Oxidative stress generated during ischemia/reperfusion injury has been shown to augment cellular responsiveness. Whereas oxidants are themselves known to induce several intracellular signaling cascades, their effect on signaling pathways initiated by other inflammatory stimuli remains poorly elucidated. Previous work has suggested that oxidants are able to prime alveolar macrophages for increased NF-κB translocation in response to treatment with lipopolysaccharide (LPS). Because oxidants are known to stimulate the Src family of tyrosine kinases, we hypothesized that the oxidants might contribute to augmented NF-κB translocation by LPS via the involvement of Src family kinases. To model macrophage priming in vitro, the murine macrophage cell line, RAW 264.7, was first incubated with various oxidants and then exposed to low dose LPS. These studies show that oxidant stress is able to augment macrophage responsiveness to LPS as evidenced by earlier and increased NF-κB translocation. Inhibition of the Src family kinases by either pharmacological inhibition using PP2 or through a molecular approach by cell transfection with Csk was found to prevent the augmented LPS-induced NF-κB translocation caused by oxidants. Interestingly, while Src kinase inhibition was able to prevent the LPS-induced NF-κB translocation in oxidant-treated macrophages, this strategy had no effect on NF-κB translocation caused by LPS in the absence of oxidants. These findings suggested that oxidative stress might divert LPS signaling along an alternative signaling pathway. Further studies demonstrated that the Src-dependent pathway induced by oxidant pretreatment involved the activation of phosphatidylinositol 3-kinase. Involvement of this pathway appeared to be independent of traditional LPS signaling. Together, these studies provide a novel potential mechanism whereby oxidants might prime alveolar macrophages for altered responsiveness to subsequent inflammatory stimuli and suggest different cellular targets for immunomodulation following ischemia/reperfusion.

Civilian trauma remains a leading cause of disability and mortality in North American society (1). Late deaths are related to the development of multiple organ failure in up to two-thirds of these patients. Many studies have suggested that the global ischemia-reperfusion resulting from resuscitated hemorrhagic shock predisposes to organ injury by priming for an exaggerated immune response to a delayed inflammatory stimulus (2). The synergistic effect of sequential stimuli has been coined the “two-hit” hypothesis for the development of organ injury in trauma patients (2). We have previously reported an animal model wherein antecedent resuscitated shock was shown to prime for increased LPS1-induced lung injury by augmenting alveolar macrophage NF-κB nuclear translocation and gene transcription of the chemokine, cytokine-induced neutrophil chemotactic factor (CINC), the rodent orthologue of interleukin-8. The exaggerated generation of CINC by alveolar macrophages in this model was primarily responsible for increased lung neutrophilia and consequent lung injury in animals exposed to the combined stimuli of resuscitated shock followed by LPS (3).

Ischemia/reperfusion produces oxidative stress through the irreversible conversion of xanthine dehydrogenase to xanthine oxidase (XO) during ischemia with consequent production of oxygen-free radicals and H2O2 during reperfusion (4, 5). Several lines of evidence suggest a role for oxidative stress as contributing to activation of hematopoietic cells. In the in vitro setting, oxidants have been shown to induce activation of multiple signaling pathways in hematopoietic cells. The best studied of these involves signaling cascades culminating in NF-κB translocation and induction of transcription of NF-κB-dependent genes. Early reports documented the ability of oxidants to induce NF-κB translocation and also defined the role of oxidant stress in NF-κB translocation by various inflammatory stimuli including LPS and tumor necrosis factor. In vivo studies have generally supported a role for oxidative stress in the induction of NF-κB translocation following ischemia/reperfusion. Schwartz et al. (6) reported that high expression of NADPH oxidase generation or activity using a tungsten-enriched diet or allopurinol, respectively, prevented induction of NF-κB-dependent gene expression such as tumor necrosis factor in a murine hemorrhagic shock model. The potential priming role of oxidant stress generated by ischemia/reperfusion is well demonstrated in our previous reports. In these studies, neither shock/resuscitation nor low dose LPS was able to induce significant

1 The abbreviations used are: LPS, lipopolysaccharide; CINC, cytokine-induced neutrophil chemotactic factor; XO, xanthine oxidase; PI, phosphatidylinositol; NF-κB, nuclear factor-κB; IF, immunofluorescence; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; IF, immunofluorescence; PP2, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine.
NF-κB translocation in alveolar macrophages, whereas the combined presence of these two stimuli culminated in a robust response, far greater than the additive effect of the separate responses. Importantly, supplementation of resuscitation fluid with the antioxidant N-acetylcysteine prevented the synergistic rise in NF-κB translocation (3). These observations are consistent with a role for oxidative stress in the priming of macrophages for increased responsiveness to a delayed second inflammatory stimulus. However, whereas oxidative stress is known to activate a number of signaling pathways in inflammatory cells, the alterations in cell signaling leading to enhanced responsiveness following exposure to oxidants have not been elucidated.

