Requirement of Inducible Nitric-oxide Synthase in Lipopolysaccharide-mediated Src Induction and Macrophage Migration

Previously, we have demonstrated the induction of Src in lipopolysaccharide (LPS)-stimulated macrophages. In this study, we observed that pharmacological blockade or knockout of inducible nitric-oxide synthase (iNOS) reduced LPS-mediated Src induction and macrophage migration. Either SNAP (a NO donor) or 8-Br-cGMP (a cGMP analogue) could rescue these defects in iNOS-null macrophages, which indicated the participation of NO/cGMP in LPS-elicited Src expression and mobilization. In addition, Src family kinase (SFK)-specific inhibitor, PP2, inhibited SNAP- and 8-Br-cGMP-evoked motility implicating the involvement of SFKs downstream of NO/cGMP. Analysis of the expression of SFKs indicated LPS dramatically induced Src, which could be attributable to the increased level of the src transcript. Attenuation of Src by src-specific siRNA reduced LPS- and SNAP-evoked mobilization in Raw264.7 macrophages, and reintroduction of avian Src could rescue their motility. Furthermore, LPS-mediated Src induction led to increased FAK-Pi-Tyr-397 and Pi-Tyr-861, which was also iNOS-dependent. With these findings, we concluded that iNOS was important for LPS-mediated macrophage locomotion and Src was a critical player in this process.

Macrophages are important participants in innate immunity. Due to their ability to eliminate opsonized pathogens through a spectrum of surface receptors and antigen presentation to cells of the adaptive immunity, macrophage recruitment to sites of infection is an important physiological process in host defenses. Disturbed regulation of this event results in a variety of diseases such as sepsis, atherosclerosis, and autoimmune disorders.

Nitric oxide (NO), a unique diffusible messenger molecule, is produced via the oxidation of l-arginine by enzymes known as nitric-oxide synthases (NOSs) (1). Three distinct isoforms of the enzyme have been identified and characterized. Whereas Ca^{2+}/calmodulin can regulate the activity of neuronal (n) and endothelial (e) NOS that are constitutively expressed, the activity of inducible (i) NOS is independent of Ca^{2+}/calmodulin and only induced by bacterial products as well as inflammatory cytokines. A low level of constitutively produced NO is a crucial mediator for a spectrum of physiological functions such as regulation of neurotransmission, vasodilation, smooth muscle relaxation, and inhibition of platelet aggregation. In contrast, a high level of NO generated by macrophages and other effector cells under inducible conditions mediates host defense, including antibacterial and antitumor functions (2, 3). However, it is also well documented that some pathological processes such as inflammation and tumor can be induced by sustained, chronically produced NO (4, 5). As a main target of NO, sGC is a cytosolic, heme-containing heterodimer of α and β subunits (6). When NO binds to the sGC heme prosthetic group, it activates the enzyme to convert guanosine 5′-triphosphate to cGMP, an intracellular second messenger (7). Accumulation of cGMP results in transmission of NO signals to the downstream effectors such as cGMP-dependent protein kinase, cGMP-regulated phosphodiesterase, and cGMP-gated cation channels, leading to physiological and pathological responses (8).

The SFKs are membrane-associated, nonreceptor tyrosine kinases that have been implicated in multiple signaling pathways.

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The abbreviations used are: NO, nitric oxide; AG, aminoguanidine hemisulfate; cGMP, cyclic guanosine monophosphate; gapdh, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric-oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; ODQ, 1H[1,2,4]oxadiazolo[4,3-x]quinoxalin-1-one; PEM, peritoneal macrophage; PP2, 4-amino-5-(4-chlorophenyl)-7-(4-butyloxy)pyrazolo[3,4-d]pyrimidinamide; RT, reverse transcription; SFK, Src family kinase; sGC, soluble guanylyl cyclase; siRNA, small interfering RNA; SNAP, sodium nitroprusside; SNAP, S-nitroso-N-acetylpenicillamine; 8-Br-cGMP, 8-bromo-cyclic guanosine monophosphate.
ways, dictating a wide spectrum of cell physiology (9, 10). The SFKs comprise nine members that share similar structural and biochemical properties. Although Src, Yes, and Fyn are widely expressed in most tissues, the distribution of Lck, Lyn, Fgr, Hck, Blk, and Yrk exhibits a more tissue-specific fashion, mainly in hematopoietic cells (11). For example, the myeloid-specific Src members, Lyn, Fgr, and Hck, are predominantly expressed in macrophages (12). The presence of alternatively spliced isoforms is quite common for SFKs (13). Mounting evidence indicates that regardless of the stimulus and receptor type, these SFKs amplify and diversify the signal leading to proliferation, cell survival, adhesion, migration, and phagocytosis in leukocytes (13).

