THE EXPRESSION OF C-SRC IS LIPOPOLYSACCHARIDE-INDUCIBLE THAT PLAYS A ROLE IN NITRIC OXIDE PRODUCTION AND TNF SECRETION IN MACROPHAGES[1]

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ABSTRACT

As tyrosine kinases are indispensable in Lipopolysaccharide (LPS)-induced macrophage activation, the myeloid-

INTRODUCTION

reactive nitrites as well as enhances their tumoricidal activity that similar to those bacteria have on macrophages *in vivo*.

Cellular Src (c-Src) is a ubiquitious, membrane-associated nonreceptor tyrosine kinase belonging to a family of related kinases including Yes, Fyn, Hck, Lyn, Lck and Blk.[11] Accumulated evidence suggests that members of Src family function as co-transducers of transmembrane mitogenic signals in a variety of cell types such as fibroblasts and cells of hematopoietic lineage.[11,12] Notably, Lyn, Hck and Fgr are three predominant Src family members expressed in macrophages that become activated after LPS stimulation[13,14] and chronic LPS exposure (24 to 48 hr) further results in increased synthesis of Lyn and Hck.[15] Due to the release of eicosanoid mediators from LPS-stimulated Raw 264.7 macrophages[16] and the development of tumoricidal activity of LPS-treated murine peritoneal macrophages (PEMs)[17] was hampered by herbimycin A, a tyrosine kinase inhibitor, therefore, it was speculated that Lyn, Hck and Fgr were essential signal transducers in LPS-triggered tyrosyl phosphorylation and required for the anti-bacterial responses elicited by macrophages. However, macrophages derived from mice with three-combined deficiency of Hck, Fgr and Lyn still retained full LPS responsiveness.[18] This result not only highlighted the activation of the myeloid-specific Src family members was not obligatory for LPS-induced macrophage activation, but also raised the question of what was the compensating tyrosine kinase(s)?

In this study, we present evidence demonstrating the induction of c-Src in Raw 264.7 and PEMs in response to LPS. And similar result could also be observed in macrophages recovered from LPS-challenged rats. Remarkably, the LPS-elicited responses (i.e. enhanced COX-2 expression as well as increased release of NO and TNF) were abrogated by PP2 both *in vitro* and *in vivo*, implicating the requirement of Src family kinases in transmitting LPS signaling in macrophages. Thus, our results indicate that Src might functionally compensate for its myeloid-specific relatives and restore LPS-induced activation in $lyn^{-/-}fgr^{-/-}hck^{-/-}$ macrophages.

MATERIALS AND METHODS

Reagents and antibodies

LPS purified from Escherichia coli serotype 0111:B4 and thioglycollate were obtained from Sigma (St. Louis, Missouri, USA) and Merck (Darmstadt, Germany) respectively. PP2, pyrrolidinedithiocarbamate (PDTC), LY294002 and PD98059 were purchased from Calbiochem (La Jolla, California, USA). Src-specific mouse mAb (GD11) was provided by Dr. Sarah J. Parsons in University of Virginia. Antibodies specific for COX-2, tubulin and actin were purchased from Upstate Biotechnology (New York, USA). HRP-conjugated anti-phosphotyrosine antibody (PY20) was obtained from Santa Crutz (California, USA).

Cell culture

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 $37^{o}C$ in humidified atmosphere of 5% CO_2 and air.

Collection and cultivation of rat peritoneal macrophages (PEMs)

PEMs were collected by peritoneal lavage from rats (Sprague-Dawley) given an i.p. injection of 8 ml of thioglycollate broth 4 days before harvest. The PEM were

Lysate preparation and immunoblot analysis

Lysis of the cells was carried out with modified RIPA buffers as described before (Maa et al., 1999) and protein concentration was determined by protein assay kit (Bio-Rad) (Hercules, California, USA). Methods for immunoblotting analysis have been described.[19] The cells lysates were resolved on 8% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and probed with respective antibodies followed by horseradish peroxidase (HRP)-conjugated protein A or HRP-conjugated rabbit anti-mouse IgG and detected by Enhanced Chemiluminescence method (Amersham) (Rockford, Illinois, USA).

Nitrite assays

At indicated time points the culture medium was collected for nitrite measurements, which was used as a measure of NO production. Culture medium (100 μl) was incubated with the same volume of Griess reagent and the absorbance was measured at 540 nm. The concentration of nitrite was calculated with sodium nitrite as a standard.

Measurement of serum TNF

Mice (8-10 weeks old) received PP2 (20 μg/mice) or vehicle were injected with sterile PBS or LPS (20 mg/kg) intraperitoneally. The animals were bled retro-orbitally (approximately 100 μl) 2 hrs after LPS injection and the amount of TNF in the serum was determined with the ELISA Kits from Bioscience International (Camarillo, California, USA).