The Src family of tyrosine kinases is involved in many signal transduction pathways. The Src tyrosine kinases have been shown to be activated by oxidant stress such as UV irradiation as well as H$_2$O$_2$ (7, 8). Relevant to the activation of NF-κB and induction of NF-κB-dependent proinflammatory genes, Src has also been shown to phosphorylate inhibitory κB (IκB) on tyrosine residues, an event that activates NF-κB without inducing IκB degradation itself (9). In the present studies, we tested the hypothesis that oxidant stress might contribute to augmented NF-κB translocation in response to LPS through involvement of members of the Src family of kinases. To examine this hypothesis, an in vitro culture system was established wherein macrophages were sequentially exposed to an oxidative stress followed by LPS as a means of modeling the in vitro “two-hit hypothesis” of cellular activation and organ injury following shock/resuscitation. The major findings in the present studies are that Src family kinases are not only involved in macrophage priming by oxidants, but also that antecedent oxidant stress reprograms LPS-NF-κB signaling such that it changes from a Src-independent pathway to one that is Src-dependent.

**EXPERIMENTAL PROCEDURES**

**Buffers and Reagents—**Hydrogen peroxide (30%) (H$_2$O$_2$) was purchased from University of Toronto Medstore (Toronto, ON). Antibodies against p-Lyn, c-Fgr, p-Hck, and IκBα were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The polyclonal anti-IκBα, anti-phospho-IκBα-specific monoclonal antibody (G410), Pl 3-kinase antibody (clone UB93-3), and an in vitro tyrosine kinase assay kit were purchased from Upstate Biomedical Institute (Lake Placid, NY). An inhibitor of the Src family of protein tyrosine kinases, IκBα, was purchased from Calbiochem, Inc. (San Diego, CA). The PI substrate was purchased from Avanti Polar Lips, Inc. (Alabaster, AL). The Protein A-Sepharose beads, horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody, and ECL was purchased from Amersham Biosciences. The 50 mM dithiothreitol buffer was purchased from Amersham Biosciences, which is complementary to nucleotides 134–164 of CINC cDNA (kindly provided by Dr. Timothy S. Blackwell, Vanderbilt University School of Medicine, Nashville, TN) (12). Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens. Blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase, which is a ubiquitously expressed housekeeping gene to control for loading. Expression of mRNA was quantitated using a PhosphorImager and accompanying ImageQuant software (Molecular dynamics), and was normalized to the glyceraldehyde-3-phosphate dehydrogenase signal.

**Immunoprecipitation—**After treatments, cells were lysed for 5 min on ice with buffer containing 100 mM NaCl, 30 mM Hepes, 20 mM NaF, 1 mM EDTA, and 1% Triton X-100 to which protease mixture inhibitor had been added. Cell lysates were then centrifuged at 13,000 × g for 10 min, and the supernatants were then collected and precleared with protein A-Sepharose beads. Equal amounts of cellular proteins were immunoprecipitated with 2 μg of rabbit polyclonal anti-IκBα, anti-Hck, anti-Fgr, or anti-Lyn antibody for 1 h rotating at 4°C. Subsequently, 30 μg of protein A-Sepharose beads were added to the samples and incubated at 4°C for 1 h. The immunoprecipitates were then washed three times with the same lysis buffer and then resuspended in 25 μl of Laemmli buffer.

