Published in final edited form as:

Biochem J. 2008 May 1; 411(3): 531–541. doi:10.1042/BJ20071534.

Endosomal NADPH oxidase regulates c-Src activation following hypoxia/reoxygenation injury

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Abstract

c-Src has been shown to activate NF- κ B (nuclear factor κ B) following H/R (hypoxia/reoxygenation) by acting as a redox-dependent I κ B α (inhibitory κ B) tyrosine kinase. In the present study, we have investigated the redox-dependent mechanism of c-Src activation following H/R injury and found that ROS (reactive oxygen species) generated by endosomal Noxs (NADPH oxidases) are critical for this process. Endocytosis following H/R was required for the activation of endosomal Noxs, c-Src activation, and the ability of c-Src to tyrosine-phosphorylate I κ B α . Quenching intra-endosomal ROS during reoxygenation inhibited c-Src activation without affecting c-Src recruitment from the plasma membrane to endosomes. However, siRNA (small interfering RNA)-mediated knockdown of Rac1 prevented c-Src recruitment into the endosomal compartment following H/R. Given that Rac1 is a known activator of Nox1 and Nox2, we investigated whether these two proteins were required for c-Src activation in Nox-deficient primary fibroblasts. Findings from these studies suggest that both Nox1 and Nox2 participate in the initial redox activation of c-Src following H/R. In summary, our results suggest that Rac1-dependent Noxs play a critical role in activating c-Src following H/R injury. This signalling pathway may be a useful therapeutic target for ischaemia/reperfusion-related diseases.

Keywords

c-Src; endomembrane; hypoxia/reoxygenation; NADPH oxidase; Rac1; reactive oxygen species

INTRODUCTION

ROS (reactive oxygen species) have been implicated in a variety of pathophysiological signalling responses to diverse types of cellular injury. Superoxide radical ($O_2^{\bullet-}$) and its dismutation product, H_2O_2 , are two important forms of ROS known to act as second messengers in signal transduction. For example, these ROS are generated following PDGF (platelet-derived growth factor), LPS (lipopolysaccharide), TNF α (tumour necrosis factor α) and IL-1 β (interleukin-1 β) stimulation and play critical roles in determining downstream signals invoked by activation of their respective receptors [1–3]. ROS induction is also observed in various cells and organs following both H/R (hypoxia/reoxygenation) and I/R (ischaemia/reperfusion) injuries [4,5]. However, the mechanisms of action of H/R- and I/R-induced ROS are far less clear than those of ligand-activated receptor pathways.

The reoxygenation phases of both H/R and I/R are known to lead to the acute production of ROS, and clearance of such ROS can abrogate detrimental signals [5–7]. Numerous sources

of induced cellular ROS following H/R and I/R have been implicated in cell signalling. Among them, XO (xanthine oxidase) and MPO (myeloperoxidase) have been reported to produce extracellular ROS, as well as associated endothelial cell damage, during the reperfusion injury phase that follows ischaemia [8,9]. Cytochrome P450 and the mitochondria have also been implicated as important sources of ROS following H/R [10,11]. Finally, Noxs (NADPH oxidases) appear also to serve as a source for ROS following H/R [12,13]. In the present study, we investigated whether Nox-derived ROS are important in the redox-dependent activation of c-Src following H/R and the extent to which such a pathway might influence the activation of NF- κ B (nuclear factor κ B).

The Noxs characterized to date incorporate one of the seven known catalytic subunits: Nox1, Nox2 (gp91^{phox}), Nox3, Nox4, Nox5, Duox1 (dual oxidase 1) and Duox2 (dual oxidase 2) [14]. In their active forms, these complexes generate O₂^{•−} by transferring an electron from NADPH to molecular oxygen. The active form of the most widely characterized phagocytic Nox (Nox2) is a multi-subunit membrane complex for which recruitment of p67^{phox}, p47^{phox}, p22^{phox} and Rac1/2 plays an important role in activating O₂^{•−} production [14,15]. Among these components, the Rac GTPase is believed to be requisite for activation of Nox1 and Nox2. A link between Nox protein activation and cellular signalling has become increasingly recognized [16]. The localized production of O₂^{•−} appears to be a major regulatory component of pathways that depend on Nox-derived ROS [16].

c-Src, a member of the SFKs (Src family of protein tyrosine kinases), is involved in a wide spectrum of cellular functions, including cell proliferation, cellular transformation, cell migration and responses to H/R and I/R injury [5,17–19]. c-Src is myristoylated at the N-terminus, and palmitoylation may also occur downstream of the myristoylation site. These fatty acid moieties are required for c-Src localization to cellular membranes [20]. The activity of c-Src is tightly regulated in normal cells; aberrant regulation can lead to constitutive activation, which is an underlying cause of many diseases. c-Src activation requires Tyr⁴¹⁶ phosphorylation to occur within a segment of the kinase domain termed the activation loop [21]. Despite numerous observations that have associated the activation of Noxs with the c-Src pathway following cytokine and growth-factor stimulation [22,23], the mechanisms that control ROS production and downstream activation cascades remain poorly understood. Furthermore, in the context of H/R, links between Nox activation and the redox regulation of c-Src have yet to be explored.

We have previously demonstrated that the redox-dependent activation of c-Src following H/R injury is required for the activation of NF- κ B, the latter event relying on a non-canonical pathway that involves c-Src-mediated tyrosine phosphorylation of I κ B α (Tyr-42) (I κ B is inhibitory κ B) [5,19]. This pathway of I κ B α tyrosine phosphorylation imparts context-specific NF- κ B activation in the liver following I/R, but not LPS injury [19]. In the present study, we sought to determine the mechanism of redox-mediated c-Src activation following H/R injury. Our results indicate that both Nox1 and Nox2 participate in the induction of endosomal ROS that follows H/R injury, and that this is required for c-Src activation and c-Src-mediated I κ B α tyrosine phosphorylation. This process required endosomal recruitment of both Rac1 and c-Src. These results suggest that Rac1-dependent activation of Nox1 and Nox2 plays critical roles in activating c-Src following H/R injury.

EXPERIMENTAL

Materials

A number of E1-deleted recombinant adenoviral (Ad) vectors were used in the studies. They included: (i) Ad.DynK44A, which encodes a dominant-negative mutant (K44A) of dynamin

and inhibits endocytosis [24]; (ii) Ad.GPx-1, which encodes GPx-1 (glutathione peroxidase-1) and degrades cytoplasmic H₂O₂ [25]; (iii) Ad.NF- κ BLuc, which encodes an NF- κ B-responsive promoter that drives luciferase expression and has been used to assess NF- κ B transcriptional activation *in vivo* [26]; (iv) Ad.BglIII (an empty vector with no insert) or Ad.LacZ (which encodes a β -galactosidase expression cassette) as a control for viral infection [25]; and (v) Ad.L61Rac1, which encodes the dominant-positive L61 mutation in Rac1. Ad.L61Rac1 was generated using previously described methods [27]. siRNAs (small interfering RNAs) against human c-Src and Rac1 were obtained from Santa Cruz Biotechnology, and their transfection was performed using methods and reagents as described by the manufacturer.

