Arch. Pharm. Res. Vol.18, No.4, pp. 262-266, 1995

The Involvement of Protein Tyrosine Kinase in the Bacterial Lipopolysaccharide-induced Arachidonic Acid Metabolism in Rat Alveolar Macrophages

Ji-Young Kim*, Soo-Hwan Lee*, Ji-Young Lee, Chang-Hyun Moon*, Jong-Seok Lim and Chang-Kiu Moon**

*College of Pharmacy, Seoul National University, Seoul 151-742, Korea, *Department of Physiology, School of Medicine, Ajou University, Suwon 442-749, Korea and **Korea Cancer Center Hospital 215-4, Gongneung-Dong, Nowon-Gu, Seoul 139-240, Korea*

(Received June 13, 1995)

Bacterial lipopolysaccharide (LPS) is one of the most potent inducers of various cytokines and other proinflammatory mediators .in macrophages. Although pathophysiological consequences of LPS-induced responses are well established, the mechanisms through which LPS-generated signals are transduced remain unclear. In the present study, we attempted to determine early intracellular events after LPS binding which transduced the signal for the induction of arachidonic acid metabolism in rat alveolar macrophages. While H-7, a protein kinase C (PKC) inhibitor, did not affect LPS-stimulated prostaglandin synthesis, staurosporine enhanced arachidonic acid metabolism in macrophages treated with LPS. Phorbol-12-myristate-13 acetate (PMA) significantly inhibited the action of LPS. In high glucose media, macrophages were less sensitive to LPS compared with control group. PMA and H-7 did not alter the effect of glucose. Pertussis toxin did not show any effect, thus pertussis toxin sensitive G-protein pathway appears not to play a role in this experimental system. Genistein and tyrphostin 25, protein tyrosine kinase (PTK) inhibitors, markedly inhibited prostaglandin synthesis in macrophages stimulated with LPS. These data suggest the roles of PKC and PTK activation in endotoxin signal transduction events leading to increased macrophage arachidonic acid metabolism.

Key words : Lipopolysaccharide, Arachidonic acid metabolism, Alveolar macrophage, Protein kinase C, Protein tyrosine kinase

INTRODUCTION

Lipopolysaccharide (LPS), a major component of the cell wall of gram negative bacteria, elicits various pathophysiologic responses including hypotension, disseminated intravascular coagulation, multiple organ failure, etc. (Morrison, 1987). While the action of LPS appears to have serious clinical implications, the path of signal transduction between the interaction of LPS with cell membrane and the synthesis and release of the mediators is yet poorly understood. The best charcterized mechanism of LPS induced cell activation is a soluble LPS receptor system. LPS binding protein (LBP) and CD14 exert a crucial role in triggering cellular responses induced by LPS (Gallay *et al.*, 1993). The routes along which LPS stimulates various cellular responses are not all understood, but it would appear that signaling is not identical for all LPS responsive cells. Furthermore, individual cell types may respond through more than one signaling pathway (Waga *et al.*, 1993; Weinhold *et al.*, 1991; Matsunaga *et al.*, 1990).

Recently, we and others reported that LPS selectively induced the expression of prostaglandin H synthase-2 (PGHS-2) in rat alveolar macrophages by which it markedly enhanced prostaglandin synthesis (Lee *et al.*, 1992). Although several putative regulators have been presented, the specific cellular and molecular mechanisms leading to induction of PGHS-2 expression in response to endotoxin and endotoxin priming are not fully elucidated. In the present study, we attempted to determine the early intracellular signal transduction pathway after LPS binding that induces the expression of PGHS-2 in rat alveolar macrophages.

Correspondence to: Chang-Kiu Moon, College of Pharmacy, Seoul Natinoal University, Seoul 151-742, Korea.

MATERIALS AND METHODS

Materials

Lipopolysaccharide from *Escherichia coli* (0111:B4), genistein, staurosporine, H-7, pertussis toxin, phorbol-12-myristate-13-acetate (PMA) and tyrphostin 25 were purchased from Sigma Chemical Co. (St. Louis, MO). Medium RPMI 1640 and heat inactivated fetal calf serum were obtained from GIBCO (Grand Island, NY). [^{3}H]-thromboxane B_2 was from DuPont NEN (Boston, MA). Other reagents were of reagent grade.