**Western Blot Analysis—**Cells were pelleted and lysed in ice-cold cell lysis buffer. Whole cell lysates were prepared with 2× Laemmli, 0.1 M dithiothreitol buffer followed by immediate boiling at 100°C for 5 min. Cytosolic fractions were isolated with 1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 2 mM sodium orthovanadate, 10 μg/ml leupeptin, 50 mM NaF, 5 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride. Postnuclear supernatants were collected following centrifugation at 13,000 × g for 5 min and diluted with 2× Laemmli buffer, 0.1 M dithiothreitol followed by immediate boiling for 5 min. Lysates prepared from 100,000 cells were separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were then probed with 1:1000 dilution of anti-phospho-IκBα-specific monoclonal antibody (G410), anti-IκBα antibody, or anti-phosphospecific antibody for 1 h at room temperature. Following incubation with the horseradish peroxidase-conjugated donkey IgG secondary antibody at 1:3000 dilution for 1 h at room temperature, blots were developed using an ECL-based system.

**Tyrosine Kinase Assay—**Immunoprecipitation of Hck was performed as previously described. The tyrosine kinase assay kit (non-radioactive) was used. Biotinylated substrate peptide containing poly(GLu Tyr), which is complementary to nucleotides 134–164 of CINC cDNA, was incubated with the tyrosine kinase, in the presence of non-radioactive PI substrate peptide containing poly(Glu4-Tyr) was added to the kinase reaction, and the reaction was then placed in a microplate coated with streptavidin. Horseradish peroxidase-conjugated monoclonal anti-phosphotyrosine antibody was used to detect the phosphorylated substrate. Chemiluminescence was used for colorimetric detection with a spectrophotometric plate reader set at wavelength of 450 nm. Reference wells were used to determine the background of peptide/lysis buffer.

**Pl 3-Kinase Activity Assay—**After treatments, cells were lysed with 600 μl of ice-cold lysis buffer containing 100 mM NaCl, 30 mM Hepes, 20 mM NaF, 1 mM EDTA, and 1% Triton X-100 to which protease mixture inhibitor had been added. Samples were left on ice for 10 min then cellular debris was removed by centrifugation at 13,000 × g for 10 min at 4°C. Supernatant was then plated using green fluorescent protein (GFP) pFR-hrGFP plasmid (Stratagene) and Csk plasmid. The Csk plasmid was a kind gift from Dr. I. Mucsi (Semmelweis University, Budapest, Hungary). The full-length coding sequence of murine Csk was subcloned into pcDNA3 vector using HindIII and XbaI restriction enzymes. Transient transfection with the corresponding plasmids was performed using Superfect reagent (Qiagen Ltd., West Sussex, UK) according to manufacturer’s instructions. Routinely, cells were transfected with 4 μg of plasmid DNA per well (for 6-well plates). The DNA was diluted in OptiMEM medium (Invitrogen). The ratio of total plasmid DNA to Superfect reagent was 4 μg to 20 μl, respectively. After 3 h of incubation the medium containing the remaining complexes was removed and replaced with fresh growth medium with 10% fetal calf serum and PS.

**Stable Transfection of Cell Line—**Stable transfection of RAW 264.7 cell lines expressing Csk were kindly provided by Dr. Z. Honda (University of Tokyo, Japan) (10). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, PS, and neomycin antibiotic G418 (Invitrogen).

**Northern Blot Analysis—**Total RNA was extracted from cells using the guanidine-isothiocyanate method (11). Hybridization was conducted using a [γ-32P]dATP end-labeled 30-base oligonucleotide probe for CINC with the sequence 5’-GGCGCATACCTTTAAAATCTCGATGTTCT-3’, which is complementary to nucleotides 134–164 of CINC cDNA (kindly provided by Dr. Timothy S. Blackwell, Vanderbilt University School of Medicine, Nashville, TN) (12). Blots were then washed under conditions of high stringency, and specific mRNA bands were detected by autoradiography in the presence of intensifying screens. Blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase, which is a ubiquitously expressed housekeeping gene to control for loading. Expression of mRNA was quantitated using a PhosphorImager and accompanying ImageQuant software (Molecular dynamics), and was normalized to the glyceraldehyde-3-phosphate dehydrogenase signal.
performed as previously described (13). Briefly the immunoprecipitates were resuspended in 30 μl of the same buffer to which 1× protein mixture inhibitor has been added in a reaction mixture containing the 30 μl of beads in 70 μl ATP, 20 μCi of [γ-32P]ATP, and 5 μg of t-α-phosphatidylinositol, mixed gently, and incubated for 20 min at 37 °C. The reaction was stopped with 20 μl of 6 n HCl and the organic layer was extracted with 160 μl of CHCl3/CH3OH (1:1) and separated on a silica gel thin layer chromatography plate in solvent containing CHCl3/CH3OH/H2O/NH4OH (60:47:11.3:2). Radiolabeled phosphatidylinositol phosphates were visualized by autoradiography on X-Omat film.

