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Short Communication

Detection of the Activities of Multiple Protein Kinases in Lipopolysaccharide-Stimulated Macrophages by Renaturation

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Past studies of individual kinases have demonstrated that protein phosphorylation plays a crucial role in the intracellular signaling pathway of bacterial lipopolysaccharide (LPS). However, no one has determined how many kinases may be activated collectively by LPS stimulation. We examined the spectrum of protein kinases activated in macrophages in response to LPS. Activity was assessed by a renaturation method that exploited the ability of proteins denatured with sodium dodecyl sulfate and then blotted onto a membrane to regain enzymatic activity after guanidine treatment. Seven electrophoretically-distinct protein kinases with apparent molecular masses of 78, 74, 62, 59, 58, 52, and 48-kDa were detected in lysates from unstimulated murine peritoneal macrophages. An additional three kinases, with apparent molecular masses of 82, 55, and 46-kDa, were detected when the macrophages were stimulated with LPS. The activation of these protein kinases may be dictated by complex signals that are delivered by receptor complexes, including Toll-like receptor 4. These results should provide a clue to clarifying the pleiotropic action of LPS on macrophages.
Key words lipopolysaccharide, macrophage, phosphorylation, protein kinase

INTRODUCTION

Bacterial lipopolysaccharide (LPS) has an unusually wide range of biological effects on the host[1–3], and the principal target of LPS is the macrophage. LPS is one of the most potent bacterial components in stimulating macrophages to produce a variety of mediators, such as interleukin-1, tumor necrosis factor, and arachidonic acid metabolites. These mediators lead to inflammatory vascular changes, fever, and, in severe cases, septic shock[4,5]. Understanding of the molecular mechanisms whereby LPS stimulates macrophages should be helpful in the development of clinical measures to cope with septic shock.

Over the past ten years it has been demonstrated by several groups, including ours, that protein phosphorylation plays a crucial role in the intracellular signaling pathway of LPS[6–12]. Many different kinases, including some yet to be identified, have been shown to contribute to LPS-induced phosphorylation in cells. However, each study investigated a particular kinase exclusively, and no examination has been made of how many kinases are activated by LPS stimulation. This prompted us to explore the whole spectrum of kinases that are activated in macrophages upon exposure to LPS.

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MATERIALS AND METHODS

Preparation of peritoneal macrophages

Macrophages were obtained from specific pathogen-free 7–8 wk-old C3H/HeN mice (Clea Japan Inc., Osaka, Japan), and cultured in RPMI 1640 medium supplemented with 0.2% $NaHCO_3$, 10 mM HEPES, 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 1%

fetal calf serum (FCS) (low endotoxin ; Hyclone Laboratories, Logan, UT) (FCS-RPMI), as previously described[6–8]. The use of uninfected mice was essential because spontaneously activated macrophages show an enhanced level of protein phosphorylation similar to that induced by LPS. The LPS content in the media was less than 0.1 ng/ml as tested by the Toxicolor Test (Seikagaku Kogyo Co., Tokyo, Japan).

Kinase renaturation assay

To examine the effect of LPS stimulation on the activities of renaturable protein kinases in macrophages, the modified kinase renaturation assay method of Ferrell and Martin[13] was used. Macrophages were cultured in FCS-RPMI (10^6 ml/well) for periods of 0 to 2 h in the presence of 1 μg/ml of chromatographically-purified protein-free LPS from *Salmonella typhimurium* (Sigma Chemical Co., St. Louis, MO). After incubation, the medium was drained thoroughly and 100 μl of boiled sodium dodecyl sulfate (SDS) lysis buffer (2.3% SDS, 62.5 mM Tris, 5 mM EDTA, 10% glycerol, plus 5% 2-mercaptoethanol, pH 6.8) was added to the cells. The lysates were scraped into microcentrifuge tubes, submerged in boiling water for 3 min, vortexed briskly, and centrifuged for 5 min to pellet the insoluble debris.