Isolation of macrophages

Sprague-Dawley rats, weighing 160-180 g, were supplied by experimental animal center of Seoul National University. Alveolar macrophages were collected by bronchoalveolar lavage as described by Chandler and Fulmer (1987). Cell viability as determined by trypan blue dye exclusion was greater than 90%. More than 95% of lavaged cells were macrophages as determined by differential counting. Cells in RPMI 1640 media (GIBCO) were allowed to attach to 24 well plates (NUNC) for 24 hours (37°C, 5% CO_2).

Assay for PGH synthase activity

Cells were allowed to adhere in the presence of aspirin (500 M) in RPMI 1640 media for 2 hours. Cells were washed twice with cold phosphate buffered saline and incubated in RPMI 1640 containing 3% fetal calf serum with indicated treatment for 16 hours otherwise mentioned. Spent medium was stored at -20°C and cells were incubated in the fresh medium containing arachidonic acid (30 M) for 10 min to determine PGH synthase activity as described by Fu *et al* (1990). Levels of TXB_2 were determined by radioimmunoassay.

Statistical analysis

Each result represents the mean±SEM and statistical analysis was performed by an analysis of variance and subsequent Duncan's Multiple Range Tests.

RESULTS AND DISCUSSION

Bacterial lipopolysaccharide triggers a variety of cellular and extracellular host responses including production and release of mediators such as cytokines and arachidonic acid, which are believed to be responsible for the pathogenesis of septic shock (Morrison, 1987). Mechanism by which LPS activates macrophages and other cells to induce gene expression are now being explored. The best characterized mechanisms of LPS mediated cellular events

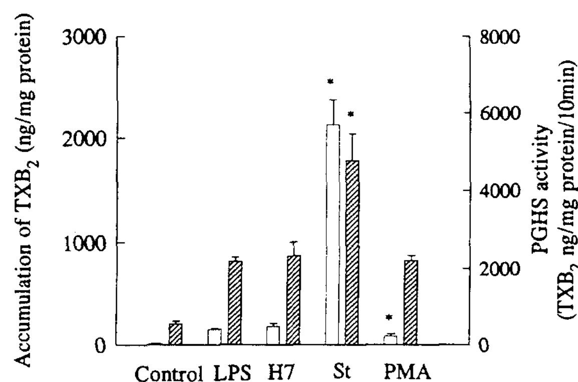

Fig. 1. Effects of protein kinase C (PKC) regulators on TXB_2 synthesis and PGHS activity in alveolar macrophages stimulated with LPS. Isolated macrophages were incubated with H-7 (50 μM), staurosporine (St, 50 nM) or phorbol-12-myristate-13-acetate (PMA, 50 nM) in RPMI 1640 media containing LPS (10 μg/ml) for 2 hrs. And then, cells were washed with cold phosphate buffered saline twice and further incubated in the same media without PKC regulators for 14 hrs. Accumulated levels of TXB_2 synthesized from endogenous arachidonic acid were determined from spent media (□). After removal of the spent media, cells were incubated with arachidonic acid (30 μM) for 10 min, and the levels of TXB_2 were measured by radioimmunoassay (▨). Results represent mean±SEM of triplicate samples. *$p<0.01$ vs LPS treated control

are LPS-binding protein (LBP) and CD14 molecule system (Gallay *et al.*, 1993). The interaction of LPS-LBP complex and membrane associated CD14 results in increased expression of several cytokines through effects on the rate of transcription and mRNA stability. However, the early intracellular events after LPS binding that cause LPS-mediated gene expression have not been characterized in detail.