LPS, an outer membrane component of Gram-negative bacteria, activates macrophages via Toll-like receptor 4 signaling (14, 15). Because tyrosine kinases are important in LPS-mediated macrophage signaling (16, 17), the myeloid-specific Src members are speculated to play crucial roles in this process. However, macrophages derived from mice with three-combined deficiency of Lyn, Fgr, and Hck still retain full LPS responsiveness (12), suggesting the presence of one or more elusive, compensating tyrosine kinase(s). Indeed, in our previous report, we demonstrated the up-regulation of Src in LPS-stimulated macrophages both in vitro and in vivo. The abrogation of LPS-elicited responses by PP2 (the inhibitor for SFKs) implicated that Src might functionally compensate for its myeloid-specific relatives and restored LPS-induced activation in lyn−/−/fgr−/−/hck−/− macrophages. In other words, albeit Src is barely detectable in resting macrophages, its ready enhancement upon LPS exposure implicates that it might have an unperceived role in transmitting LPS signaling (18).

Given that 1) NOS inhibitors attenuated human monocyte chemotaxis (19); 2) reduced polymorphonuclear granulocyte extravasation in peritoneal cavity was observed in zymosan-challenged iNOS-deficient mice (20), NO was speculated to modulate leukocyte locomotion during inflammation and tissue injury. In this study, we demonstrated that iNOS was required in LPS-mediated macrophage movement, which was PP2-sensitive. Unlike its myeloid relatives, the expression of Src was LPS-, NO−, and cGMP-inducible, which was required for FAK-Pi-Tyr-397 and Pi-Tyr-861. Around 80% of LPS-, SNAP-initiated motility was inhibited by src siRNA in Raw264.7 macrophages, which could be recovered by ectopic avian c-Src, corroborating the importance of Src in this process. Studies conducted with wild-type (WT) and iNOS knockout (iNOS−/−) macrophages provided further definitive evidence regarding the concomitant induction of Src and increased motility by the NO/cGMP pathway. These data establish a role for Src in LPS-induced macrophage motility that depends on iNOS.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—1400W dihydrochloride (1400W), aminoquinidine hemisulfate (AG), 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ), 5-nitroso-N-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), 8-bromo-cyclic guanosine monophosphate (8-Br-cGMP), and LPS purified from *Escherichia coli* serotype 0111:B4 were obtained from Sigma. Thioglycollate was obtained from Merck (Darmstadt, Germany). 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was purchased from Calbiochem. The primary antibodies used were actin, COX-2, iNOS, Pi-Y397 FAK, and FAK (Upstate); Pi-Y861 FAK and Pi-Y416 Src (BIO SOURCE International); Pi-Y416 Src and Pi-Y527 Src (Cell Signaling Technology); Lyn, Fgr, Hck, FAK (A-17), and horseradish peroxidase-conjugated anti-phosphotyrosine antibody (PY20) (Santa Cruz Biotechnology). Src-specific mouse monoclonal antibody (2–17) was provided by Dr. Sarah Parsons in the University of Virginia.

**Animals**—Rats (Sprague-Dawley) were utilized to prepare peritoneal macrophages (PEMs). C57BL/6 inducible nitric-oxide synthase knockout (C57BL/6-Nos<sup>smIlau</sup>) (iNOS−/−) and WT mice of the same age and sex were used to assess the role of iNOS in LPS-induced Src expression in macrophages. These mutant mice were normal in appearance, histology, growth rate, and reproduction. But their PEMs failed to generate NO when stimulated with LPS (21). All experiments using laboratory animals were done in accordance with China Medical University guidelines.

**Cell Culture and Collection of PEMs**—The murine macrophage cell line, Raw264.7 (American Type Culture Collection), was cultured and propagated in RPMI 1640 medium supplemented with 10% fetal calf serum (HyClone, Logan, UT) and 2 mM l-glutamine at 37 °C in humidified atmosphere of 5% CO<sub>2</sub> and air. PEMs were collected by peritoneal lavage from rats (Sprague-Dawley) or wild-type and iNOS-null mice given an intraperitoneal injection of 8 ml and 1 ml of thioglycollate broth, respectively, 4 days before harvest. The PEMs were washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline and plated in fetal calf serum-containing RPMI medium overnight. Then the cells were washed with medium to remove non-adherent cells. In addition, according to morphological and phagocytic criteria, the resultant macrophage monolayer was >98% pure and ready for experimentation.