Cell culture and viral infections

HeLa cells or primary dermal fibroblasts were cultured in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10 % (v/v) FBS (fetal bovine serum) and 100 μ g/ml penicillin and streptomycin. The culture medium for primary fibroblasts was supplemented with 2 mM L-glutamine. In various assays, HeLa cells were infected with recombinant adenoviral vectors at an MOI (multiplicity of infection) of 1000 particles per cell. For the NF- κ B-responsive luciferase reporter assay, cells were infected with Ad.NF- κ BLuc at an MOI of 500 particles per cell. Adenoviral infections were performed as previously described [25]. Essentially, cells were infected for 2 h in DMEM without FBS, after which an equal volume of 20 % (v/v) FBS/DMEM was added and incubation was continued for 22 h. At 24 h post-infection, virus-containing medium was replaced with 10 % (v/v) FBS/DMEM. Typically, experiments were initiated at 48 h post-infection [24–27].

Generation of Nox-deficient primary dermal fibroblasts

Nox2^{gp91^{phox}}-knockout mice [28] were obtained from Jackson Laboratories (strain name: B6.129S6-*Cybb*^{tm1Din/J}; stock number: 002365) and were inbred on the C57BL/6 background. Nox1-knockout mice were previously reported [29]. This line has an undefined mixed genetic background containing C57BL/6 and 129SvJ lineages. For the generation of primary dermal fibroblasts, 1-day-old pups were killed. Their skins were immediately removed and placed with dermal-side down in a sterile Petri dish and floated on 0.25 % trypsin/EDTA overnight at 4 °C. The epidermis was then peeled away and the dermis was incubated in 0.2 % collagenase in DMEM for 1 h at 37 °C and was then shaken to release the fibroblasts. The cell population was pelleted by centrifugation at 2324 g for 10 min at room temperature (21 °C) and plated in DMEM with 10 % (v/v) FBS, 1 % Fungizone, 2.5 units/ml amphotericin B and 2 mM L-glutamine. Calcium was raised to 6 mM to induce calcium-dependent differentiation and detachment of contaminating keratinocytes. Following the expansion of primary fibroblasts, a subset of cells from each isolate was used to generate genomic DNA for genotyping.

Cell H/R experiments

H/R injury to HeLa cells and primary fibroblasts was performed as previously described [5]. Briefly, the hypoxia and reoxygenation media were produced by equilibrating DMEM (without glucose or FBS) in 95 % N₂/5 % CO₂ or 95 % O₂/5 % CO₂ respectively. Cells were covered with minimal hypoxia medium and incubated at 37 °C for 6 h in an airtight chamber equilibrated with 5 % CO₂ and 95 % N₂. The medium was then replaced with a minimal amount of reoxygenation medium, and the cells were further incubated at 37 °C in a chamber perfused with 5 % CO₂ and 95 % O₂. Cells were then harvested at different times for various assays as specified. Control cells were fed with fresh medium on the same schedule as the H/R samples, but were exposed to 5 % CO₂ and an atmospheric level of oxygen.

Western blotting and *in vitro* kinase assays

Western blotting was performed using standard protocols [30]. Immunoreactive proteins were detected using either ECL[®] (Amersham Bioscience) and exposure to an X-ray film, or an Odyssey Infrared Imaging System (LI-COR Biotech). Antibodies used for Western blotting included: anti-phosphotyrosine (PY20) and anti-Rac1 (Transduction Laboratories); anti-SOD1 (superoxide dismutase 1) and anti-catalase (The Binding Site, San Diego, CA, U.S.A.); anti-p47^{phox}, anti-c-Src, anti-Rab5, anti-Nox1 and anti-GST (glutathione transferase) (Santa Cruz Biotechnology); and anti-c-Src and anti-pY416 (phospho-Tyr⁴¹⁶)-c-Src (Abcam). All primary antibodies were used at 1:2000 dilution and all secondary antibodies were used at 1:5000 dilution. A non-radioactive *in vitro* kinase assay was used to evaluate the ability of c-Src to tyrosine-phosphorylate GST-I κ B α following H/R. Cells were lysed in 1 ml of ice-cold RIPA buffer [25 mM Tris/HCl (pH 7.6), 150 mM NaCl, 1 % Nonidet P40, 1 % sodium deoxycholate and 0.1 % SDS] and this was followed by centrifugation at 9295 *g* for 10 min at 4 °C. Protein (500 μ g) was immunoprecipitated with anti-c-Src antibody and Protein A-agarose beads. GST-I κ B α protein (1 μ g; Santa Cruz Biotechnology) was then added to the precipitant pellets in the presence of 10 μ l of kinase buffer [40 mM Hepes, 1 mM 2-glycerophosphate, 1 mM PNPP (*p*-nitrophenyl phosphate), 1 mM sodium orthovanadate, 10 mM MgCl₂ and 2 mM dithiothreitol] and 0.3 mM ATP, after which the samples were incubated at 30 °C for 30 min. The reactions were terminated by heating at 98 °C for 2 min in the presence of SDS/PAGE buffer, followed by Western blotting of supernatants sequentially with anti-phosphotyrosine (PY20) and anti-GST (as a loading control) antibodies.

Generation of endomembranes and endosomes

Cell pellets (scraped into ice-cold PBS) were suspended in 0.5 ml of homogenization buffer (0.25 M sucrose, 10 mM triethanolamine, 1 mM PMSF and 100 μ g/ml aprotinin), homogenized in a Dullc tissue grinder and centrifuged at 3000 *g* at 4 °C for 10 min. The supernatant was designated the PNS (postnuclear supernatant). The PNS was subsequently centrifuged at 100 000 *g* for 2 h at 4 °C to pellet the endomembranes, which were then collected and used immediately for O₂^{•−} production assays or frozen for later analysis. In selected experiments, intact endosomes were isolated on iodixanol gradients as previously described [31] to assess endosomal loading with purified SOD1 and catalase proteins. For the endosomal loading experiments, purified bovine Cu/ZnSOD (SOD1) (Oxis Research) and catalase (Sigma–Aldrich) were added to fresh medium (1 mg of protein/ml for each) and applied to the cells at the time hypoxia or reoxygenation was initiated. PNS was generated and combined with 60 % (w/v) iodixanol (OptiPrep[™], Axis-Shield) solution to obtain a final concentration of 32 %, and loaded into an SW55Ti centrifuge tube. The PNS was then bottom loaded under two-step gradients of 24 and 20 % (w/v) iodixanol in homogenization buffer. Samples were centrifuged at 88 195 *g* for 2 h at 4 °C. Typically, 13 fractions were collected from the top to the bottom of the centrifuge tube at 4 °C (~ 300 μ l per fraction). The endosomal fractions were combined and used for various studies.