It has been suggested that activation of protein kinase C (PKC) play a role in endotoxin stimulated arachidonic acid metabolism (Matsunaga *et al*, 1990; Weinhold *et al.*, 1991; Geisel *et al.*, 1991). But their works were primarily on the priming effects of LPS and they did not correlated these results with specific enzymatic steps involved in eicosanoid metabolism. Thus, we examined the effects of PKC inhibitors and stimulator on LPS-induced arachidonic acid metabolism in relation to PGHS activity in rat alveolar macrophages. As shown in Fig. 1, PMA, a PKC stimulator, significantly inhibited the prostaglandin synthesis stimulated by LPS regardless of treating time. But PMA alone could not stimulate the prostaglandin synthesis (data are not shown, Kim, 1995). This result coincides with other reports in which PMA increased arachidonic acid metabolism in peritoneal macrophages but this was not the case in alveolar macrophage (Marc *et al.*, 1990). In contrast, staurosporine, a PKC inhibitor, stimulated thromboxane synthesis in macrophages activated with LPS and it enhanced the activity of PGHS (Fig. 1). But because

staurosporine induced the expression of PGHS-2 even in the absence of LPS (Moon *et al.*, 1995), it could not be addressed whether these effects were specifically due to the inhibtion of PKC. H-7, another type of PKC inhibitor, showed minimal effects, if any, on thromboxane synthesis and PGHS activity in the same system (Fig. 1). In our preliminary study, the addition of glucose to the culture media decreased LPS-stimulated prostaglandin synthesis in a dose dependent manner in rat alveolar macrophages (data are not shown, Kim, 1995). It is well known that PKC could be activated by the elevation of glucose concentration (Lee *et al.*, 1989). Thus, these data implicated glucose sensitive PKC isozyme might have regulatory role in LPS elicited responses, especially, the increase of arachidonic acid metabolism. PMA and H-7 did not alter the effect of glucose on prostaglandin synthesis and the activity of PGHS in this particular system (Fig. 2). Taken together, it could be concluded that H-7 insensitive, glucose sensitive PKC isozyme(s) is somehow involved in the regulation of LPS-induced arachidonic acid metabolism, but detailed nature of responsible PKC(s) remains to be elucidated.

The G-protein serves as signal transducers, linking extracellularly oriented receptors to membrane bound effectors. Activation of certain cell types by LPS is dependent upon specific binding sites on cellular membrane. Moreover, the mechanism underlying the cellular action of LPS on macrophages involves activation of GTP-binding protein (G-protein) that is sensitive to pertussis toxin inhibition (Coffee, 1990). Therefore, in order to explore the possible involvement of G-protein in LPS mediated prostaglandin sysnthesis, the effects of pertussis toxin were examined. Pertussis toxin did not showed any noticeable effect on prostaglandin synthesis and the activity of PGHS under our experimental system (Fig. 3). Our data did not coincide with other reports. (Wang *et al.*, 1988) suggested that LPS-induced PGE_2 sysnthesis in rat glomerular mesengial cells was mediated through G-protein coupled phospholipase A_2 activation. And also, it was reported that pertussis toxin inhibited TXB_2 synthesis in mice peritoneal macrophages stimulated with LPS and cholera toxin, a Gs-protein activator, showed opposite result (Coffee *et al.*, 1990). These apparent discrepancies may be explained by differences in experimental conditions. It has been suggested that LPS exerts differential effects depending on the cell types. Alveolar macrophages are usually insensitive to phorbol ester, but peritoneal macrophages increase arachidonic acid metabolism upon stimulation of PMA (Marc *et al.*, 1990). The dosage of LPS is another factor to be considered. We used somewhat high cocentration of LPS (10 μg/ml), on the other hand, others used low doses (10-100 ng/ml) to stimulate cells. Usually LPS requires soluble binding proteins to elicit biological responses. However, very high concentration of LPS (more than 1 μg/ml) could stimulate cytokine production in the absence of LBP or CD14 (Raetz *et al.*, 1991). Anyhow, pertussis toxin sensitive G protein ap-