**Generation of Raw264.7 Macrophages Expressing src siRNA**—To generate cells expressing src siRNA (siRNA) or the nonspecific siRNA (Ctrl), Raw264.7 macrophages were transfected with plasmid DNA pLKO.1-msrc (puro) (targeted sequence: 5′-CGAGCGGCGCAAATGCTCTGTA-3′) or the negative control pSilencer plasmid (which contains sequences not present in mouse genome and provided by Ambion Inc.) by the Lipofectamine Plus method (Invitrogen) followed by puromycin or hygromycin selection. To generate Src-attenuated cells expressing ectopic Src, Raw264.7 cells expressing src siRNA were cotransfected with pBabe (hygro) DNA and a plasmid DNA encoding avian c-Src (22) followed by hygromycin selection.

**Pretreatment with PP2**—Raw264.7 cells (or PEMs) were cultured with PP2 (10 μM) for 30 min prior to the addition of SNAP and 8-Br-cGMP. These SNAP (or 8-Br-cGMP)-stimulated or -nonstimulated cells were then harvested, and their migratory ability was determined.

**Administration of SNP and 8-Br-cGMP**—SNP and 8-Br-cGMP were dissolved in autoclaved phosphate-buffered saline. Rats were injected with SNP (6 mg/kg) (23) or 8-Br-cGMP (5
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mg/kg) intraperitoneally. 48 h after SNP or 8-Br-cGMP injection, PEMs were harvested and analyzed.

**Lysate Preparation, Immunoprecipitation, and Immunoblottting Analysis**—Lysis of the cells was carried out with modified radioimmune precipitation assay buffers as described before (24), and protein concentration was determined by protein assay kit (Bio-Rad, Hercules, CA). Methods for immunoblotting analysis have been described (25). The cell lysates were resolved in an 8% SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and probed with respective antibodies followed by horseradish peroxidase-conjugated protein A or horseradish peroxidase-conjugated secondary antibodies and detected by Enhanced Chemiluminescence method (Amer sham Biosciences). For immunoprecipitation, lysates of interest were immunoprecipitated with Src or Hck antibody as described previously (24).

**RT-PCR**—The amount of src and gpdh transcript was semi-quantitated by RT-PCR as previously described (26). The following program is for both src and gpdh PCR reaction: the cDNA was denatured for 3 min at 95 °C and amplified for 30 cycles under the following conditions: 95 °C, 30 s; 60 °C, 30 s; and 72 °C, 45 s; followed by a 5-min elongation step at 72 °C. Sequences of primer pairs used were as follows: src: forward, 5′-CTGCTGGACTTTCTCAAGGG-3′; reverse, 5′-GTACAGGAGCAGCTTCAGGGG-3′; gpdh: forward, 5′-CCATCACCTTCTCAGGAG-3′; reverse, 5′-CCATCACCTTCTCAGGAG-3′; PCR products were resolved in 2% agarose gel and detected by ethidium bromide staining.

**Migration**—The migration of cells of interest exposed to LPS, SNAP, SNP, or 8-Br-cGMP was determined by using a modified Boyden chamber as described before (27). Briefly, cells with or without 48-h LPS, SNAP, SNP, or 8-Br-cGMP exposure were added to the upper wells (48-multiwell Boyden microcham bers) at 2 × 10^5 cells per well. The migrated cells will traverse a polycarbonate filter (8 μm) from the upper chamber to the lower chamber, which contains 10% fetal bovine serum as a chemoattractant. After 5 h at 37 °C in 5% CO_2_, non-migratory cells on the upper membrane surface were removed with a cotton swab, and the cells that traversed and spread on the lower membrane surface were fixed with methanol and stained with Giemsa stain (Modified solution, Sigma). By utilizing a microscope with a 40× objective, the number of migratory cells per membrane was enumerated. Four random fields in each filter were examined. Each experiment was performed in triplicate, and migration was expressed as the mean ± S.D. of total cells counted per field. The motility of Raw264.7 and its derived Ctrl, siRNA-1, siRNA-1/Src6, siRNA-1/Src15 in response to LPS, and SNAP was also monitored using a Leica AS MDW system equipped with a Coolsnap HQ camera (Roper Scientific). Video images were collected at intervals of 20 min for 3 h. The positions of nuclei were tracked to quantify cell motility and analyzed with Metamorph Software (Universal Imaging Corp.).