Statistical analysis

Each experiment was performed at least three times. The results were expressed as means±S.D. Data on nitrite concentration and TNF were analyzed using Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Sustained activation of Src family kinases is required for macrophage activation in response to LPS

Accumulated evidence demonstrated that tyrosine kinase(s) was required for LPS-induced macrophage activation.[17, 20] However, to date, only the early profile of protein tyrosyl phosphorylation in LPS-stimulated macrophages was analyzed.[16,17, 21] In order to get its whole picture, lysates from Raw264.7 cells stimulated with LPS up to 48 hrs were examined. As demonstrated in Figure 1, the tyrosyl phosphorylation pattern of different proteins in response to LPS varied. For example, while a protein with its mass around 35 kDa had its tyrosyl phosphorylation increased by 0.5 hr, subsided by 1 hr and re-appeared by 24 hr after LPS exposure, the proteins around 110 to 130 kDa had theirs emerged after 1.5 hr LPS stimulation and sustained afterwards. To assess the importance of Src family kinases in transmitting LPS signals after they were initiated, PP2 was added into the cells that have been incubated with LPS for 1.6 hr (Figure 1A). Notably, accompanied with the abrogated protein tyrosyl phosphorylation following PP2 addition was the concomitant suppression of LPS-induced COX-2 expression and NO release (Figure 1B). Thus, for Src family kinases, in addition to their well-documented immediate, transient activation, their sustained activity was also indispensable for LPS-elicited responses.

Upregulation of c-Src in LPS-stimulated macrophages

Given that Src family kinases were essential for LPS-provoked signaling and knockout of Lyn, Fgr, and Hck did not hamper macrophage activation, therefore, we postulated that non-myeloid-specific Src family members might compensate for the loss of their myeloid-predominant relatives. However, considered their low expression and the requirement for their sustained activation, the most likely tactic utilized by macrophages was to have these proteins become LPS-inducible. Indeed, when the increase of COX-2 expression was utilized as an internal control for LPS-mediated macrophage activation, a significant time-dependent upregulation of c-Src was detected when the expression of tubulin was constant (Figure 2A). Importantly, similar phenomenon could also be observed in PEMs after LPS stimulation (Figure 2B). To further substantiate that the LPS-mediated c-Src induction in macrophages is a *bona fi c*

Figure 3A, PEMs recovered from LPS-challenged rats exhibited an enhancement of COX-2 expression as compared to control, reflecting their activation. Notably, while the actin amount kept constant, the expression of c-Src turned to be LPS-inducible (Figure 3A) and this LPS-mediated c-Src induction could reach two to three fold (Figure 3B). Therefore, the induction of c-Src in macrophages by LPS *in vivo* was corroborated.

this issue, LPS-mediated TNF secretion in Raw264.7 cells that were pretreated with or without PP2 was analyzed. As shown in Figure 6A, PP2 deteriorated LPS-induced TNF secretion. To further elucidate the role of Src family kinases in LPS-induced TNF secretion *in vivo*, the effect of PP2 on serum TNF in animal models of endotoxemia was assessed. Mice were injected intraperitoneally with PBS or a high dose of LPS (20 mg/kg) with or without prior PP2 administration. Two hours after LPS challenge, the TNF levels in serum samples of all mice were determined. As shown Figure 6B, serum from mice receiving PP2/LPS contained significantly lower levels of TNF than that from mice receiving LPS alone. Collectively, these observations highlighted a pivotal role of Src family kinases in LPS-induced TNF secretion.

DISCUSSION

Macrophages are not only important players in innate immunity against pathogens, but also are the major effectors of inflammation and tissue injury whose activation can be triggered by a variety of pro-inflammatory agonists as well as the pathogen-derived factors such as LPS. Like other cytokine receptors, the LPS receptor complex does not contain intrinsic tyrosine kinase activity, thus, activation of appropriate membrane-associated tyrosine kinases is required for its signal transduction. Accumulated evidence has indicated that Lyn, Fgr and Hck, the myeloid-specific Src family members, were crucial in LPS-initiated events.[15, 22, 23] Surprisingly, this process occurred normally in their absence, implicating that they were not obligatory for LPS-elicited macrophage activation and their exerted effects might be compensated by other tyrosine kinases.[18] Due to the barely detectable other Src family kinases by Western immunoblotting in $lyn^{-/-}fgr^{-/-}hck^{-/-}$ macrophages as well as the unaltered LPS signaling in macrophages lacking Src or Syk,[18] therefore, these tyrosine kinases were not considered as the ones that readily compensated for the loss of Lyn, Fgr and Hck to rescue the LPS responses.