Subcellular localization of c-Src in HeLa cells following H/R

Immunofluorescent localization of c-Src and/or Rab5 was performed following 6 h of hypoxia and the indicated times of reoxygenation. Cells were washed with ice-cold PBS and then fixed for 10 min in 4 % (w/v) paraformaldehyde, after which they were exposed to 10 % (v/v) methanol for 10 min at −20 °C. The cells were then treated with 0.2 % Triton X-100 for 5 min at room temperature. Following blocking in 20 % (v/v) donkey serum/PBS for 20 min, the cells were incubated with anti-c-Src monoclonal and/or anti-Rab5 polyclonal antibodies (1:100 dilution; Santa Cruz Biotechnology) for 30 min at room temperature, and this was followed by incubation with a Texas Red-conjugated donkey anti-mouse antibody

(1:200 dilution; Jackson ImmunoResearch Laboratories) for c-Src single label studies or Alexa Fluor®488 donkey anti-mouse and Alexa Fluor®594 donkey anti-rabbit antibodies (1:200 dilution; Invitrogen) for co-localization studies. Cells were then mounted in DAPI (4',6-diamidino-2-phenylindole)-containing antifade (Citifluor) and examined by fluorescence microscopy.

NF- κ B activation and Nox activity assays

NF- κ B transcriptional activity was evaluated using an Ad.NF- κ BLuc reporter vector as previously described [26]. Cells were infected with Ad.NF- κ BLuc at an MOI of 500 particles per cell 24 h prior to H/R treatment. The luciferase activity (an indicator of NF- κ B transcriptional activity) was assessed in 5 μ g of total protein from each sample, following the manufacturer's protocols (Promega). Nox activity was assessed as the rate of NADPH-dependent $O_2^{\bullet-}$ production, by using a lucigenin-based chemiluminescent assay as previously described [31]. Prior to initiation of the assay, 1 μ g of endomembranes was combined with 5 μ M lucigenin (Sigma-Aldrich) in PBS, and incubated in darkness at room temperature for 10 min. The reaction was initiated by the addition of 100 μ M of NADPH (Sigma-Aldrich), and changes in luminescence were used to calculate the rate of $O_2^{\bullet-}$ formation as an index of Nox activity. To assess the specificity of Nox-dependent $O_2^{\bullet-}$ production, DPI (diphenyleneiodonium; 100 μ M) was added to the reaction 10 min prior to the addition of NADPH.

Statistical analysis

Statistical analyses were performed using ANOVA followed by the Dunnett's post-hoc test. Statistical significance was set at $P < 0.05$. Results are reported as the means $\pm$ S.E.M.

RESULTS

c-Src-mediated NF- κ B activation following H/R is accompanied by redistribution of c-Src to the endosomal compartment

Previous studies have demonstrated that the redox activation of c-Src following reperfusion injury induces NF- κ B activation by tyrosine phosphorylation of I κ B α [5,19]. Although H_2O_2 is known to facilitate activation of c-Src following reoxygenation injury, the source of H_2O_2 that facilitates this process remains largely unknown. To better understand this mechanism, we first sought to validate the involvement of c-Src in H/R-mediated NF- κ B activation using a HeLa cell model. Using an siRNA-mediated knockdown approach, a significant reduction (~ 50 %) of cellular c-Src expression was achieved (Figure 1A). As anticipated, c-Src knockdown reduced the ability of immunoprecipitated c-Src to tyrosine-phosphorylate GST-I κ B α following H/R treatment (Figure 1B), which was accompanied by a concordant reduction in transcriptional activation of NF- κ B (Figure 1C). These effects on I κ B α tyrosine phosphorylation and NF- κ B activation were specific for the c-Src-targeting siRNA and were not observed in scrambled siRNA-transfected controls. Collectively, these studies confirmed the importance of c-Src in I κ B α tyrosine-phosphorylation and NF- κ B activation following H/R in HeLa cell.

We next sought to investigate the mechanism of H/R-responsive c-Src activation in the HeLa cell model. c-Src, whose N-terminus contains myristoylation and palmitoylation sites, is principally a membrane-associated factor [20]. The intracellular localization of c-Src is also known to be finely controlled by environmental signals and its association with the actin cytoskeleton [32]. However, it remains unclear whether changes in c-Src subcellular localization influence its ability to phosphorylate I κ B α following H/R. To investigate this issue, we sought to determine whether the subcellular localization of c-Src was altered during either the hypoxic or reoxygenation stage of injury and whether any change in its

localization correlates with its activation. Using immunofluorescent localization, we found that c-Src was predominantly localized to the plasma membrane of HeLa cells grown under normoxic conditions (Figure 2A), and that this plasma-membrane localization predominated throughout 6 h of hypoxia, with a slight shift to punctate intracellular staining at later time points. Following reoxygenation, however, c-Src localization dramatically shifted to a vesicular pattern that was most evident at 30 min of reoxygenation (Figure 2A). This reoxygenation-induced endosomal relocation of c-Src was confirmed by immunocolocalization of c-Src with the early endosomal marker Rab5 (Figure 2B) and by biochemical isolation of endomembranes (Figure 2C). Whereas total c-Src levels remained unchanged throughout H/R injury, endomembrane-associated c-Src dramatically increased at 15–30 min of reoxygenation. In contrast, the endomembrane-associated c-Src fraction did not appear to change during the 6 h of hypoxia. An antibody that specifically recognizes the active, Tyr⁴¹⁶-phosphorylated, form of c-Src was used to demonstrate that recruitment of c-Src to the endomembrane fraction directly correlated with its activation (Figure 2C). These results suggested that c-Src recruitment to endosomes during the reoxygenation phase of injury is likely to be important for its activation.

Endocytosis is required for c-Src and Nox-activator (Rac1 and p47^{phox}) recruitment to the endosomal compartment following H/R

We next sought to better correlate the timing of c-Src recruitment to endomembranes following H/R with its activation (as evident by Tyr⁴¹⁶ phosphorylation and c-Src I κ B α tyrosine kinase activity). As shown in Figure 3(A), c-Src recruitment to the endomembrane fraction was maximal by 20 min post-reoxygenation. c-Src activation slightly lagged its recruitment to the endomembranes fraction, peaking by 40 min of reoxygenation. This lag in c-Src activation was even more pronounced in assays assessing c-Src-mediated tyrosine phosphorylation of I κ B α , which increased predominantly between 40 and 60 min of reoxygenation. These studies suggested the intriguing possibility that the activation of c-Src as an I κ B α tyrosine kinase occurs only after it has been recruited to the endomembrane compartment.