**Statistical Analysis**—Each experiment was performed at least three times. Unless indicated, the results were presented as means ± S.D. from a representative triplicate experiment. The significance of difference was assessed by Student’s t test. Bonferronic correction was used for controlling type I error in multiple comparisons.

**RESULTS**

**iNOS Participates in LPS-induced Macrophage Migration**—Stimulation of macrophages with LPS can provoke a spectrum of cellular activities including cell motility (13, 28). Interestingly, this LPS-mediated migration was inhibited in the presence of 1400W, an iNOS-selective inhibitor (Fig. 1A). To confirm the importance of iNOS in macrophage motility by LPS, the migratory potential of PEMs derived from WT and iNOS−/− mice treated with or without LPS was analyzed. As shown in Fig. 1B, compared with WT PEMs, reduced migration evoked by LPS was observed in iNOS null PEMs. This iNOS deficiency caused defect of LPS-induced motility was not due to the general inability of cells to mobilize, because analogues of iNOS downstream mediators, SNAP (a NO donor) and 8-Br-cGMP (a permeable cGMP analogue), could augment the migratory ability of iNOS-deficient macrophages comparable to that in WT macrophages (Fig. 1, C and D). These findings indicate the participation of the NO/cGMP pathway in LPS-mediated macrophage movement.

**LPS-induced Src Expression in Macrophages Is Sensitive to iNOS- and sGC-selective Inhibitors**—In accordance with the participation of SFKs in cell motility, 1400W significantly suppressed LPS-elicited protein tyrosyl phosphorylation and Pi-Tyr-416 of SFKs in Raw264.7 cells (Fig. 2A). To further substantiate the importance of SFKs in cell motility, alteration of tyrosyl-phosphorylated proteins and Pi-Tyr-416 of SFKs in WT and iNOS knockout PEMs in response to LPS, SNAP, and 8-Br-cGMP was examined. As shown in Fig. 2B, unlike WT PEMs, LPS failed to increase the content of protein tyrosyl phosphorylation and Pi-Tyr-416 of SFKs in PEMs derived from iNOS null mice (Fig. 2B, left). In addition, these defects did not occur when SNAP and 8-Br-cGMP were used as stimulants (Fig. 2B, right).
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A, Raw264.7 macrophages were pretreated without or with 1400W for 1 h, and then cells were stimulated without or with LPS for 48 h. B, PEMs from wild-type (WT) and iNOS−/− mice were treated without or with LPS (left) or SNAP and 8-Br-cGMP (cGMP, right) for 48 h. Total lysates (100 μg) from each group were resolved by SDS-PAGE and probed with antibodies against phosphotyrosine (pTyr) and Src Pi-Tyr-416. C, Raw264.7 cells were either untreated or with PP2 (10 μM) for 1 h, and then cells were stimulated without or with SNAP (100 μM) or 8-Br-cGMP (cGMP, 100 μM) for 48 h. C, the migratory ability of each group was determined by using a Boyden chamber as described under “Experimental Procedures.” ***p < 0.001. D, the lysates (100 μg) from each group were resolved by SDS-PAGE and probed with antibodies against Src Pi-Tyr-416 and actin. The star indicates the position of Src.

**FIGURE 2.** The participation of Src family kinase(s) in NO/cGMP-mediated macrophage migration. A, Raw264.7 macrophages were pretreated without or with 1400W for 1 h, and then cells were stimulated without or with LPS for 48 h. B, PEMs from wild-type (WT) and iNOS−/− mice were treated without or with LPS (left) or SNAP and 8-Br-cGMP (cGMP, right) for 48 h. Total lysates (100 μg) from each group were resolved by SDS-PAGE and probed with antibodies as indicated. 8-Br-cGMP (cGMP, 100 μM) was added to the cell culture medium. C, Raw264.7 cells were either untreated or with PP2 (10 μM) for 1 h, and then cells were stimulated without or with SNAP (100 μM) or 8-Br-cGMP (cGMP, 100 μM) for 48 h. D, the migratory ability of each group was determined by using a Boyden chamber as described under “Experimental Procedures.” ***p < 0.001. E, the lysates (100 μg) from each group were resolved by SDS-PAGE and probed with antibodies against Src Pi-Tyr-416 and actin. The star indicates the position of Src.