Samples containing equivalent amounts of protein or prestained molecular weight standards were first separated on a 13% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli[14]. Then the samples were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Bedford, MA) using a transfer apparatus in 192 mM glycine-25 mM Tris transfer buffer without SDS or methanol. The PVDF membrane was incubated for 1 h at 4°C by gentle rocking in 7 M guanidine HCl (Sigma, grade I)-50 mM Tris-50 mM dithiothreitol-2 mM EDTA (pH 8.3), washed briefly with Tris-buffered saline, and allowed to renature overnight at 4°C in the renaturation buffer (140 mM NaCl, 10 mM Tris, 2 mM EDTA, 2 mM dithiothreitol, 0.1% Nonidet P-40, 1% bovine serum albumin, pH 7.4).

The blot was blocked with 5% bovine serum albumin in 30 mM Tris at room temperature for 1 h, and overlaid with kinase reaction buffer (30 mM Tris, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 50 μCi/ml of [γ-^{32}P] ATP, pH 7.4 ; 1 ml/10 cm^2 blot area) for 30 min at room temperature. The reaction was stopped by removing the kinase buffer and rinsing the blot with 30 mM Tris, pH 7.5. To reduce background radioactivity, the blot was washed with 1 M KOH for 10 min at 37°C. The membrane was rinsed several times with water followed by 10% acetic acid and air dried. For detection, the membrane was subjected to autoradiography for an appropriate time at -80°C. The bands were quantified by densitometric analysis (NIH Image). The PVDF blot was stained with Coomassie blue after autoradiography to ascertain the transfer of proteins.

RESULTS AND DISCUSSION

After macrophages were cultured in presence or absence of LPS, the macrophage samples were lysed and subjected to denaturing gel electrophoresis. Proteins were transferred to a blotting membrane, treated with guanidine, allowed to renature, and assayed for kinase activity by incubation with [γ-^{32}P] ATP. Radiolabels were detected in at least seven electrophoretically-distinct bands of 78, 74, 62, 59, 58, 52, and 48-kDa in unstimulated macrophages (Fig. 1A, lanes 1, 3, and 5). We designated these putative kinases as PK78, PK74, and so on, according to their apparent molecular masses. Each band appeared to correspond to a distinct protein kinase. In addition to these kinases, the activities of three other kinases, PK82, PK55, and PK46, were detected in macrophages as early as 15 min after LPS stimulation and declined after 60 min of stimulation (Fig. 1A, lanes 2, 4, and 6 ; and Fig. 1B).

This study revealed for the first time that macrophages possess many protein kinase activities, even when they are not stimulated with LPS. At least seven electrophoretically-distinct kinases were routinely detected in samples from unstimulated macrophages by the renaturation method, and three additional kinases were detected after LPS stimulation. Because not all protein kinases can regain activity after renaturation, we think more than three kinases may be activated by LPS stimulation. □The renaturation method has an advantage over the usual kinase assay methods in being able to detect many distinct kinases simultaneously[13]. Although it is not yet known whether any of these kinases are related

