Lysophosphatidylcholine Stimulates the Expression and Production of MCP-1 by Human Vascular Endothelial Cells

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Lysophosphatidylcholine (LPC) increased monocyte chemoattractant protein-1 (MCP-1) messenger RNA concentrations in human umbilical vein endothelial cells (HUVECs). A time-course study showed that the increase in MCP-1 mRNA levels peaked at 6 hours after treatment with LPC. The effect of LPC on the accumulation of MCP-1 mRNA levels in HUVECs depended on LPC concentration, and the maximal effect was obtained at 50 μmol/L LPC, which induced a sixfold increase in MCP-1 mRNA levels. The amount of MCP-1 released from HUVECs measured using an enzyme-linked immunosorbent assay (ELISA) showed a 38% increase in the presence of 50 μmol/L LPC, but not in the presence of phosphatidylcholine or lysophosphatidylethanolamine. Coincubation with staurosporine, a potent inhibitor of protein kinase C (PKC) activity, attenuated the LPC-induced increase in MCP-1 mRNA levels by 53%. These results indicate that LPC can induce an increase in MCP-1 mRNA concentrations and stimulate the release of MCP-1 protein from HUVECs, and that the effect of LPC on the MCP-1 gene may be mediated through activation of the PKC pathway.

THE EARLIEST recognizable lesion in atherosclerosis is the fatty streak, characterized by the accumulation of foam cells in the subendothelial space.[1] It has already been shown that many of these cells are derived from circulating monocytes that have migrated to and invaded the subendothelial space in the initial stages of the disease.[2] Therefore, it is postulated that the migration of monocytes may be induced by certain monocyte chemotactic factors released from the endothelium.

Monocyte chemoattractant protein-1 (MCP-1) is a secreted molecule with specific chemotactic activity for monocytes.[3] MCP-1 mRNA has been shown to be expressed in human atherosclerotic plaques in vivo.[4] Furthermore, arterial tissues isolated from cholesterol-fed animals also show strong expression of MCP-1 mRNA as compared with that in control animals.[5] These data indicate that MCP-1 plays a significant role in mediating monocyte recruitment in atherosclerotic lesions. Recently, Takeya et al[6] reported that endothelial cells in the fatty streak showed strong positive immunostaining for MCP-1, although endothelial cells in advanced atherosclerotic lesions stain only weakly. These results suggest that MCP-1 is expressed in the endothelium at a time during evolving atheroma formation.

Previous studies have suggested that oxidized low-density lipoprotein (LDL) exists in vivo and is atherogenic.[7,8] These atherogenic effects can be mediated partly through the alteration of vascular endothelial cell functions. For example, oxidized LDL inhibits endothelium-dependent arterial relaxation,[9] stimulates monocyte–endothelial cell interactions,[10] and abolishes prostacyclin release by endothelial cells.[11] However, it has been reported that both phospholipid and protein moieties can be modified during the oxidation of LDL.[12] Of these modifications, lysophosphatidylcholine (LPC) has been shown to be specifically produced through the activation of phospholipase A_2 activity during oxidation of LDL,[13,14] and this molecule can modulate endothelial cell functions. Therefore, in the present study, we investigated whether LPC could modulate MCP-1 gene expression and its secretion from human vascular endothelial cells. Moreover, we also examined a possible signal transduction pathway for MCP-1 gene expression in endothelial cells.

MATERIALS AND METHODS

Materials

LPC (palmitoyl), phosphatidylcholine, lysophosphatidylethanolamine, and platelet-activating factor (PAF) were obtained from Sigma Chemical (St Louis, MO). Restriction endonucleases and DNA-modifying enzymes were purchased from Toyobo (Osaka, Japan), and recombinant Taq DNA polymerase was purchased from Perkin-Elmer Cetus (Norwalk, CT). Oligonucleotide primers were synthesized on an Applied Biosystem 380A synthesizer (Obu Medical, Osaka, Japan). Anti–human MCP-1 antibody (polyclonal) was purchased from Peprotech (Rocky Hill, NJ).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured according to the method described by Jaffe et al.[15] Cells were grown in minimal essential medium (MEM) supplemented with 10% fetal calf serum ([FCS] Gibco, Grand Island, NY), 90 μg/mL heparin (NOVO Nordisk, Copenhagen, Denmark), and 50 μg/mL endothelial cell growth factor (Boehringer, Mannheim, Germany) under a humidified atmosphere containing 5% CO_2. The cells were used before passage 6. Cells were incubated with LPC complexed to fatty acid–free bovine serum albumin ([BSA] A-8806; Sigma) at an LPC to BSA molar ratio of 2 in phosphate-buffered saline (PBS). LPC-BSA complex was dissolved in the above medium, except with 5% FCS instead of 10%. After incubation for the indicated periods, total RNA was isolated as described later.

Polymerase Chain Reaction and Subcloning of MCP-1 cDNA

Total RNA from HUVECs was isolated by acid guanidium thiocyanate-phenol-chloroform extraction (AGPC)[16] and converted to single-stranded cDNA by oligo-dT priming and M-MLV reverse transcriptase (Superscript preamplification system; BML, Gaithersburg, MD). Polymerase chain reaction (PCR) products were amplified with Taq DNA polymerase for 30 cycles consisting of denaturation at 95°C (15 seconds), annealing at 58°C (5

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seconds), and extension at 72°C (20 seconds). The synthetic oligomers used for amplification were based on the published human MCP-1 nucleotide sequence[17]:(5′) (5′) 5′GCGGATCCCCTCCAGCATGAAAGTCTCT3′ and (3′) 5′ACGAATTCTTCTTGGGTTGTGGAGTGAG3′. PCR products were subcloned into a pUC119 vector, and their sequences were determined using an automated laser fluorescent sequencer (Pharmacia, Milwaukee, WI). A 331-bp human MCP-1 cDNA containing the complete coding sequence was obtained.

