_2$ consistent with previous findings (22). There was no significant effect of CasΔSD on H$_2$O$_2$-mediated ERK1/2 and p38 activation (Fig. 5, C–F). These results demonstrate an essential role for Cas in H$_2$O$_2$-mediated JNK activation but not ERK1/2 or p38 activation.

H$_2$O$_2$ Stimulates Cas Tyrosine Phosphorylation and Cas-Crk Complex Formation via a Src-dependent, Fyn-independent Mechanism—We next investigated H$_2$O$_2$-mediated Cas tyrosine phosphorylation in Src−/− and Fyn−/− fibroblasts. In wild type fibroblasts, Cas tyrosine phosphorylation was maximally stimulated (8.1 ± 1.5-fold increase, n = 3) by H$_2$O$_2$ at 5 min (Fig. 6A). In Fyn−/− fibroblasts, Cas tyrosine phosphorylation increased (maximum 8.0 ± 1.5-fold increase at 5 min) after H$_2$O$_2$ stimulation to an extent similar to wild type (Fig. 6, B and C). In contrast, in Src−/− fibroblasts, H$_2$O$_2$-mediated Cas tyrosine phosphorylation was inhibited significantly (1.6 ± 0.6-fold increase at 30 min, p < 0.01) (Fig. 6, B and C). No difference in Cas protein expression was observed in lysates from control and H$_2$O$_2$-stimulated cells as determined by immunoprecipitation and Western blot analysis with anti-Cas antibody (Fig. 6B). These results indicate that H$_2$O$_2$-mediated Cas tyrosine phosphorylation in fibroblasts is dependent on Src but not on Fyn.

To gain further insight into the functional significance of Cas tyrosine phosphorylation, we determined whether H$_2$O$_2$ stimulates the association of Cas with Crk. Fibroblasts were exposed to H$_2$O$_2$, and lysates were immunoprecipitated with anti-Crk antibody and immunoblotted with anti-Cas antibody or anti-phosphotyrosine antibody (Fig. 7). Following H$_2$O$_2$ treatment there was a significant increase in coimmunoprecipitation of Cas with Crk (Fig. 7A, top). Immunoblotting with anti-phosphotyrosine antibody showed that coimmunoprecipitated Cas was tyrosine-phosphorylated (Fig. 7A, middle).

To confirm the role of Src family kinases in Cas-Crk complex formation by H$_2$O$_2$ we also studied Cas-Crk complex formation in Src−/− and Fyn−/− cells. In Fyn−/− fibroblasts, Cas-Crk complex formation was stimulated with peak 5 min after H$_2$O$_2$ stimulation (6.9 ± 1.4-fold increase) (Fig. 7, C and D). In contrast, in Src−/− fibroblasts, H$_2$O$_2$ failed to stimulate Cas-Crk complex formation at any time (Fig. 7, B and D). No difference in Crk protein expression was observed in lysates from control and H$_2$O$_2$-stimulated cells as determined by immunoprecipitation and Western blot analysis with anti-Crk antibody. These results indicate that stimulation of Cas-Crk complex formation by H$_2$O$_2$ is also dependent on Src but not Fyn in fibroblasts.

**DISCUSSION**

The major findings of this study are that H$_2$O$_2$ stimulates JNK via a pathway that is dependent on Src and p130Cas but not Fyn. Redox-sensitive regulation of Cas/JNK is thus a new function for Src. Data to support an essential role for Src and Cas in H$_2$O$_2$-mediated JNK activation include the following findings. 1) PP2, a specific Src family kinase inhibitor, prevented H$_2$O$_2$-mediated activation of JNK, but not ERK1/2 or p38, in VSMC and fibroblasts. 2) In Src−/− fibroblasts, there was no JNK activation in response to H$_2$O$_2$. In contrast, in Fyn−/− fibroblasts, H$_2$O$_2$-mediated JNK activation was similar to wild type fibroblasts. 3) Cas tyrosine phosphorylation and Cas-Crk complex formation by H$_2$O$_2$ were also inhibited in Src−/− fibroblasts but not in Fyn−/− fibroblasts. 4) A dominant negative mutant of Cas which fails to bind Crk blocked H$_2$O$_2$-mediated activation of JNK but not ERK1/2 and p38. Our results are the first to show that Src, but not Fyn, is involved specifically in oxidative stress-mediated JNK activation, via a Cas/Crk signaling pathway.