In this study, we demonstrated that for Src family kinases, in addition to their well-documented immediate and transient activation, their sustained activity was also essential to achieve LPS responses in macrophages (Figure 1). Intriguingly, we found that LPS enhanced the expression of Src in both Raw264.7 and PEMs (Figure 2) and this enhancement exhibited a time-dependent manner and relied on the signaling proteins of Src family kinases, NF- B and PI-3 kinase (Figure 4). Though the detailed mechanism underlying this phenomenon was still unclear, the upregulation of Src detected in macrophages recovered from LPS-challenged rats (Figure 3) implicated its physiological significance. Indeed, the impairment of LPS-provoked nitrite production and TNF secretion in PP2-treated macrophages supported this speculation (Figure 5 and 6). Complementary to the results obtained from $lyn^{-/-}fgr^{-/-}hck^{-/-}$ macrophages, our findings unequivocally suggested that Src, with its expression induced by LPS, was an attractive, compensating candidate for the defect of Lyn, Fgr and Hck. Likewise, Lyn, Fgr and Hck could also rescue the deficiency of Src. Thus, the structural similarity and functional redundancy among Src family members might explain why the LPS responses in $src^{-/-}$ and $lyn^{-/-}fgr^{-/-}hck^{-/-}$ macrophages were still intact.[18] Further verification of this hypothesis requires the generation of quadruple mutant $src^{-/-}lyn^{-/-}fgr^{-/-}hck^{-/-}$ mice with aberrant LPS responses. However, we would like to point out here that despite their low expression in macrophages, the ubiquitous Src, Yes and Fyn was readily detectable in murine bone marrow-derived macrophages (BMDMs),[24] and at least Src, has been demonstrated to

be essential in TNF -induced NF- B activation in macrophages.[25] Furthermore, the expression of Fyn was also LPS-inducible despite with less extent than Src (data not shown). Thus, under this circumstance, normal LPS signaling in *src*$^{-/-}$*lyn*$^{-/-}$*fgr*$^{-/-}$*hck*$^{-/-}$ macrophages might still be possible. Nevertheless, the suppression of LPS-induced secretion of nitrite by herbimycin A in *lyn*$^{-/-}$*fgr*$^{-/-}$*hck*$^{-/-}$ macrophages[18] as well as the abrogation of LPS-elicited macrophage activation by PP2 in our study manifested the indispensability of Src family kinases in LPS-evoked signaling.

The generation and investigation of *lyn*$^{-/-}$*fgr*$^{-/-}$*hck*$^{-/-}$ mice have provided affluent information concerning the physiological roles of those myeloid-expressed Src family kinases. The normal myeloid cell development and LPS-induced macrophage responses observed in the triple knockout mice indicated the existence of a tremendous functional overlap between these kinases and their relatives. In contrast, a dramatic block in *in vivo* macrophage migration in mice deficient of these three kinases indicating their stringent, non-displaceable role in 1-integrin signal transduction.[26] These results disclosed that some functions of Lyn, Fgr and Hck were redundant and could be compensated while some turned to be unique and could not be replaced.

As one of the cytokines generated by overwhelming immune responses triggered by LPS, TNF was a critical mediator of septic shock.[27] Though Lyn, Fgr and Hck were not obligatory in LPS-induced TNF secretion as exhibited in triple knockout mice,[18] PP2 did diminish LPS-mediated TNF induction in Raw264.7 cells in this

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FIGURE LEGENDS

Figure 1. Involvement of Src family kinases in the late stage of LPS-induced macrophage activation. (A) Flow diagram describing the administration of PP2 and

Figure 5. The inhibitory effect of PP2 on LPS-induced COX-2 expression and NO release. (A) Rat PEMs were pretreated with PP2 for 20 min and then stimulated with or without LPS (100 ng/ml) for 24 hrs. The control was pretreated with vehicle, dimethyl sulfoxide (5 μg/ml) only. The expression of COX-2 and actin were analyzed by direct Western immunoblotting. Nitrite concentration in the culture supernatants was also determined. The results were representatives of four different experiments with similar results. Data were analyzed and were presented as mean ± S.D. **$P<0.01$ compared with cells treated with LPS. (B) Rats with or without prior PP2 administration were injected with sterile PBS or LPS (1 mg/ml) intraperitoneally as described in Materials and Methods. Twenty-four hours after LPS injection, PEMs were harvested as described in Materials and Methods. The NO release and the expression of COX-2 and actin in each sample were determined by analysis of culture supernatants and cell lysates prepared from adherent PEMs respectively. Each sample represented an individual rat. The results were representatives of four different experiments with similar results. Data were analyzed and were presented as mean ± S.D. ***$P<0.001$ compared with cells treated with LPS.

Figure 1

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