The production of ROS during the reoxygenation stage of H/R is known to be critical for activating the I κ B α tyrosine kinase function of c-Src [5,19]. Noxs are an important cellular source of ROS; however, their involvement in c-Src activation following H/R remains unclear. Recognizing that c-Src requires both recruitment to the endosome (Figures 2 and 3A) and the presence of H₂O₂ [5,19] for its activation as an I κ B α tyrosine kinase, we hypothesized that c-Src may be endocytosed from the plasma membrane along with known Nox activators (Rac1 and p47^{phox}) and that this may be required for its redox-dependent activation on endosomes. To test this possibility, we assessed whether inhibiting endocytosis would alter c-Src endosomal recruitment, c-Src activation and, ultimately, NF- κ B activation. Using an adenoviral expression vector carrying a dominant-negative (K44A) dynamin mutant that is known to inhibit clathrin- and caveolin-mediated endocytosis, we tested this hypothesis. As previously observed for other cell lines [31], infection of HeLa cells with Ad.DynK44A inhibited ~ 60 % of clathrin-mediated endocytosis when biotin-conjugated transferrin was used as a general endocytic marker (results not shown). As expected, infection with Ad.DynK44A, but not a control LacZ adenovirus, significantly inhibited c-Src recruitment to the endomembrane fraction at 30 min of reoxygenation (Figure 3B). Recruitment of Rac1 and p47^{phox} to the endomembrane fraction was also inhibited to a similar extent (Figure 3B). These results suggested that endocytosis during the reoxygenation phase of H/R injury is critical for the co-recruitment of c-Src and two known Nox activators to the endosomal compartment. We next sought to determine whether inhibiting endocytosis following H/R also affects events downstream of c-Src activation. As shown in Figure 3(C), the ability of immunoprecipitated c-Src to tyrosine-phosphorylate

GST-I κ B α following H/R was indeed reduced when Ad.DynK44A was used to inhibit endocytosis. Similarly, NF- κ B transcriptional activation was reduced ~ 50 % by dynamin-K44A expression (Figure 3D). These results demonstrate that endocytosis is required for the movement of c-Src to the endosome following H/R and that this event is required for the tyrosine phosphorylation of I κ B α and for the functional activation of NF- κ B. Together with the changes in the immunolocalization of c-Src following H/R (Figure 2A), these findings suggest that c-Src bound to the plasma membrane is recruited into the endosome during the reoxygenation stage of injury.

c-Src-mediated NF- κ B activation following H/R is dependent on NADPH-oxidase-derived ROS in the endosomal compartment

Expression of an H₂O₂-scavenging enzyme, GPx-1 or catalase, prior to H/R injury is known to significantly reduce both c-Src activation and NF- κ B transcriptional activation in HeLa cells [5], implicating H₂O₂ in the H/R-induced response. Given that Rac1 and p47^{phox} are recruited to endosomes with c-Src following H/R injury (Figure 3B), we hypothesized that NADPH-dependent ROS production by the endosomal compartment might be responsible for activating c-Src following H/R. To test this, we first assessed whether H/R injury led to an increase in the capacity of endosomes to generate NADPH-dependent O₂^{•-}. Indeed, we observed a 4-fold increase in endosomal NADPH-dependent O₂^{•-} production following H/R injury (Figure 4A). Furthermore, this enhanced level of endosomal O₂^{•-} production was significantly blocked (~ 91 %) by the addition of the general Nox inhibitor DPI (Figure 4A).

To assess the contribution of Noxs to the transcriptional activation of NF- κ B following H/R, we also performed H/R experiments in the presence of DPI. In these experiments, DPI did not affect baseline level NF- κ B activation in the absence of H/R injury (Figure 4A). Following H/R injury, however, DPI reduced NF- κ B activation by ~ 30 %. Using an alternative approach that neutralizes ROS from within endosomes, we performed similar experiments to specifically assess the contribution of endosomal O₂^{•-} and H₂O₂ to NF- κ B activation. This approach utilized endosomal loading of purified SOD1 and catalase enzymes (referred to as SOD1/catalase hereinafter) at the time of H/R injury. As shown in Figure 4(B), SOD1/catalase added to the HeLa cell medium was indeed endocytosed into the endosomal compartment. As expected, such an endosomal loading during the reoxygenation phase of injury significantly inhibited (by >50 %) both endosomal NADPH-dependent O₂^{•-} production and NF- κ B activation following H/R (Figure 4C). These findings demonstrated that NADPH-dependent ROS production within the interior of the endosomal compartment following H/R was critical for NF- κ B activation.

We next sought to examine how endosomal ROS production influenced c-Src activation following H/R injury. We hypothesized that endosomal ROS induced following H/R directly activates endosome-recruited c-Src to become an I κ B α tyrosine kinase. Indeed, endosomal loading with SOD1/catalase significantly inhibited the ability of immunoprecipitated c-Src to tyrosine-phosphorylate GST-I κ B α , from either whole cell lysates or endomembranes (Figure 4D). The addition of SOD1/catalase to the medium at the beginning of hypoxia or at the onset of reoxygenation inhibited I κ B α tyrosine kinase activity of c-Src to a similar extent (Figure 4D), suggesting that the functional effect of endosomal loading with ROS scavengers was the most critical in endosomes formed during the reoxygenation stage of injury. Cumulatively, these findings suggest that H/R-induced, NADPH-dependent ROS production by the endosomal compartment leads to c-Src activation, I κ B α tyrosine-phosphorylation and, ultimately, NF- κ B activation.

To test whether NADPH-dependent ROS also played a role in the translocation of c-Src to endomembranes following H/R injury, we inhibited H/R-induced ROS production by the endosomal compartment – either by overexpressing GPx-1 or adding DPI to the medium –

and then assessed the effect of this treatment on c-Src recruitment to and activation on endomembranes (Figure 4E). Neither GPx-1 expression nor DPI treatment reduced the recruitment of c-Src to endomembranes following H/R injury, suggesting that ROS have little effect on this process. In contrast, both GPx-1 and DPI significantly inhibited c-Src activation, as indicated by a significant decrease in the levels of endomembrane-associated pY416-c-Src. From these studies, we conclude that NADPH-dependent H_2O_2 production is required for c-Src activation on endosomes following H/R, but that it plays little role on c-Src recruitment. Consistent with this hypothesis, both GPx-1 expression and DPI treatment also significantly attenuated NF- κ B activation following H/R (Figure 4F).