Nuclear Protein Extraction—Nuclear protein extracts were prepared from the cells by the method of Deryckere and Gannon (14). The cells were resuspended in 1 ml of solution A (150 mmol/liter NaCl, 10 mmol/liter Hepes, pH 7.9, 1 mmol/liter EDTA, and 0.5 mmol/liter phenylmethylsulfonyl fluoride). The cells were centrifuged at 2,000 × g for 5 min and resuspended in solution A + 0.1% Nonidet P-40. Cells were incubated on ice for 10 min, vortexed gently, and centrifuged at 13,000 × g for 10 min. Nuclear pellets were resuspended in 30 μl of solution B (25% glycerol, 20 mmol/liter Hepes, pH 7.9, 420 mmol/liter NaCl, 1.2 mmol/liter MgCl2, 0.2 mmol/liter EDTA, 0.5 mmol/liter dithiothreitol, 0.5 mmol/liter phenylmethylsulfonyl fluoride, 2 mmol/liter benzamidine, 5 μg/ml aprotinin) and incubated on ice for 15 min. Nuclei were then obtained by centrifugation at 13,000 × g, and proteins were measured with the Bio-Rad protein assay dye reagent (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)—The probe used for EMSA is a 30-μm double-stranded construct (5′-CCCTGTTGCTCCGG-GAATTTCCTGGCTGGA-3′) corresponding to a sequence (−72 to −42) in the CINC-proximal promoter region containing the NF-κB motif (12). End labeling was performed by T4 kinase in the presence of [γ-32P]dATP. Labeled oligonucleotides were purified on a Sephadex G-50M column (Amerham Biosciences).

An aliquot of 5 μg of nuclear protein was incubated with the labeled double-stranded probe (~50,000 cpm) in the presence of 5 μg of non-specific blocker, poly(dI-dC) in binding buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40, and 0.5 mM dithiothreitol) at 25 °C for 20 min. Specific competition was performed by adding 100 ng of unlabeled double-stranded CINC oligonucleotide probe to the nuclear extract from the sample with the greatest nuclear binding. The probe used for EMSA was an oligonucleotide corresponding to a sequence corresponding to the NF-κB-binding sequence to the CINC promoter site. EMSA was performed at the given LPS exposure times. D, XO primers for increased LPS responsiveness. RAW 264.7 cells were exposed to XO (50 millionunits/ml), LPS (0.1 μg/ml), or the combination of pretreatment with H2O2 for 1 h then LPS exposure. B, increased nuclear translocation with the combination of H2O2/LPS compared with H2O2 or LPS alone at all doses of H2O2. RAW 264.7 cells were exposed to either increasing doses of H2O2, LPS (0.01 μg/ml), or the combination of pretreatment with H2O2 for 1 h then LPS exposure. C, XO primes for increased LPS-induced NF-κB translocation. RAW 264.7 cells were exposed to XO (50 millionunits/ml), LPS (0.1 μg/ml), or the combination of pretreatment with XO for 2 h and then LPS treatment. Electrophoretic mobility shift assays were carried out with an oligonucleotide probe containing an NF-κB-binding sequence to the CINC promoter site. EMSA was performed at the given LPS exposure times. D, XO primes for increased LPS responsiveness. RAW 264.7 cells were either untreated or exposed to XO (100 millionunits/ml), LPS (0.1 μg/ml), or pre-treated 2 h with XO then stimulated by LPS. The cells were examined for CINC mRNA by Northern blot. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA is identified as a control for RNA loading. A representative of three independent experiments is shown.