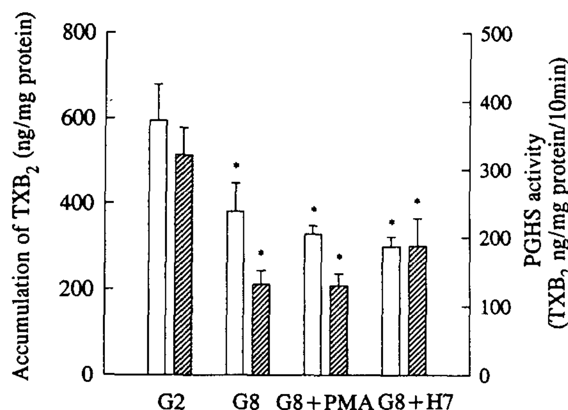

Fig. 2. Effects of PMA and H-7 on TXB_2 synthesis and PGHS activity in alveolar macrophages incubated in high glucose medium in the presence of LPS. Isolated macrophages were incubated with phorbol-12-myristate-13 - acetate (PMA, 50 nM) or H-7 (50 μM) in the presence of LPS (10 μg/ml) in RPMI 1640 containing 8 mg/ml glucose (G8) for 2 hrs. And then, cells were washed with cold phosphate buffered saline twice and further incubated in the same media without PMA or H-7 for 14 hrs. Accumulated levels of TXB_2 synthesized from endogenous arachidonic acid were determined from spent media (□). After removal of the spent media, cells were incubated with arachidonic acid (30 M) for 10 min, and the levels of TXB_2 were measured by radioimmunoassay (▨). Results represent mean±SEM of triplicate samples. *$p<0.05$ vs 2 mg/ml glucose treated group (G2).

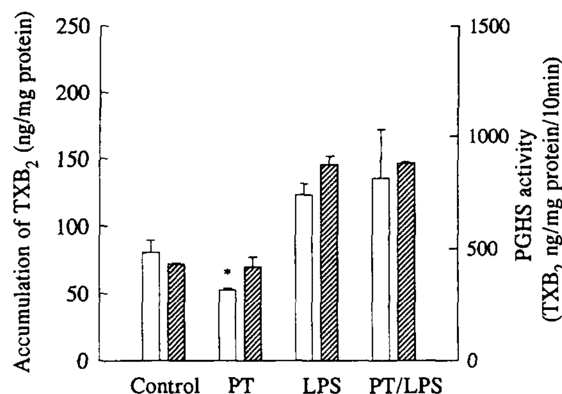

Fig. 3. Effects of pertussis toxin on TXB_2 synthesis and PGHS activity in alveolar macrophages stimulated with LPS. Isolated macrophages were incubated with pertussis toxin (PT, 2 μg/ml) in the presence or absence of LPS (10 μg/ml) for 16 hrs. Accumulated levels of TXB_2 synthesized from endogenous arachidonic acid were determined from spent media (□). After removal of the spent media, cells were incubated with arachidonic acid (30 μM) for 10 min, and the levels of TXB_2 were measured by radioimmunoassay (▨). Results represent mean±SEM of triplicate samples. *$p<0.05$ vs control.

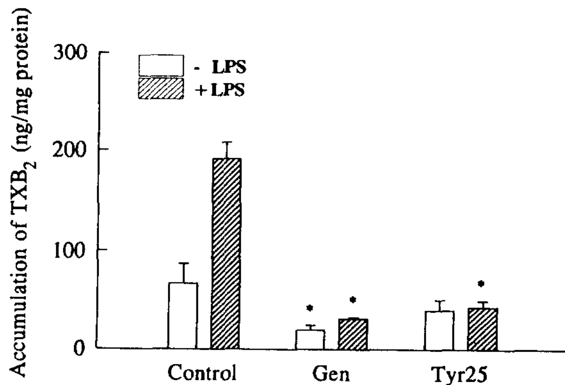

Fig. 4. Effects of genistein and tyrphostin 25 on TXB_2 synthesis in alveolar macrophages stimulated with LPS. Isolated macrophages were incubated with genistein (Gen, 100 μM) or tyrphostin 25 (Tyr25, 100 M) in the presence or absence of LPS (10 μg/ml) for 16 hrs. Accumulated levels of TXB_2 synthesized from endogenous arachidonic acid were determined from spent media. Results represent mean± SEM of triplicate samples. *p<0.01 vs control