Nox1 is primarily responsible for H/R-induced endosomal ROS and c-Src activation following H/R

Two closely related Noxs (Nox1 and Nox2) have been shown to be activated by Rac1 and p47^{phox} [14,33–35]. Given our finding that Rac1 and p47^{phox} are both recruited to redox-active endosomes following H/R injury, we hypothesized that Nox1 and/or Nox2 might play key roles in activating c-Src following H/R injury. To approach this question, we generated primary dermal fibroblasts from both Nox1- and Nox2-deficient mice, as well as from their wild-type littermates, and studied their capacity to induce endosomal ROS and to activate c-Src following H/R. Findings from these studies demonstrated that NADPH-dependent $\text{O}_2^{\bullet-}$ production by endomembranes following H/R was significantly blunted in Nox1-, but not Nox2-, deficient fibroblasts (Figures 5A and 5B). To assess what fraction of endosomal $\text{O}_2^{\bullet-}$ was produced by Nox, we next performed an analysis of $\text{O}_2^{\bullet-}$ production in the presence or absence of DPI. As shown in Figures 5(A) and 5(B), DPI-insensitive NADPH-dependent $\text{O}_2^{\bullet-}$ production rose approx. 2-fold in all fibroblasts (both wild-type and knockout) following H/R injury. In this context, a clearer delineation of the relative differences in Nox-dependent $\text{O}_2^{\bullet-}$ production can be seen when DPI-sensitive $\text{O}_2^{\bullet-}$ production is compared between the various Nox-deficient cell lines (Figure 5C). This analysis uncovered a 70 % reduction in H/R-induced, DPI-sensitive $\text{O}_2^{\bullet-}$ production by endomembranes in Nox1-deficient fibroblasts. Surprisingly, an assessment of DPI-sensitive $\text{O}_2^{\bullet-}$ production also demonstrated that a smaller but significant reduction also occurred in Nox2-deficient fibroblasts. The reason for this difference from the findings presented in Figure 5(A) was variation in the extent of change in the DPI-insensitive background between samples.

Overall, these findings suggest that both Nox1 and Nox2 influence endosomal ROS production following H/R, although Nox1 plays a more major role. As anticipated, the reduced ability of Nox1-deficient fibroblasts to stimulate endosomal ROS production following H/R resulted in a concordant decrease in the ability of c-Src to tyrosine-phosphorylate I κ B α (Figure 5D). This reduction in c-Src kinase activity was also seen to a lesser extent in Nox2-deficient fibroblasts (Figure 5D), consistent with the lower reduction in DPI-sensitive endosomal $\text{O}_2^{\bullet-}$ following H/R in these cells as compared with Nox1-deficient cells.

Data supporting a functional role for Nox1 in the redox activation of c-Src was also obtained in HeLa cells following transient transfection of a Nox1 expression plasmid. As shown in Figure 5(E), ectopic expression of mNox1 (mouse Nox1) led to a significant increase in baseline (normoxic) endosomal NADPH-dependent superoxide production and total cellular c-Src and NF- κ B activation. Overexpression of Nox1 also enhanced the H/R-mediated induction of these three endpoints, although to a lesser extent than baseline changes. These findings strengthen the evidence that Nox1 can directly activate c-Src and NF- κ B.

A reciprocal relationship between Rac1 and c-Src is required for the recruitment of both factors into the endosomal compartment following H/R

Our findings suggest that NADPH-dependent ROS production by the endosomal compartment is required for the activation of c-Src, the tyrosine phosphorylation of I κ B α and, ultimately, the NF- κ B activation that occurs in response to H/R. However, studies of other signalling systems have suggested that c-Src activates Nox through its ability to phosphorylate p47^{phox} [36]. Thus we sought to evaluate whether c-Src could also influence Nox activation following H/R. We approached this question by evaluating endomembrane production of O₂^{•-} following H/R, in cells that were transfected with a c-Src or scrambled siRNA (Figure 6A). The inhibition of c-Src protein levels significantly reduced (30 %) the ability of the endomembrane fraction to generate NADPH-dependent O₂^{•-} following H/R. Together with the results described above, these findings suggest that c-Src activation is both responsive to NADPH-dependent O₂^{•-} and capable of amplifying Nox-dependent ROS production in the endosomal compartment following H/R.

Previous studies have implicated c-Src in the activation of the small GTPase Rac1 during TGF (transforming growth factor) signalling and cell transformation [37,38]. Given that Rac1 has been shown to activate Nox1 and Nox2 [14,34,35] and that Rac1 and c-Src co-recruit to endomembranes following H/R (Figure 4B), we hypothesized that c-Src might influence ROS production by the endosomal compartment through its interaction with Rac1. However, if ROS production is required for c-Src activation, it remained unclear how c-Src could directly influence Rac1-dependent ROS production. We hypothesized that c-Src and Rac1 interactions might be required to facilitate the formation of redox-active endosomes following H/R, and hence could mutually affect the process of Nox activation. To address this question, we asked whether inhibition of either Rac1 or c-Src would alter the recruitment of both factors into the endosomal compartment following H/R. As shown in Figures 6(B) and 6(C), this was indeed the case. Rac1 siRNA, but not the scrambled siRNA, inhibited Rac1 protein levels by approx. 50 % and led to a concordant decrease in c-Src recruitment into the endomembrane fraction following H/R (Figure 6B). Similarly, c-Src siRNA, but not the scrambled control siRNA, reduced H/R-induced recruitment of Rac1 to the endomembrane fraction (Figure 6C). These findings demonstrate a reciprocal relationship between c-Src and Rac1 in terms of their recruitment to the endosomal compartment following H/R. Hence, c-Src siRNA-mediated reduction in endomembrane NADPH-dependent O₂^{•-} production following H/R (Figure 6A) is likely to be due to reduced levels of Rac1 (a co-activator of Nox1 and Nox2) in this compartment.

We next sought to investigate how Rac1 activation might influence c-Src endocytosis. To this end, we examined whether a constitutively active form of Rac1 (L61Rac1), which maintains Rac1 in the GTP bound state, might alter the distribution of c-Src in the endosomal compartment and the capacity of this compartment to produce NADPH-dependent O₂^{•-}. As shown in Figure 7(A), expression of L61Rac1 using recombinant adenovirus (but not the empty vector control Ad.BglIII) led to enhanced NADPH-dependent O₂^{•-} production by endomembranes in the absence and presence of H/R. This L61Rac1-induced increase in endosomal ROS correlated with increases in total and activated (pY416) c-Src in the endomembrane fraction as well as cellular NF- κ B activation (Figure 7A). Additionally, the extent of HA (haemagglutinin)-tagged L61Rac1 recruitment to the endosomal fraction correlated with the extent of c-Src recruitment, suggesting that the recruitment of these two factors is functionally linked. These findings indicate that constitutive activation of Rac1 can mimic the H/R response, leading to endosomal recruitment of c-Src and activation of Nox in the endosomal compartment.