**FIGURE 3.** LPS-mediated Src induction was 1400W and ODQ sensitive. A, Raw264.7 cells were stimulated with LPS (100 ng/ml) for various time as indicated. B, Raw264.7 cells were pretreated without or with 1400W (100 μM) for 1 h and then stimulated with LPS (100 ng/ml) for 48 h. Raw264.7 cells treated with 1400W alone were used as controls. C, Raw264.7 cells were either untreated as control or pretreated with or without ODQ (100 μM) for 1 h and then stimulated with LPS for 48 h. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated.

These data suggested that one or more SFKs might contribute to the macrophage mobilization triggered by the LPS/iNOS pathway. Indeed, while PP2 (an SFK inhibitor) could abolish SNAP and 8-Br-cGMP-induced motility (Fig. 2C), it also reduced SNAP- and 8-Br-cGMP-elevated Pi-Tyr-416 of SFKs in rat PEMs (Fig. 2D). To pinpoint which SFK was required for LPS/iNOS-evoked macrophage migration, the expression of myeloid-specific SFKs (i.e. Lyn, Fgr, and Hck) as well as Src in LPS-stimulated Raw264.7 cells was analyzed. Although the expression of Lyn, Fgr, and Hck was almost unaltered, a significant time-dependent up-regulation of Src was detected (Fig. 3A). Further analysis of the expression profile of the four SFKs in LPS-stimulated Raw264.7 cells revealed that only the expression of Src was labile to 1400W (Fig. 3B). Similarly, compared with the three myeloid-specific Src relatives, only Src was ODQ (a sGC inhibitor)-sensitive (Fig. 3C). These findings suggested that Src might be the SFK critical in LPS/iNOS-elicited macrophage movement.

Up-regulation of Src in SNAP- and 8-Br-cGMP-stimulated Raw264.7 Cells and Rat Peritoneal Macrophages—Because NO has been implicated in regulating gene expression (29) and LPS-mediated Src induction could be impaired by iNOS- and sGC-selective inhibitors, we wondered whether NO as well as cGMP might directly augment Src expression in macrophages. To address this, Raw264.7 cells were stimulated with 100 μM SNAP for various time points. As exhibited in Fig. 4A, compared with the constant expression of actin, a time-dependent up-regulation of Src was observed in SNAP-treated macrophages. Similar results were also obtained in Raw264.7 cells treated with another NO donor, SNP (data not shown). To exclude the possibility that NO-elevated Src expression is an in vitro artifact that only occurs in Raw264.7 cells, thioglycolate-elicited PEMs were utilized to address this issue. Similar Src induction could also be detected in PEMs after SNAP and SNP exposure (Fig. 4A and data not shown).

Next, we utilized 8-Br-cGMP to stimulate both Raw264.7 cells and PEMs and studied its effect on Src expression. As demonstrated in Fig. 4B, a time-dependent increase of Src was also detected. Notably, up-regulation of Src did occur in PEMs recovered from SNP- and 8-Br-cGMP-challenged rats. These PEMs also exhibited an increased migratory potential as compared with those recovered from rats challenged with phosphate-buffered saline (supplemental Fig. S1). Thus, the induction of Src by the NO/cGMP signaling pathway was confirmed. Further RT-PCR analysis utilizing specific primers for src revealed that LPS, SNAP, and 8-Br-cGMP led to an increase in the expected 273-bp fragment, whereas AG (another iNOS
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**FIGURE 4.** Up-regulation of Src in SNAP- and 8-Br-cGMP-stimulated Raw264.7 cells and rat PEMs. Raw264.7 cells and rat PEMs were stimulated with SNAP (100 μM) (A) and 8-Br-cGMP (cGMP; 100 μM) (B) for various time points as indicated. Equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies against Src and actin, respectively. C, Raw264.7 cells were pretreated without or with AG (2 mM), ODQ (100 μM) for 30 min, and then cells were stimulated without or with LPS, SNAP, and 8-Br-cGMP for 48 h. The amount of src transcript was analyzed by RT-PCR. gapdh was utilized as an internal control for amplification efficiency.

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**FIGURE 5.** Src induction was abrogated in LPS-exposed iNOS knock-out macrophages but was restored by SNAP and 8-Br-cGMP. PEMs from wild-type (WT) and iNOS−/− mice were stimulated without or with LPS (100 ng/ml) (A), SNAP (100 μM) (B), and 8-Br-cGMP (cGMP; 100 μM) (C) for 48 h, and then equal amounts of lysates (100 μg) from each sample were resolved by SDS-PAGE and probed with antibodies as indicated.