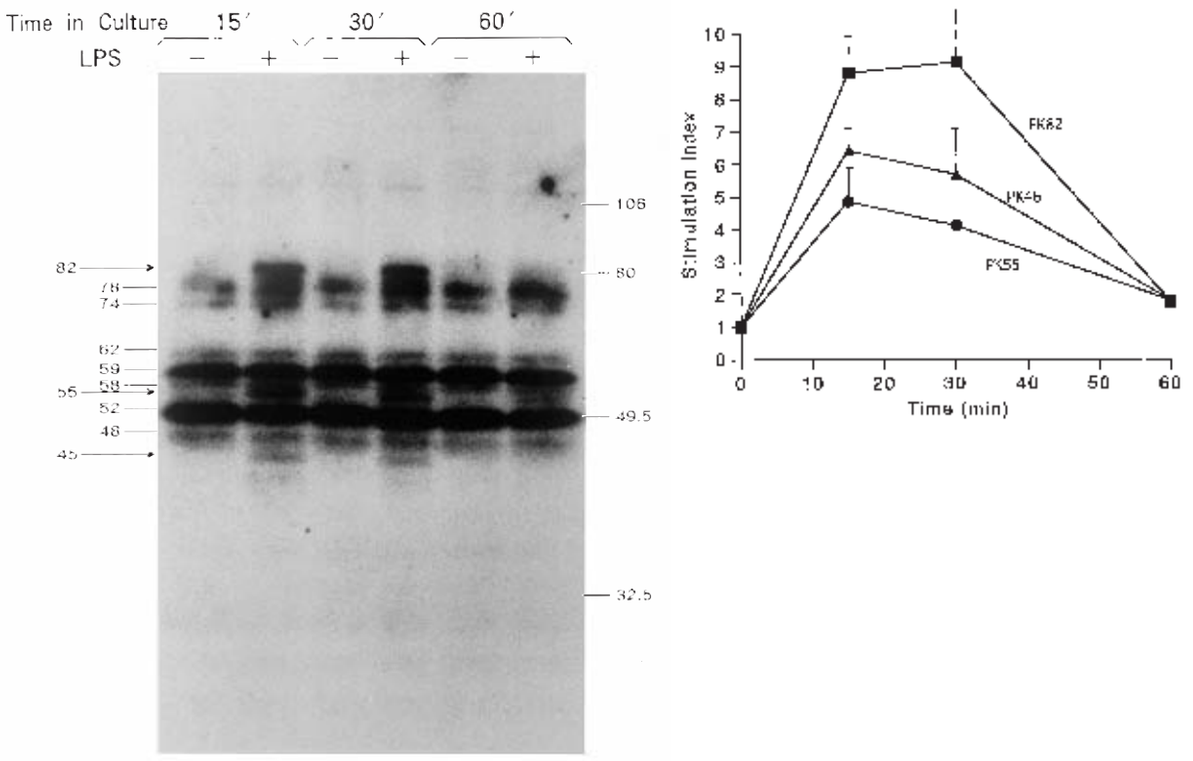

Fig. 1. Renaturation of protein kinases in LPS-stimulated macrophages. Lysates from macrophages cultured in presence or absence of LPS (1 μg/ml) for 15, 30, and 60 min were prepared and subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane, treated with guanidine, allowed to renature, and overlaid with [γ-^{32}P] ATP for 30 min. The PVDF blot was washed, air dried, and subjected to autoradiography. (A) Resultant autoradiogram. Molecular mass markers are indicated in kilodaltons on the right side of the autoradiogram. (B) The bands were quantified by densitometric analysis. Data are expressed as a stimulation index compared with the unstimulated value, which was set at 1.0. Values are expressed as the mean + SD of three separate experiments.

to known kinases, it is intriguing that the apparent molecular masses of the three kinases, PK82, PK55 and PK46, are similar to those of IkappaB kinase 1 (IKK1 ; 85-kDa), protein kinase C (PKC ; 77-kDa), interleukin-1 receptor-associated kinase 1 (IRAK-1 ; 76-kDa), protein kinase A (PKA ; 55-kDa), two mitogen-activated protein kinases, Erk-1 (44-kDa) and c-Jun N-terminal kinase (JNK ; 44-kDa), or casein kinase II (CKII ; 45-kDa). Each of the kinases has been shown or suggested to be rapidly activated by LPS in respective□ reports[6−12,15−18] ; so they may correspond to the three kinases.

Regardless, this study revealed activation of multiple protein kinases, an interesting and previously undocumented finding. Protein phosphorylation induced by LPS has been studied for at least ten years. Many studies, performed under various experimental conditions, have shown that, as a whole, LPS activates many kinases. But none established that multiple kinases are activated by LPS within a single type of cell since each of the studies focused on a particular kinase. Therefore, we examined the spectrum of the LPS-inducible kinases in macrophages. We believe that the findings of the present study are important to understanding the pleiotropic action of LPS on macrophages[2], and could explain why a variety of protein kinase inhibitors differentially suppress the various biological activities of LPS[15−17].

Mechanisms by which LPS can stimulate multiple kinases in macrophages have not been clarified. In this respect, Toll-like receptor 4 (TLR4) has recently been identified as a functional receptor for LPS[18,19]. Several different kinases may exist downstream of the TLR4-initiated pathway, or TLR4-initiated signals may cross-activate kinases in other pathways. Alternatively, as shown by PY Perera, et al. [20], LPS-inducing multimeric receptor complexes, which can elicit complex patterns of signaling, com-

posed of CD14, TLR4 and CD11b/CD18 may lead to the activation of multiple kinases.

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