To confirm that Rac1 activation was solely required for the redistribution of c-Src to endosomes in the absence of H/R, we localized c-Src in the presence and absence of

L61Rac1. As shown in Figure 7(B), expression of L61Rac1 led to the redistribution of c-Src from the plasma membrane to endosomes in the absence of H/R, in a pattern that mirrored c-Src localization following H/R in the absence of L61Rac1. Furthermore, treatment of cells with DPI to inhibit vesicular ROS production (Figure 4A) did not alter the redistribution of c-Src following H/R (Figure 7B), confirming earlier results demonstrating that c-Src recruitment to the endomembrane fraction following H/R is not dependent on ROS (Figure 4E). Cumulatively, the results of this set of experiments demonstrate that Rac1 plays a key role in recruiting c-Src to endosomes following H/R, as well as in the induction of Nox-dependent endosomal ROS that are required for c-Src activation in this compartment.

DISCUSSION

Noxs have been implicated in a number of redox-dependent signalling pathways, where they can act by inhibiting PTPs (protein tyrosine phosphatases) [2,14]. Notably, the temporospatial activation of Noxs following ligand/receptor stimulation appears to be a key event that facilitates specificity of redox signals [16]. For example, localized inactivation of the antioxidant enzyme peroxiredoxin II by H_2O_2 has been shown to regulate PDGF signalling by controlling the redox-dependent inactivation of PTPs that are required for PDGF receptor inactivation [39]. Additionally, endosomal activation of Nox2 has been demonstrated to be important for the localized activation of IL-1R (IL-1 receptor) signalling by H_2O_2 [31]. Despite these defined examples of redox-dependent receptor control, it remains unclear how other ligand-independent, redox-regulated signalling pathways such as those stimulated by H/R are regulated by Noxs.

H/R stimulates the production of cellular ROS during the reoxygenation phases of injury. This burst of ROS formation has been shown to be dependent on Rac1 [40], implicating Noxs in the process. Inhibitors of Noxs have also been shown to suppress ROS production following H/R [41]. ROS production following reperfusion injury has been shown to be important in the activation of NF- κ B [6], and activation of this pathway can determine cell fate responses to injury [19]. NF- κ B activation following H/R and I/R is largely dependent on the redox-dependent activation of c-Src and the subsequent tyrosine phosphorylation of I κ B α . However, the mechanisms of c-Src activation during the reperfusion phases of injury remain largely unknown. The present study has begun to piece together the manner in which c-Src and Rac1 co-operatively regulate Nox during reoxygenation injury to facilitate the redox-dependent tyrosine phosphorylation of I κ B α .

Our study is the first to demonstrate that the induction of Nox1- and Nox2-derived endosomal ROS following H/R injury is a key event in c-Src-dependent NF- κ B activation (Figure 8). Several observations suggest that this process requires the endocytosis of certain plasma-membrane components. First, inhibiting dynamin-dependent endocytosis significantly reduced H/R-induced movement of c-Src, Rac1 and p47^{phox} into the endosomal compartment, as well as activation of NF- κ B. Secondly, during the reoxygenation phases of injury, c-Src moved from the plasma membrane to the endosomal compartment. Together, these observations suggest that redox-active endosomes form in response to H/R, by the co-endocytosis of c-Src with components of the Nox complex. Alternatively, endocytosis of c-Src may be required for the subsequent recruitment of components of the Nox complex shortly after endocytosis. Using siRNA to inhibit either Rac1 or c-Src, we have shown that these two factors are mutually dependent on one another for recruitment into the endosomal compartment following H/R. Inhibition of Rac1 has previously been shown to inhibit ROS production following H/R [40]. Interestingly, even in the absence of H/R, the expression of a constitutively active form of Rac1 (L61) was capable of enhancing endosomal ROS, c-Src recruitment to endosomes, and c-Src activation

within endosomes (Figure 7). Hence, the initiating event that leads to enhanced endosomal ROS following H/R injury may primarily depend on Rac1 activation.

Our findings suggest that Nox1/2 and its co-activator Rac1 play a major role in the redox-mediated activation of c-Src following H/R injury (Figure 8). The redox activation of c-Src involves its phosphorylation on Tyr⁴¹⁶, and it is this form of c-Src that is responsible for tyrosine phosphorylation of I κ B α and subsequent NF- κ B activation following H/R. The mechanism whereby Nox1/2-derived ROS leads to activation of c-Src in the endosomal compartment following H/R likely involves H₂O₂-mediated inhibition of PTPs at the endosomal surface. General PTP inhibitors such as pervanadate are well known to activate c-Src as an I κ B α tyrosine kinase, and to lead to transcriptional activation of NF- κ B [5]. Hence, it is reasonable to hypothesize that Nox-1/2-derived H₂O₂ facilitates c-Src activation by inhibiting a PTP that is required to maintain c-Src in an inactive state.

The characterization of endosomal pathways required for the redox activation of c-Src and NF- κ B following reperfusion injury has important potential therapeutic implications. Of note, our studies suggest that targeted redox modulation of the intra-endosomal space may inhibit this pathway very effectively. c-Src-mediated induction of NF- κ B following liver I/R injury has been shown to be detrimental to hepatocyte survival, because it promotes TNF α -mediated inflammation [19]. As in the case of H/R injury, hepatic activation of NF- κ B following I/R injury is largely dependent on c-Src-mediated tyrosine phosphorylation of I κ B α . Hence, inhibition of Nox1/2-derived endosomal ROS following I/R may aid in preventing deleterious NF- κ B activation that promotes downstream inflammatory destruction of tissue.

Acknowledgments

This work was supported by NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases; part of the National Institutes of Health) (DK067928 and DK51315; to J. F. E.) and core facilities from the Center for Gene Therapy (P30 DK54759). We also gratefully acknowledge Dr Christine Blaumueller for editorial assistance.