**RESULTS**

**Effect of Oxidative Stress on Macrophages Responsiveness**—To examine the effect of oxidants on LPS responsiveness, H2O2 was examined for its effect on LPS-induced NF-κB translocation. As shown in Fig. 1A, low dose H2O2 (100 μM), which itself was able to provoke only a modest delayed NF-κB translocation, significantly enhanced the ability of LPS to induce NF-κB translocation, both by inducing an earlier response as well as a heightened response during the LPS stimulation period. Fig. 1B illustrates a dose response of the effect of varying doses of H2O2 on LPS-induced NF-κB translocation. At t = 30 min of LPS stimulation, H2O2 ranging from 15 to 100 μM was able to augment LPS-induced NF-κB translocation. The
higher doses of H2O2 alone caused modest NF-κB translocation in this model, whereas 1000 μM resulted in cytotoxicity. The dose of LPS chosen in these studies was based on its ability to induce relatively slow and modest changes in NF-κB translocation, as a means of highlighting the ability of oxidants to augment this activation. Xanthine oxidase is a proximal generator of oxidants following ischemia/reperfusion and was also tested for its ability to augment LPS responsiveness. In Fig. 1C, cells were exposed to XO (50 milliunits/ml) alone, LPS (0.1 μg/ml) alone, or the combination of pretreatment with XO followed by LPS treatment. Whereas XO pretreatment alone induced little NF-κB translocation, it markedly augmented the responsiveness to LPS at all time points studied. XO doses from 25 to 500 milliunits/ml were studied and optimal priming occurred at the 100 milliunits/ml concentration (data not shown).

Because CINC gene expression is dependent on NF-κB translocation and its transcription is augmented in vivo by shock plus LPS (3), we evaluated a functional correlate of the increased NF-κB translocation in this model by following the expression of the chemokine CINC. Pretreatment of cells with XO significantly enhanced levels of CINC mRNA expression following LPS treatment compared with LPS alone (Fig. 1D). The levels of CINC mRNA following XO treatment alone were modestly increased compared with control levels. Together, these studies established a cellular model of oxidant priming for increased LPS responsiveness, representing an in vitro correlate of the priming of alveolar macrophages observed after shock/resuscitation in vivo. Based on the dose-response studies, we chose to use 100 μM H2O2 as the priming dose of oxidative stress in subsequent experiments.

Oxidants Augment Tyrosine Phosphorylation of the Src Family Members—To determine whether members of the Src family of kinases might contribute to oxidant-induced signaling, we evaluated tyrosine phosphorylation as a measure of their activation. The three major Src family members expressed in the macrophage are Hck, Fgr, and Lyn (15). H2O2 (100 μM) induced phosphorysorylation accumulation in all three of these kinases within 10 min following exposure (Fig. 2). Therefore, the ability of oxidative stress to cause tyrosine phosphorylation of the three Src kinases is consistent with their potential contribution to the activation of downstream signaling (16).
FIG. 4. Oxidant primes for earlier LPS-induced NF-κB translocation in a Src-dependent manner. A, earlier p65 translocation in cells exposed to the combination of H$_2$O$_2$/LPS compared with H$_2$O$_2$ or LPS-treated cells. IF staining of the macrophage RAW 264.7 cell line at LPS exposure at $t = 5$, 15, and 30 min. The left panel at each time point displays IF staining for anti-p65 NF-κB antibody (red). The right panel displays dual staining for p65 (red) and 4′,6′-diamidino-2-phenylindole (DAPI) staining for nuclei (blue). Translocation is represented by purple because of the co-localization of the red and blue within the nucleus. There is little nuclear translocation in control, H$_2$O$_2$-, and LPS-treated cells, shown with p65 on the periphery/cyttoplasm of the cell (red) and distinct nuclei stain (blue). There is marked nuclear translocation of p65 in H$_2$O$_2$/LPS-treated cells demonstrated by the p65 in the nucleus of the cell (red) and purple color with dual staining by $t = 15$ min. Both LPS and H$_2$O$_2$/LPS had complete translocation at $t = 30$ min. B, pretreatment with PP2 inhibited the early translocation of H$_2$O$_2$/LPS shown by p65 in the periphery of the cell (red) and blue nuclei staining ($t = 15$ min). Control cells and PP2-treated cells had no translocation. There was no p65 translocation in cells treated with either H$_2$O$_2$ or LPS alone shown in Fig. 4A.
was no effect of PP2 (data not shown). We also employed a molecular approach wherein cells were transfected with the C-terminal Src kinase (Csk), an endogenous inhibitor of Src family kinases. Co-transfection with GFP was used to identify transfected cells. As illustrated above, in Fig. 5 at $t = 15$ min, p65 translocation had occurred in cells treated with H$_2$O$_2$/LPS. Co-transfection had no effect in control, H$_2$O$_2$-, and LPS-induced p65 nuclear translocation in the absence of H$_2$O$_2$ pre-treatment. Cells were co-transfected with Csk and GFP. Cells were transfected cells treated with LPS alone. In aggregate, these studies suggest that exposure of cells to oxidant stress causes an alteration in LPS signaling for NF-$\kappa$B, such that it becomes dependent on activation of Src family kinases.