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inhibitor) and ODQ abrogated its enhancement initiated by LPS (Fig. 4C). These findings implied that iNOS as well as sGC were involved in LPS-augmented Src expression, and this NO/cGMP-mediated increase in Src expression could be partly attributable to the increment of src transcript.

LPS-, but not SNAP- or 8-Br-cGMP-induced Src Expression Is Abrogated in iNOS Knockout Macrophages—To confirm that Src induction by LPS is iNOS-dependent, the expression of Src and its myeloid relatives in WT and iNOS−/− macrophages in the presence or absence of LPS was examined. As exhibited in Fig. 5A, concurrent with the absence of iNOS, diminished expression of Src (but not the myeloid-specific SFKs) was observed in LPS-stimulated PEMs devoid of iNOS. Interestingly, the expression of Src in iNOS null PEMs could be restored by SNAP and 8-Br-cGMP to a level comparable to that detected in WT PEMs (Fig. 5B). Again, the expression of Lyn in WT and iNOS−/− PEMs was not affected. Thus, our results further bolstered the up-regulation of Src by NO/cGMP pathway in LPS-exposed macrophages.

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**Src Attenuation Reduces LPS/iNOS-elicited Macrophage Migration**—Because SFKs were required for NO/cGMP-induced motility (Fig. 2, C and D), and there was a concomitant up-regulation of Src in LPS-, SNAP-, and 8-Br-cGMP-stimulated macrophages (Figs. 3–5); therefore, Src could be the main SFK required for macrophage locomotion. To prove this hypothesis, nonspecific and src-specific siRNA-bearing plasmids were introduced into Raw264.7 macrophages to obtain the corresponding control (Ctrl-1 and -2) and siRNA (siRNA-1 and -2) cells, respectively. As shown in Fig. 6, remarkable Src induction was observed in parental and control but not in Src-attenuated Raw264.7 following 48-h LPS (Fig. 6A) and SNAP (Fig. 6B) stimulation. Notably, like parental and control cells, the expression of Lyn, Fgr, and Hck was not significantly altered in Src-attenuated cells in the presence or absence of LPS and SNAP. Further analysis of the mobility of these cells by Transwell assay, we observed that LPS- and SNAP-induced cell motility was significantly suppressed. To verify this result, we performed a rescue experiment. By time-lapse microscopy, reduced LPS-, and SNAP-mediated macrophage migration was observed in Src-attenuated (siRNA-1) cells, which could be restored by ectopic Src (Fig. 7). Similar results were observed in siRNA-2 and its derived Src-expressing cells (data not shown). Thus, the pivotal role of Src in macrophage movement elicited by LPS/iNOS pathway was confirmed.

Src Is the Main SFK Responsible for FAK Pi-Tyr-397 and Pi-Tyr-861 in LPS-treated Macrophages—In addition to analysis of the protein expression of SFKs in LPS-exposed macrophages, we further analyzed the level Pi-Tyr-416 (an indicator of active SFKs) and Pi-Tyr-527 (an indicator of inactive SFKs) of Src relative to that of Hck in Raw 264.7 cells before and after LPS stimulation. Shown in Fig. 8A, both Pi-Tyr-416 and Pi-Tyr-527 in Src immunoprecipitates were elevated, whereas only increased Pi-Tyr-416 in Hck immunoprecipitates was detected in LPS-treated macrophages. Given that Src could mediate FAK Pi-Tyr-861 that promotes its autophosphorylation at Tyr-397 (30), therefore, we examined the level of FAK Pi-Tyr-861 and Pi-Tyr-397 in Raw264.7 and its derived cells in response to LPS. Shown in Fig. 8B, LPS-induced FAK Pi-Tyr-861 and Pi-Tyr-397 was inhibited by Src attenuation and could be restored by ectopic Src. In addition, the induction of Src was iNOS-dependent; LPS-mediated FAK Pi-Tyr-861 and Pi-Tyr-397 were also sensitive to the iNOS inhibitor, AG (Fig. 8C), and were suppressed in macrophages devoid of iNOS (Fig. 8D). Together, we concluded that Src was the major SFK responsible for FAK Pi-Tyr-861 and Pi-Tyr-397 in LPS-stimulated macrophages, and this process required the participation of iNOS.