Abbreviations used

DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DPI	diphenyleneiodonium
FBS	fetal bovine serum
GPx-1	glutathione peroxidase-1
GST	glutathione transferase
HA	haemagglutinin
H/R	hypoxia/reoxygenation
IκB	inhibitory κ B
IL	interleukin
I/R	ischaemia/reperfusion
LPS	lipopolysaccharide
MOI	multiplicity of infection
Nox	NADPH oxidase

NF-κB	nuclear factor κ B
PDGF	platelet-derived growth factor
PNS	postnuclear supernatant
PTP	protein tyrosine phosphatase
pY416	phospho-Tyr ⁴¹⁶
ROS	reactive oxygen species
siRNA	small interfering RNA
SOD	superoxide dismutase
TNFα	tumour necrosis factor α

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Figure 1. c-Src is required for IκBα tyrosine-phosphorylation and NF-κB activation following H/R

(A) HeLa cells were transfected with a c-Src or scrambled control (Scrm) siRNA 48 h prior to examination of cellular c-Src expression by Western blotting (cellular actin is used as a loading control). (B) HeLa cells were transfected with a c-Src or scrambled siRNA 48 h prior to H/R injury by 6 h of hypoxia and 30 min of reoxygenation. c-Src was immunoprecipitated from 500 μg of lysate of normoxic or H/R-treated cells. The ability of immunoprecipitated c-Src to tyrosine-phosphorylate GST-IκBα directly was then evaluated using a non-radioactive *in vitro* kinase assay. Tyrosine phosphorylation of IκBα was evaluated by Western blotting with anti-phosphotyrosine (PY20) antibody, and anti-GST antibody was used as a loading control. (C) HeLa cells were transfected with the c-Src or scrambled siRNA 48 h prior to H/R injury, and infected with Ad.NF-κBLuc 24 h prior to H/R injury. Luciferase activities were assessed following 6 h of hypoxia and 6 h of reoxygenation (mean ± S.E.M., *n* = 3). * indicates a significant difference using ANOVA followed by the Dunnett's post-hoc test (*P* < 0.01). IP, immunoprecipitation.

Figure 2. c-Src redistributes to the endosomal compartment following H/R injury

(A) HeLa cells were fixed for immunolocalization of c-Src following the indicated periods of hypoxia (H) and reoxygenation (R). They were then mounted with DAPI-containing antifadent and examined by fluorescence microscopy (magnification $\times 40$). Red fluorescence indicates the presence of c-Src, and blue fluorescence (DAPI) marks nuclei. Boxed regions labelled a' and b' are enlarged in the bottom panels. (B) Immunoco-localization of c-Src and Rab5 in normoxic or H/R-treated HeLa cells. Cells were mounted with DAPI-containing antifadent, and examined by fluorescent microscopy. FITC fluorescence (green) indicates the presence of c-Src, Texas Red fluorescence (red) indicates the presence of Rab5, and DAPI fluorescence (blue) demarcates nuclei. Lower panels are enlargements of the boxed regions in the top panels. (C) Cells from the H/R procedures shown in (A) were used to generate total endomembranes, and the endomembrane pellets were evaluated by Western blotting with anti-c-Src, anti-pY416-c-Src antibodies (indicative of c-Src activation) and anti-Rab5 (early endosomal marker). Whole cell lysates from these samples were also evaluated by Western blotting against anti-c-Src to rule out any H/R-induced changes in total c-Src. Bottom panel: quantification of endomembrane c-Src/Rab5 and pY416-c-Src/Rab5 protein intensity ratios. The data points in the graph line up with various H/R conditions shown in the top Western blot panels. The '*' and '†' symbols indicate significant differences ($P < 0.01$) as determined by ANOVA followed by the Dunnett's post-hoc test. Each set of symbols shows the conditions being compared.

Figure 3. Dynamin-dependent endocytosis is required for the redistribution of c-Src following H/R injury

(A) HeLa cells were exposed to 6 h of hypoxia and the indicated periods of reoxygenation (Reoxy) prior to cell harvesting. A 1 μ g portion of each endomembrane sample (100k pellet) was used to evaluate both total c-Src recruitment to endomembranes and c-Src activation (using an anti-pY416-c-Src antibody). In addition, c-Src was immunoprecipitated from 500 μ g of total cell lysate, and its ability to tyrosine-phosphorylate GST-I κ B α was then evaluated using a non-radioactive *in vitro* kinase assay. Tyrosine phosphorylation of I κ B α (pYI κ B α) was evaluated by Western blotting with an anti-phosphotyrosine (PY20) antibody. (B) HeLa cells were infected with Ad.DynK44A (Dydn, a dominant-negative mutant of dynamin) or Ad.LacZ (viral infection control) 48 h prior to H/R injury (by 6 h hypoxia and 30 min reoxygenation). Endomembrane recruitment of the indicated proteins

was examined by Western blotting. (C) HeLa cells were infected with Ad.DynK44A or Ad.LacZ 48 h prior to H/R injury (6 h hypoxia and 30 min reoxygenation). c-Src was immunoprecipitated and its ability to tyrosine-phosphorylate GST-I κ B α directly was evaluated using a non-radioactive *in vitro* kinase assay followed by Western blotting with anti-phosphotyrosine (PY20) antibody. Anti-GST antibody was used as a loading control. (D) HeLa cells were infected with Ad.DynK44A or Ad.BglIII 48 h prior to H/R injury, and with Ad.NF- κ BLuc 24 h prior to H/R injury. Luciferase activities were assessed following 6 h of hypoxia and 6 h of reoxygenation (mean $\pm$ S.E.M., $n = 3$). The '*' symbol indicates a statistically significant difference using ANOVA followed by the Dunnett's post-hoc test ($P < 0.01$). IP, immunoprecipitation.

Figure 4. ROS are required for the activation of both c-Src and NF- κ B following H/R injury (A) HeLa cells were exposed to 6 h of hypoxia and reoxygenation for 30 min prior to isolating endosomes on iodixanol gradients. The peak endosomal fraction was incubated in the presence and absence of 100 μ M DPI for 20 min prior to measuring $O_2^{\bullet -}$ production using the standard lucigenin assay. In separate experiments, cells were also infected with Ad.NF- κ BLuc 24 h prior to 6 h of hypoxia and 6 h of reoxygenation in the presence and absence of 100 μ M DPI for the entire H/R period. Luciferase activities were assessed at 6 h of reoxygenation (mean $\pm$ S.E.M., $n = 3$). (B) HeLa cells were treated with purified SOD1/catalase (SOD + Cat) proteins (1 mg/ml each) and biotin-conjugated transferrin (10 μ g/ml) throughout the hypoxia (6 h) and reoxygenation (30 min) treatment. Cells were then washed to remove excess SOD1/catalase and transferrin prior to cell harvesting and preparation of PNS. Endosomes were isolated from the PNS on an iodixanol gradient and the endosomal fractions were Western blotted for the indicated proteins. (C) HeLa cells were treated with purified SOD1 and catalase proteins in the culture medium during H/R as described in (B). Endosomes were isolated from the PNS by iodixanol fractionation following 6 h hypoxia/30 min reoxygenation and used to assess NADPH-dependent superoxide production using a lucigenin-based chemiluminescent assay. In a second set of experiments, cells were infected with Ad.NF- κ BLuc and luciferase activities were assessed following 6 h of hypoxia and 6 h of reoxygenation (mean $\pm$ S.E.M., $n = 3$). (D) Purified SOD1/catalase were added to the culture medium at the start of hypoxia (6 h) or reoxygenation (30 min) as indicated. c-Src was then immunoprecipitated from total cell lysates or endomembranes and its ability to tyrosine-phosphorylate GST-I κ B α directly was evaluated using a non-radioactive *in vitro* kinase assay followed by Western blotting with anti-phosphotyrosine (PY20) antibody (anti-GST antibody served as a loading control). (E) HeLa cells were infected with Ad.GPx-1 or