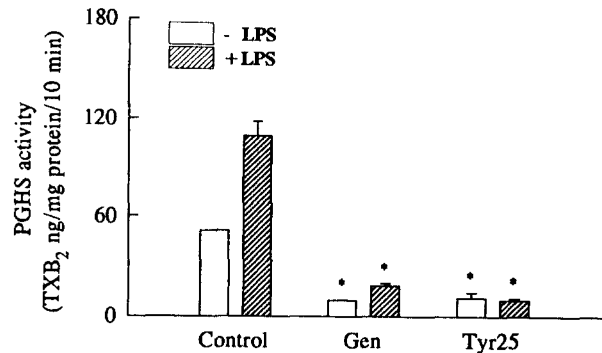

Fig. 5. Effects of genistein and tyrphostin 25 on PGHS activity in alveolar macrophages stimulated with LPS. Isolated macrophages were incubated with genistein (Gen, 100 μM) or tyrphostin 25 (Tyr25, 100 μM) in the presence or absence of LPS (10 μg/ml) for 16 hrs. After removal of the spent media, cells were incubated with arachidonic acid (30 μM) for 10 min, and the levels of TXB_2 were measured by radioimmunoassay. Results represent mean±SEM of triplicate samples. *p< 0.01 vs control.

parently have no role in LPS-stimulated prostaglandin synthesis in rat alveolar macrophages under our experimental conditions.

Recently, it has been demonstrated that LPS increases protein tyrosine phosphorylation and protein tyrosine kinase (PTK) activity in macrophages (Weinstein *et al.*, 1992; Boulet *et al.*, 1992). Protein tyrosine phosphorylation is required for the induction of NF-κB which has important regulatory roles in the induction of cytokine gene expression by LPS (Geng *et al.*, 1993). However, the protein kinases that are responsible for the LPS induction of cytokine genes in macrophages have not been identified and it is not clear which macrophage functional responses to LPS are associated with the activation of PTK. In the present study, we attempted to determine whether PTK is involved in the induction of arachidonic acid metabolism by LPS. Genistein and tyrphostin are well known inhibitors of PTK. Both of them markedly inhibited thromboxane synthesis and the activity of PGHS in LPS-stimulated macrophages under our experimental conditions (Fig. 4, Fig. 5). In a separate experiment, they did not show cytotoxic activity against alveolar macrophages in concentration range used in this experiment (data are not shown, Kim, 1995). Our result is supported by other report that tyrphostin dose-dependently inhibited PGE_2 synthesis in peritoneal macrophages primed with LPS (Glaser *et al.*, 1993). It is generally accepted that PTK is required for LPS induction of broad spectrum of cytokines and NF-κB activation in monocytes (Geng *et al.*, 1993). Recent evidences also support the role of mitogen activated protein (MAP) kinases in LPS induced cellular responses (Han *et al.*, 1994, Cordle *et al.*, 1993). In our experimental system, LPS selectively induces PGHS-2 gene expression by which it enhances the prostaglandin synthesis (Lee *et al.*, 1992). Simmons *et al.* showed that putative NF-κB binding element is located at the promotor region of PGHS-2 gene (Simmons *et al.*, 1993). Therefore, it is conceivable that LPS activates NF-κB through stimulating PTK such as MAP kinase cascade, by which it induces the expression of PGHS-2.

In summary, PTK appears to be essential in macrophage activation responses to LPS including induction of arachidonic acid metabolism. On the other hand, pertussis toxin sensitive G-protein pathway seems not to play a role in signal transduction in this particular experimental system. Protein kinase C appears to be involved in the regulation of prostaglandin synthesis in alveolar macrophages stimulated with LPS, but detailed nature of its role remains to be elucidated.

ACKNOWLEDGEMENTS

This work was partly supported by the grant from the Ministry of Health and Welfare, Korea.

REFERENCES CITED

Boulet, I., Ralph, S., Stanley, E., Lock, P., Dunn, A.R., Green, S.P. and Philips, W.A. Lipopolysaccharide and interferon gamma induced expression of hck and lyn tyrosine kinases in murine bone marrow derived macrophages. *Oncogene.* 7, 703-711 (1992).

Chandler, D.B. and Fulmer, J.D. Prostaglandin synthesis and release by subpopulations of rat alveolar macrophages. *J. Immunol.* 139, 893-898 (1987).

Coffee, K.A. Halushka, P.V., Wise, G.E. and Cook, J.

A. Altered responses to modulators of guanine nucleotide binding protein activity in endotoxin tolerance. *Biochim. Biophys. Acta.* 1035, 201-205 (1990).

Cordle, S.R., Donald, R., Read, M.A. and Hawiger, J. Lipopolysaccharide induces phosphorylation of MAD3 and activation of c-Rel and related NF-κB proteins in human monocytic THP-1. *J. Biol. Chem.* 268, 11803-11810 (1993).