**Oxidant Stress Primed for Increased PI 3-Kinase Activity in a Src-dependent Manner**—Recent reports have shown that the PI 3-kinase pathway may be a downstream effector of cellular response to oxidative stress (19). During its activation, this kinase is phosphorylated on tyrosine residues, and thus might serve as a possible substrate for the Src family kinases (20). We hypothesized that LPS signaling following oxidant stress involves the PI 3-kinase pathway and is activated by Src kinases. We first examined tyrosine phosphorylation of PI 3-kinase following oxidant stress with and without pre-exposure to H$_2$O$_2$. As shown in Fig. 7A, H$_2$O$_2$/LPS exhibited an increased phosphotyrosine accumulation by 5 min compared with LPS alone. This rise was prevented by treatment with the Src tyrosine kinase inhibitor PP2. To confirm that this was associated with increased activation of PI 3-kinase, kinase assay was performed by immunoprecipitating the p85 subunit of PI 3-kinase and incubating with phosphatidylinositol and $[^{32}$P]dATP. The combination of H$_2$O$_2$ plus LPS caused a marked increase in PI 3-kinase activity at 5 and 15 min of LPS stimulation compared with either LPS or H$_2$O$_2$ alone. The Src family kinase inhibitor, PP2, was able to prevent this activation at these time points (Fig. 7B). As reported by others (21, 22), at higher doses, LPS alone (0.5–1.0 $\mu$g/ml) was able to cause activation of PI 3-kinase, but again this activation was not inhibited by PP2 (data not shown). Furthermore, consistent with the idea that activation of Src family kinases is upstream of PI 3-kinase following oxidant stress, phosphorylation of Fgr kinase is phosphorylated on tyrosine residues, and thus might serve as a possible substrate for the Src family kinases (20). We hypothesized that LPS signaling following oxidant stress involves the PI 3-kinase pathway and is activated by Src kinases. We first examined tyrosine phosphorylation of PI 3-kinase following oxidant stress with and without pre-exposure to H$_2$O$_2$. As shown in Fig. 7A, H$_2$O$_2$/LPS exhibited an increased phosphotyrosine accumulation by 5 min compared with LPS alone. This rise was prevented by treatment with the Src tyrosine kinase inhibitor PP2. To confirm that this was associated with increased activation of PI 3-kinase, kinase assay was performed by immunoprecipitating the p85 subunit of PI 3-kinase and incubating with phosphatidylinositol and $[^{32}$P]dATP. The combination of H$_2$O$_2$ plus LPS caused a marked increase in PI 3-kinase activity at 5 and 15 min of LPS stimulation compared with either LPS or H$_2$O$_2$ alone. The Src family kinase inhibitor, PP2, was able to prevent this activation at these time points (Fig. 7B). As reported by others (21, 22), at higher doses, LPS alone (0.5–1.0 $\mu$g/ml) was able to cause activation of PI 3-kinase, but again this activation was not inhibited by PP2 (data not shown). Furthermore, consistent with the idea that activation of Src family kinases is upstream of PI 3-kinase following oxidant stress, phosphorylation of Fgr by LPS following H$_2$O$_2$ is potentiated (Fig. 7C). These studies suggested that the PI 3-kinase pathway is involved in the downstream signaling following Src activation. Because inhibition of the Src family inhibited NF-$\kappa$B translocation, we examined whether inhibition of the PI 3-kinase pathway might have similar effects. Immunofluorescence studies showed complete translocation of p65 by 45 min for both LPS and H$_2$O$_2$ plus LPS-treated cells (Fig. 7D). Consistent with the differential involvement of PI 3-kinase in LPS signaling in oxidant-treated cells, wortmannin had little effect on LPS-stimulated p65 translocation (10.5 ± 7.31% decrease, mean ± S.E., $n = 3$ studies with 100 cells counted in each study) while causing a 52.7 ± 9.25% reduction of p65 translocation in H$_2$O$_2$/LPS-treated cells (mean ± S.E., $n = 3$ studies with 100 cells counted in each study).
serine phosphorylation of IκBα, its degradation was augmented in cells exposed to oxidant stress plus LPS compared with LPS alone (Fig. 8B).