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**DISCUSSION**

NO is a multifunctional biomolecule. Concurrent with the notion of its contribution to leukocyte recruitment, we reported the requirement of iNOS in LPS-mediated macrophage migration. This could be evidenced by suppressed LPS-induced motility by iNOS inhibitor in wild-type PEMs (Fig. 1A) and in iNOS null PEMs exposed to LPS (Fig. 1B). Consistent with the possible involvement of protein-tyrosine kinase(s) in LPS/iNOS-induced macrophage movement, LPS failed to increase the content of total protein tyrosyl phosphorylation as
well as Pi-Tyr-416 of SFKs in PEMs preincubated with 1400W (Fig. 2A) and in iNOS-deficient PEMs (Fig. 2B). Because PP2 efficiently abrogated SNAP- and 8-Br-cGMP-elicited macrophage migration and Pi-Tyr-416 SFKs (Fig. 2, C and D), members of SFK(s) were speculated to be crucial participants. Given that 1) LPS-mediated Src induction was labile to 1400W and ODQ (Fig. 3); 2) there was a concomitant increment of Src expression (Fig. 5) and cell mobility (Fig. 1) in iNOS-deficient PEMs exposed to SNAP and 8-Br-cGMP, Src seemed to be an important player in LPS/iNOS-initiated macrophage locomotion. Indeed, Src attenuation impeded LPS- and SNAP-evoked motility of Raw264.7 cells (Fig. 6), which could be rescued by Src re-introduction (Fig. 7). Furthermore, the elevated expression of Src in PEMs recovered from SNAP- and 8-Br-cGMP-challenged rats indicated that Src up-regulation by NO/cGMP was a physiological event (supplemental Fig. S1). Consistent with Src enhancement, these PEMs retained elevated migratory potential (supplemental Fig. S1).

Src along with its family members are well documented to regulate cell motility (10, 31). However, its low basal expression conceals its possible physiological significance in macrophages. Instead, Lyn, Fgr, and Hck are the three myeloid SFKs spotlighted. The expression of Src exhibited a much more dramatic LPS-inducible manner than its relatives that involved NO/cGMP (Fig. 3). Consistently reflecting its NO-inducible characteristic, the expression of Src was deteriorated in LPS-exposed macrophages devoid of iNOS (Fig. 5A), which could be restored by SNAP and 8-Br-cGMP (Fig. 5B). By contrast, the expression of the myeloid-specific SFKs was marginally altered (Fig. 5A) in iNOS-deficient macrophages, and the expression of Lyn was almost invariable in response to SNAP and 8-Br-cGMP (Fig. 5B). These findings manifested the constitutive expression of Lyn, Fgr, and Hck that carried out routine tasks required in macrophages; whereas, the various stimuli-induced Src behaved like a sensor that was able to promote certain macrophage functions. Considering the importance of iNOS and COX-2, the two inducible enzymes in inflammation and infection, the regulation and participation of Src in macrophage physiology deserve additional study.

iNOS-induced expression of Src is required for dramatic increase of cell migration in LPS-exposed Raw264.7 macrophages (Figs. 5A and 7B). However, despite the tremendous amount of Src in siRNA-1/Src6 and siRNA-1/Src15 cells, they were still immobile in the absence of LPS or SNAP (data not shown), indicating that, without activation, Src overexpression was not sufficient to trigger macrophage motility. Indeed, an enormous amount of Src Pi-Tyr-527 was present in these Src overexpressors even though they somehow possessed elevated Src Pi-Tyr-416 (Fig. 7A). By virtue of S-nitrosylation/S–S bond formation, NO destabilizes the intramolecular interaction of Src structure and puts on the Tyr-416 phosphorylation-linked local switch for increased catalytic activity (32). Thus, NO cannot only augment the expression of Src, but also increase its activity. With a similar mechanism, the activity of myeloid-specific SFKs could also be boosted by NO. To further analyze their relative activation, Src and its myeloid relative (i.e. Hck) were immunoprecipitated, and their levels of Pi-Tyr-416 and Pi-Tyr-527 were compared. Because >80% of Src and Hck was immunoprecipitated from the lysates (data not shown), Src exhibited a greater LPS-mediated increment of Pi-Tyr-416 than that of Hck (Fig. 8A).