Fu, J.Y., Masferrer, J.L., Seibert, K., Raz, A. and Needleman, P The induction and suppression of prostaglandin H synthase in human monocytes. *J. Biol. Chem.* 265, 16737-16740 (1990).

Gallay, P., Heumann, D., LeRoy, D., Barras, C. and Glauser, M.P. Lipopolysaccharide binding protein as a major plasma protein responsible for endotoxemic shock. *Proc. Natl. Acad. Sci. USA.* 90, 9935-9938 (1993).

Geisel, J., Cook, J.A., Coffee, K.A., Wise, W.C. and Halushka, P.V. Endotoxin induced arachidonic acid metabolism requires de novo protein synthesis and protein kinase C activation. *Biochim. Biophys. Acta.* 1085, 15-20 (1991).

Geng, Y., Zhang, B. and Lotz, M. Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J. Immunol.* 151, 6692-6700 (1993).

Glaser, K.B., Sung, A., Bauer, J. and Weichman, M. Regulation of eicosanoid biosynthesis in the macrophages. *Biochem. Pharmacol.* 45, 711-721 (1993).

Han, J., Lee, J.D., Bibb, L and Ulevitch, R.J. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science* 265, 808-810 (1994).

Kim, J.Y. Studies on the prostaglandin synthesis stimulated by bacterial lipopolysaccharide in rat alveolar macrophages. Ph.D. thesis, Seoul National University (1995).

Lee, S.H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W., Zhong, H., Liou, S., Simmons, D. and Hwang, D. Selective expression of mitogen inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *J. Biol. Chem.* 267, 25934-25938 (1992).

Lee, T.A., Saltsman, K.A., Ohashi, H. and King, G.L. Activation of protein kinase C by elevation of glucose concentration. *Proc. Natl. Acad. Sci. USA.* 86, 5141-5145 (1989).

Marc, P.G. McNish, R.W., Brieland, J.K. and Fantone, J.C. Diminished protein kinase C activated arachidonate metabolism accompanies rat macrophage differentiation in the lung. *J. Immunol.* 144, 4320-4326 (1990).

Matsunaga, A., Miller, B.C. and Cottam, G.L. Pertussis toxin and H-7 distinguish mechanisms involved in eicosanoid release from lipopolysaccharide primed macrophages. *Eur. J. Biochem.* 187, 599-603 (1990).

Moon, C.K., Lee, S.H., Kim, J.Y., Kim, M.J., Lee, J.Y. and Moon C.H. Staurosporine induces de novo synthesis of prostaglandin synthase-2 in rat alveolar macrophage. *Life Sciences,* in press, (1995).

Morrison, D.C. Endotoxins and disease mechanisms. *Ann. Re. Med.* 38, 417-432 (1987).

Raetz, C.R.H., Ulevitch, R.J., Wright, S.D., Sibley C. H., Ding, A. and Nathan, C.F. Gram negative endotoxin: an extraodinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* 5, 2652-2660 (1991).

Simmons, D.L., Xie, W., Evett, G., Merrill, J., Robertson, D.L. and Bradshaw, W.S. Drug inhibition and cellular regulation of prostaglandin G/H synthase isozyme 2. *J. Lipid Mediators* 6, 113-117 (1993).

Waga, I., Nakamura, M., Honda, Z., Ferby, I., Toyoshima, S., Ishiguro, S. and Shimizu, T. Two distinct signal transduction pathways for the activation of guinea pig macrophages and neutrophils by endotoxin. *Biochem. Biophys. Res. Comm.* 197, 465-472 (1993).

Wang, J., Kester, M. and Dunn, M.J. Involvement of a pertussis toxin sensitive G-protein coupled phospholipase A_2 in lipopolysaccharide stimulated prostaglandin E_2 synthesis in cultured rat mesengial cells. *Biochim. Biophys. Acta* 963, 429-435 (1988).

Weinhold, L., Schulze-Specking, A. and Decker, K. Signal transduction in endotoxin stimulated synthesis of TNF and prostaglandin E_2 by rat kupffer cells. Biol. Chem. *Hoppe-Seyler* 372, 829-834 (1991).