Ad.BglII virus 48 h prior to 6 h of hypoxia and 30 min of reoxygenation. Alternatively, cells were treated with 100 μ M DPI at 30 min prior to initiating the same regimen of H/R injury (with DPI remaining in the medium during the entire H/R process). Endomembranes were isolated and evaluated by Western blotting with anti-c-Src and anti-pY416-c-Src antibodies. (F) HeLa cells were infected with Ad.NF- κ BLuc virus 24 h prior to H/R injury. Cells were also infected with the indicated viruses or treated with DPI under conditions identical with those described in (E). Luciferase activities were assessed following 6 h of hypoxia and 6 h of reoxygenation (mean $\pm$ S.E.M., $n = 3$). The '*' and '†' symbols indicate significant differences using ANOVA followed by the Dunnett's post-hoc test ($P < 0.01$). IP, immunoprecipitation.

Figure 5. Nox-dependent $O_2^{\bullet-}$ production is critical for c-Src activation following H/R (A, B) Nox2-knockout (KO; -/-) or Nox1-KO primary dermal fibroblasts, along with respective wild-type (WT; +/+) control fibroblasts generated from littermates, were exposed to 6 h of hypoxia and 30 min of reoxygenation. NADPH-dependent $O_2^{\bullet-}$ production in endomembranes in the presence or absence of the NADPH-oxidase inhibitor DPI (100 μ M) was then evaluated using a lucigenin-based chemiluminescent assay (mean $\pm$ S.E.M., $n = 3$). (C) DPI-sensitive $O_2^{\bullet-}$ production ($O_2^{\bullet-}$ production in the absence of DPI, minus $O_2^{\bullet-}$ production in the presence of DPI) was calculated for each experimental point from both Nox2-KO and Nox1-KO dermal fibroblast experiments shown in (A) and (B). (D) Nox2-KO and Nox1-KO dermal fibroblasts and their respective wild-type controls were exposed to 6 h of hypoxia and to reoxygenation for 30 min. c-Src was then immunoprecipitated from total cell lysates and evaluated for its ability to tyrosine-phosphorylate GST-I κ B α directly in a non-radioactive *in vitro* kinase assay. This was followed by Western blotting with anti-phosphotyrosine (PY20) and anti-c-Src antibodies. (E) HeLa cells were transfected with Nox1 expression plasmid, or control pcDNA plasmid, 48 h prior to H/R injury. For the NF- κ B activity assay, cells were also infected with Ad.NF- κ B virus 24 h prior to H/R injury. Luciferase activities were assessed following 6 h of hypoxia and 6 h of reoxygenation (mean $\pm$ S.E.M., $n = 3$). For $O_2^{\bullet-}$ generation and c-Src kinase assays, transfected cells were exposed to 6 h of hypoxia and 30 min of reoxygenation. NADPH-dependent $O_2^{\bullet-}$ production by endomembranes was evaluated using a lucigenin-based chemiluminescent assay (mean $\pm$ S.E.M., $n = 3$). Cell lysates were evaluated for the levels of pY416-c-Src and Nox1 proteins by Western blotting in the lower panels. The '#', '*' and '†' symbols indicate statistically significant differences using ANOVA followed by the Dunnett's post-hoc test ($P < 0.01$ or as indicated).

Figure 6. Rac1 influences c-Src redistribution and activation following H/R injury

(A) HeLa cells were transfected with c-Src or scrambled siRNA 48 h prior to H/R injury (6 h hypoxia and 30 min reoxygenation). Endomembranes were evaluated for NADPH-dependent $O_2^{\bullet -}$ production using a lucigenin-based chemiluminescence assay (mean $\pm$ S.E.M., $n = 3$). (B) HeLa cells were transfected with Rac1 or scrambled siRNA 48 h prior to H/R injury as described above. The abundance of c-Src and Rac1 was examined by Western blotting of whole cell lysates. c-Src redistribution and activation in the endomembrane fractions was also examined by Western blotting with anti-c-Src and anti-pY416-c-Src antibodies. (C) HeLa cells were transfected with c-Src or scrambled siRNA 48 h prior to H/R (as above). Rac1 redistribution to the endomembrane fraction was examined by Western blotting with anti-Rac1, and anti-Rab5 serving as a control. The relative intensities of Rac1 (normalized to Rab5) are plotted in the histogram (mean $\pm$ S.E.M., $n = 3$). The '*' symbol indicates a significant difference using ANOVA followed by the Dunnett's post-hoc test ($P < 0.01$).

Figure 7. L61Rac1 induces c-Src activation and redistribution following H/R

(A) HeLa cells were infected with Ad.L61Rac1 (dominant-positive Rac1 mutant) or Ad.BglII virus 48 h prior to H/R injury (6 h hypoxia and 30 min reoxygenation). Endomembranes were evaluated for NADPH-dependent O_2^- production using a lucigenin-based chemiluminescence assay and for NF- κ B activities using Ad.NF- κ BLuc virus and luciferase assay (mean $\pm$ S.E.M., $n = 3$). The '*' symbol indicates a significant difference using ANOVA followed by the Dunnett's post-hoc test ($P < 0.05$). The presence of HA-Rac1, total c-Src and pY416-c-Src in the endomembrane fraction and whole cell lysates was examined by Western blotting. (B) HeLa cells were infected with Ad.L61Rac1 virus 48 h prior to H/R injury. Additional cells were treated with 100 μ M DPI at 30 min prior to H/R injury. In both cases, H/R treatment was as described above. Cells were fixed for immunolocalization of c-Src following H/R (red). Cells were mounted with DAPI-containing antifadent and examined by fluorescence microscopy using an oil-immersion lens (magnification $\times 63$).

Figure 8. Summary of Nox-dependent redox regulation of c-Src following H/R injury

In resting cells, c-Src and Nox are plasma-membrane-associated factors. H/R injury triggers dynamin-dependent endocytosis that redistributes c-Src, Rac1 and Nox (e.g. Nox1) to the endosomes. Nox generates $O_2^{\bullet -}$ and H_2O_2 locally within the endosome. This local increase in H_2O_2 levels facilitates c-Src Tyr416 phosphorylation, potentially by inhibiting PTP. Redox-activated c-Src in turn tyrosine-phosphorylates $I\kappa B\alpha$, leading to its dissociation from $NF-\kappa B$. This allows for nuclear translocation of $NF-\kappa B$ and thereby leads to the transcriptional activation of downstream genes.