Interestingly, Pi-Tyr-527 was only detected in Src, but not in Hck, further supporting the notion that the activity of Src was under strict regulation in LPS-stimulated macrophages. Because FAK was critical in macrophage mobility (33), and FAK Pi-Tyr-861 might be involved in cell motility (34, 35), the strong association between LPS-induced Src activation (as reflected by its Pi-Tyr-416) (Fig. 7A) and FAK Pi-Tyr-861 (Fig. 8B) indicated that Src-mediated FAK Pi-Tyr-861 might be responsible for LPS/iNOS-induced cell migration. Given that Src was NO-inducible, the suppression of LPS-elicited FAK Pi-Tyr-861 in both Raw264.7 and rat PEMs pre-treated with iNOS inhibitor (Fig. 8C) as well as the decrement of LPS-increased FAK Pi-Tyr-861 in macrophages deficient in iNOS (Fig. 8D) further corroborated this speculation.

Considering that 1) 8-Br-cGMP alone was capable of inducing Src expression and facilitate cell motility and 2) concomitant Src induction and increased motility could occur even in the absence of iNOS, we concluded that sGC was the main NO sensor that whose generation of cGMP was essential for Src up-regulation and macrophage migration. Although SFKs could...
mediate the activation of NF-κB in various types of cells (36–39), including macrophages (40, 41), we perceived that, upon LPS exposure, acceleration of NF-κB activation achieved by NO-induced Src expression as well as activation of SFKs could elevate iNOS gene transcription and thus constitute a loop of signal amplification for macrophage mobility. Fig. 9 represents a simple model illustrating the mechanisms responsible for LPS-initiated, iNOS-dependent macrophage migration.

Nitric oxide/cyclic guanosine monophosphate (cGMP)-mediated cell motility was well documented. For example, elastin could induce macrophage chemotaxis via the induction of cGMP. This elastin-induced cell movement could be abolished by inhibitors of protein kinase G, but not by inhibitors of protein kinase A, protein kinase C, and tyrosine kinase (42). In addition, by virtue of time-lapse photography as well as siRNA gene knockdown, Yaroslavskiy et al. (43) demonstrated that cGMP-dependent kinase I (protein kinase G I) and VASP were involved in NO/cGMP-induced migration of osteoclasts, the specialized macrophages in bone. Recently, NO has
been implicated in the regulation of matrix metalloproteinase-9 activity of murine macrophages directly or through cGMP (44). The increased matrix metalloproteinase-9 activity could promote vascular cell migration in a wound model. Intriguingly, in addition to macrophages, NO was also reported to be involved in cellular movement of endothelial cells. Although vascular endothelial growth factor-stimulated endothelial cell migration was critical in angiogenesis (45), compelling evidence indicated that eNOS Pi-Ser-1177 mediated by Akt was essential in this process (46). Besides, Kawasaki et al. (47) demonstrated that NO increased endothelial cell migration and produced functional neovascularization via phosphatidylinositol 3-kinase/Akt signaling pathways. Thus, a phosphatidylinositol 3-kinase/Akt → eNOS → NO/cGMP → phosphatidylinositol 3-kinase/Akt cycling pathway seemed to be required for endothelial cell motility and angiogenesis. Remarkably, NO/cGMP signaling was also permissive for neuronal migration in an insect embryo (48). Considering these described physiological events as well as our observation of NO/cGMP-mediated macrophage motility, we speculate that NO/cGMP might be potent regulators of locomotion in various cell types.

Induction of Src in RAW264.7 macrophages could be regulated by the concentration and duration of LPS (Fig. 3 and data not shown). Augmented expression of Src could reach plateau around 12 h when LPS concentration was 1 μg/ml. Presumably, the production and accumulation of NO was required for Src induction. In an attempt to study mechanisms underlying the long term action of NO, Hemish et al. (29) have used DNA microarrays to determine the temporal order of gene activation induced by SNAP in NIH3T3 fibroblasts. By utilization of chemical inhibitors, they defined groups of genes that required phosphatidylinositol 3-kinase, protein kinase C, NF-κB, p53, or combinations thereof for activation by NO (29). However, Src did not appear on their list as one of the NO targets. Because MIF-2, the prominent NO-responsive gene in mesangial cells (49), was also elusive in this microarray assay (29), we believed that induction of Src by NO might depend on the type of tissues studied.

In summary, our results indicated that NO/cGMP are crucial for LPS-mediated macrophage locomotion. Src seemed to be one of the downstream targets in this process. Given the importance of Src in diverse cellular functions, its ready inducibility by NO/cGMP as demonstrated in macrophages should have an impact on multiple disciplines in life science.

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