Fig. 8. Role of IκBα in the oxidant-induced priming of the macrophage. A, oxidants did not prime for increased tyrosine phosphorylation. Cells were either exposed to LPS (0.01 μg/ml) or H2O2 (100 μM) 1 h prior to LPS treatment for 1, 5, 10, and 20 min. Whole cell lysates were prepared and subjected to immunoprecipitation with IκBα antibody. Precipitates were then analyzed by Western blots using an antibody specific for phosphotyrosine. B, oxidants prime for rapid IκBα protein degradation. Cells were exposed to LPS (0.01 μg/ml), H2O2 (100 μM), or the H2O2 (100 μM) 1 h prior the LPS treatment for 1, 5, 10, 20, and 30 min. Whole cell lysates were separated by SDS-PAGE and immunoblotted with an antibody against IκBα. A representative three independent experiments is shown.

DISCUSSION

The contribution of oxidative stress to intracellular signaling pathways has become a common theme of investigation in the area of infection/inflammation. In addition to their ability to directly activate various signaling cascades, oxidants have also been shown to prime cells for an augmented response to a second inflammatory stimulus. This concept is highly relevant to the consideration of patients with critical illness, where oxidants generated during ischemia/reperfusion have been shown to heighten the inflammatory response and consequent organ injury to a delayed stimulus (2). In the present studies, we have modeled this two-hit process in vitro to investigate the cellular mechanisms whereby oxidative stress might exert this priming effect. The major finding in this article is that antecedent oxidative stress reprograms the LPS signaling pathway leading to NF-κB translocation such that it involves activation of the Src family kinase members. Activation of PI 3-kinase appears to be a consequence of Src activation and is clearly involved in the downstream signaling events leading to NF-κB translocation. Consistent with this notion others have shown that members of the Src family can induce tyrosine phosphorylation of the p85 subunit leading to enhancement of PI 3-kinase activity (20, 24). Taken together, our studies elucidate a novel signaling pathway associated with oxidant-induced priming of macrophages and suggest consideration of alternative strategies directed at inhibiting cell activation under conditions of ischemia/reperfusion.

The diversion of LPS signaling into the PI 3-kinase pathway appears to be a critical component of the reprogramming that occurs after oxidant exposure. Whereas the mechanism of this effect is unknown, one recent report suggested that LPS-induced ceramide generation was capable of activating PI 3-kinase.
phorylation of IκBα than with tyrosine phosphorylation, be-
cause the latter has been reported to cause dissociation from
NF-κB, but without degradation (31). This conclusion would
be consistent with the contribution of PI 3-kinase-mediated
signaling, because IκB kinase is a well described substrate of
the Akt, one of the potential downstream mediators of PI 3-kinase
(32).

The present studies provide a novel mechanism for the prim-
ing effects of oxidative stress, namely through altering signal-
ning through a Src kinase-dependent pathway involving activa-
tion of PI 3-kinase. The potential importance of this pathway
was suggested recently in studies using an experimental model
of lung injury following endotoxemia where PI 3-kinase knock-
out animals exhibited reduced lung injury, neutrophil seque-
tag, and NF-κB sequestration (33). Together, these find-
ings suggest that alternative therapeutic strategies aimed at mod-
ulating oxidative stress following shock/resuscitation and/or spe-
cifically defined signaling pathways might minimize the pre-
disposition of trauma victims for developing organ injury.