Based on the present study, as well as previous work from our laboratory (4, 8) and other investigators (30–32), we propose a scheme (Fig. 8) for ROS-mediated signal transduction leading to activation of Cas and JNK. A novel aspect of this model is the specific role of Src but not Fyn. Previously we found that Fyn but not Src was required for H$_2$O$_2$-mediated janus kinase 2 activation in fibroblasts (29). In contrast, in the present study, we found that Src but not Fyn was required for H$_2$O$_2$-mediated JNK activation in fibroblasts. These results indicate that c-Src and Fyn have separate roles in ROS-medi-
Src family kinases share several functional domains including an amino-terminal myristoylation sequence for membrane targeting, a unique domain, SH2 and SH3 domains, a kinase domain, and a carboxyl-terminal non-catalytic domain. Functional properties of the amino-terminal unique region have not been well defined. Current data indicate that this domain may be required for specific interactions between particular Src family kinases and downstream targets. We have also found that Src and Fyn specifically regulate big mitogen-activated protein kinase 1 (BMK1) and janus kinase 2, respectively (8, 29). Future studies will be required to define the precise nature of the downstream substrates for c-Src and Fyn.

In addition to activation of JNK by Src, the present study demonstrates that H$_2$O$_2$-mediated Cas-Crk complex formation is dependent on Src but not Fyn (Fig. 8). Once phosphorylated, Cas acts as a docking protein to recruit Crk and its effectors (14, 33). The SH3 domains of Crk may bind several effectors capable of activating JNK, including C3G, DOCK180, and Sos (34–36) (Fig. 8). Tanaka and Hanafusa (37) have reported that the Crk-dependent stimulation of JNK requires C3G, a guanine nucleotide exchange protein for the Ras family of small G proteins. Transient expression of C3G in 293T cells induced JNK activation without a significant effect on ERK, whereas mSos activated both JNK and ERK1/2 equally. Coexpression of the dominant negative form of Ras-N17 did not suppress C3G-induced JNK activation but reduced the activity of JNK induced by mSos, suggesting that Ras is not required for JNK activation by C3G. Ras-independent activation of JNK was supported by the finding that C3G-induced JNK activation was not inhibited by the dominant negative forms of Rac or PAK, which are components of the signaling pathway from Ras leading to JNK activation (34). Dolfi et al. (22) have also found that transient expression of C3G induces JNK activation, and this activation was dependent on both the SH2 and SH3 domains of Crk. Expression of p130Cas also induced JNK activation, which was blocked by the SH2 mutant of Crk. In contrast to Tanaka and Hanafusa (37), Dolfi et al. (22) reported that JNK activation by Cas and Crk was effectively blocked by a dominant negative form of Rac, suggesting a linear pathway from the Cas-Crk complex to Rac-JNK activation. Thus, the role of C3G in JNK and cytoskeletal reorganization remains controversial. DOCK180 has been implicated previously in up-regulation of the Cas-Crk-mediated signaling pathways and was shown to activate Rac1 and JNK (22, 38). Klemke et al. (19) have demonstrated that the Cas-Crk signaling complex functions as a “molecular switch” for cell migration because overexpression of the two molecules stimulated cell migration. Significantly, Klemke et al. (19) found that dominant negative forms of Cas and Crk were able to block both integrin-mediated and cytokine-induced migration. An important role for Src in Cas-dependent cell migration is suggested by our findings that Src is the major tyrosine kinase responsible for phosphorylation of Cas by angiotensin II, thrombin, and shear stress. We have found that expression of kinase-negative Src reduced the rate of VSMC spreading on collagen, whereas wild type Src enhanced cell spreading, indicating an essential role for Src in cell adhesion and morphology (39). Cas plays a key role in cytoskeletal reorganization because it is localized to focal adhesions, binds to Src via its SH3 domain, and to other signaling molecules such as Crk, Nck, FAK, and PTP-PEST via SH2 binding motifs.

**FIG. 7.** H$_2$O$_2$-mediated Cas-Crk complex formation is inhibited in Src$^{-/-}$ cells but not in Fyn$^{-/-}$ cells. Cells were stimulated for the indicated times with 1 mM H$_2$O$_2$. Panels A, B, and C, cell lysates were incubated with Crk antibody, and immunoprecipitates from each were analyzed by anti-Cas (top) anti-phosphotyrosine (pY, middle) and anti-Crk (lower) Western blotting. Panel D, densitometric analysis of Cas-Crk complex formation. Results were normalized by arbitrarily setting the densitometry of control cells (time = 0) to 1.0 (shown is the mean ± S.D., n = 3). The asterisks represent significant differences compared with control (‘*’p < 0.05).

**FIG. 8.** Model of H$_2$O$_2$-mediated signal transduction pathways to JNK.
Tyrosine phosphorylation of Cas by H2O2 was completely dependent on Src, indicating the importance of Src in H2O2-mediated events. Further studies will be required to determine the role of JNK activation in cell migration and other downstream events activated by ROS.

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