REFERENCES

1. MacKenzie, E. J., Morris, J. A., Jr., Smith, G. S., and Fahey, M. (1990)
 J. Trauma 30, 1096–1101
2. Moore, F. A., and Moore, E. E. (1995) Surg. Clin. North Am. 75, 257–277
3. Fan, J., Marshall, J. C., Jimenez, M., Shek, P. N., Zagorski, J., and Rotstein,
 O. D. (1998) J. Immunol. 161, 440–447
4. McCord, J. M. (1985) N. Engl. J. Med. 312, 159–163
5. McCarthy, H. J., Schwappach, J. R., Enquist, E. G., Walden, D. L., Terada,
 L. S., Reiss, O. K., Leff, J. A., and Repine, J. E. (1990) Am. J. Physiol. 258,
 H1415–H1419
6. Schwartz, M. D., Repine, J. E., and Abraham, E. (1995) Am. J. Respir. Cell
 Mol. Biol. 12, 434–440
7. Akawa, R., Komuro, I., Yamazaki, T., Zou, Y., Kudoh, S., Tanaka, M.,
 Suda, T., Hori, Y., and Tanaka, Y. (1997) J. Clin. Invest. 100, 1813–1821
8. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) Cell 71, 1081–1091
9. Imbert, V., Farnahshar, D., Auberger, P., Mary, D., Rossi, B., and Peyron, J. F.
 (1996) J. Inflamm. 65, 45–77
10. Suzuki, T., Kono, H., Hirase, N., Okada, M., Yamamoto, Y., Yamamoto, K.,
 and Honda, Z. (2000) J. Immunol. 165, 473–482
11. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
12. Blackwell, T. S., Blackwell, T. R., Holden, E. P., Christman, B. W.,
 and Christman, J. W. (1996) J. Immunol. 157, 1630–1637
13. Lioubin, M. N., Algate, P. A., Tsai, S., Carlberg, K., Aebersold, A., and Rohr-
schneider, L. R. (1996) Genes Dev. 10, 1084–1095
14. Deryckere, F., and Gannon, F. (1994) BioTechniques 16, 405
15. Lowell, C. A., and Soriano, P. (1996) Genes Dev. 10, 1845–1857
16. Malolzzi, C., Di Stasi, M. A., and Minetti, M. (2001) Free Radic. Biol. Med.
 30, 1008–1117
17. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. W.,
 Weringer, E. J., Pollok, B. A., and Connellly, P. A. (1996) J. Biol. Chem. 271,
 655–701
18. Meng, F., and Lowell, C. A. (1997) J. Exp. Med. 185, 1661–1670
19. Thomas, S. R., Chen, K., and Keaney, J. F., Jr. (2002) J. Biol. Chem. 277,
 6017–6024
20. Cuervas, B. D., Lu, Y., Mao, M., Zhang, J., LaPushin, R., Siminovich, K.,
 and Mills, G. B. (2001) J. Biol. Chem. 276, 24575–24581
21. Ojaniemi, M., Kovanen, T., Aliko, J., Kaakkari, J., Oikarinen, J., Nissinen,
 and Hallman, M. (2003) Eur. J. Immunol. 33, 697–705
22. Manzoni, M., Mallampalli, R. K., Carter, A. B., Flaherty, D. M., McCoy, D.,
 Robef, P. K., Peterson, M. W., and Hunninghake, G. W. (2001) J. Immunol. 167,
 5972–5985
23. Fan, C., Li, Q., Ross, D., and Engelhardt, J. F. (2003) J. Biol. Chem. 278,
 2072–2080
24. Klopel, A., Kavazan, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell.
 Biol. 17, 338–344
25. Rice, K. L., Duane, P. G., Archer, S. L., Gilbe, D. P., and Niewoehner, D. E.
 (1992) Am. J. Respir. Cell Mol. Biol. 6, L430–L438
26. Giroix-Calle, J., Srivatsa, K., and Forman, H. J. (2002) J. Pharmacol. Exp.
 Ther. 301, 87–94
27. Su, X., Wang, P., Hitayo, A., and Bitar, K. N. (1999) Am. J. Physiol. 276,
 L853–L861
28. Triantafillou, M., Miyake, K., Golenbock, D. T., and Triantafillou, K. (2002)
 J. Cell Sci. 115, 2603–2611
29. Parat, M. O., Stachnicki, R. E., and Fox, P. L. (2002) Biochem. J. 361, 681–688
30. Mayer, S., Rothberg, K. G., and Maxfield, F. R. (1994) Science 264, 1848–1951
31. Imbert, V., Rupec, R. A., Livolis, A., Pahl, H. L., Traenckner, E. B., Mueller-
 Dickmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P. A., and
 Peyron, J. F. (1996) Cell 86, 787–798
32. Madrid, L. V., Mayo, M. W., Reutcher, J. Y., and Baldwin, A. S., Jr. (2001)
 J. Biol. Chem. 276, 18934–18940
33. Yam, H. K., Arraroli, J., Knecht, S., Tschirkov, R., Penninger, J. M., Sasaki, T.,
 Yang, K. Y., Park, J. S., and Abraham, E. (2001) J. Immunol. 167,
 6601–6608

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