Northern Blot Analysis

Total RNA isolated from HUVECs by the AGPC method was electrophoresed through 1% agarose gels containing formaldehyde, and then transferred onto Nytran membranes (NY13; Schleicher and Schuell, Dassel, Germany). The human MCP-1 cDNA was labeled with [α-^{32}P]dCTP (New England Nuclear, Boston, MA) and then hybridized at 65°C overnight in a buffer containing 0.5 mol/L Na_2HPO_4, 1% BSA, 1 mmol/L EDTA, and 7% sodium dodecyl sulfate, followed by washing and autoradiography.

MCP-1 Enzyme-Linked Immunosorbent Assay

ELISA assay of MCP-1 was performed as previously described.[18] Briefly, enzyme-linked immunosorbent assay (ELISA) plates (Immuno Plate Maxisorb; NUNC, Neptune, NJ) were coated with 50 μL/well of anti–human MCP-1 antibody diluted to 3.2 μg/mL with coating buffer (borate-buffered saline: 50 mmol/L H_3BO_3 and 120 mmol/L NaCl, pH 8.6) and incubated overnight at 4°C. The plates were then washed three times with 200 μL/well of wash buffer (PBS containing 0.05% vol/vol Tween 20), followed by blocking with 200 μL/well of a solution containing PBS and 2% BSA for 1 hour at 37°C. The plates were washed three times with 200 μL/well of wash buffer, followed by addition of 50 μL in duplicate of either MCP-1 standards (Recombinant human MCP-1; PeproTech, Rocky Hill, NJ) or samples, and incubated for 1 hour at 37°C. The plates were washed three times, followed by addition of 50 μL/well of biotinylated MCP-1 antibody diluted to 6 μg/mL in wash buffer supplemented with 2% BSA. After incubation for 45 minutes, the plates were washed three times, followed by addition of 100 μL/well of 1:5,000-diluted avidin-horseradish peroxidase (Dako, Carpinteria, CA) in wash buffer supplemented with 2% FCS, and incubated for 30 minutes at 37°C. The plates were washed three times, followed by addition of 100 μL peroxidase substrate, and color development proceeded for 6 minutes at room temperature. Absorbance at 492 nm was then measured on a Biotek-ELISA reader. A linear standard curve was obtained between 0.1 and 50 ng/mL. The coefficient of variation of this measurement was 7.4% in intraassay and 10.6% in interassay measurements. To measure intracellular MCP-1 levels, cells were incubated with 50 μmol/L LPC for 24 hours and harvested by scraping with wash buffer (100 mg protein/mL) on ice. Then cells were sonicated and centrifuged at 100,000 × *g* for 1 hour, and the resultant supernatant was used for ELISA measurement. Cellular protein was measured by a Bio-Rad protein assay kit (Richmond, CA).

Statistical Analysis

All data are expressed as the mean ± SEM. The significance of differences between means for two groups was estimated by Student's *t* test, and a *P* value less than .05 was considered significant.

RESULTS

MCP-1 mRNA Expression in HUVECs

Total cellular RNA was isolated from control or 50-μmol/L LPC–treated HUVECs and probed for MCP-1 message. MCP-1 mRNA was detectable in control HUVECs and markedly increased after treatment with 50 μmol/L LPC (Fig 1). The accumulation of mRNA peaked at 6 hours and declined to a nearly constitutive level after 24 hours. LPC had no significant effect on glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA levels in HUVECs. The effect of LPC concentration on MCP-1 mRNA induction was examined (Fig 2). The increase in MCP-1 mRNA levels depended on LPC concentration when cells were incubated for 6 hours in the presence of LPC between 0 and 50 μmol/L. Densitometric analysis of Northern blots indicated a 6.0 ± 1.9-fold increase in the expression after exposure to 50 μmol/L LPC for 6 hours.

Production of MCP-1 Protein by Endothelial Cells

To determine whether LPC significantly stimulated the production of MCP-1 protein by HUVECs, the amount of MCP-1 protein secreted into media was measured using ELISA. The secreted MCP-1 protein was accumulated linearly for 24 hours (time 0, 12, 24, and 48 hours: 0.05, 8.6, 15.0, and 14.8 ng/mL) and intracellular MCP-1 was undetectable in cells treated with or without LPC. During the incubation for 24 hours, cells untreated with LPC constitutively secreted MCP-1 protein into the media (15.2 ± 0.3 ng/24 h/mg cellular protein), and 50 μmol/L LPC significantly stimulated the production (20.9 ± 0.5 ng/24 h/mg cellular protein) (Table 1). Although it is known that LPC has detergent-like or cytotoxic properties, the release of lactate dehydrogenase from HUVECs treated with 50 μmol/L LPC for 24 hours was not increased as compared with release from control cells (control *v* 50-μmol/L LPC–treated, 22.2 ± 2.4 *v* 26.5 ± 2.5 U/L medium).

Specificity of LPC on MCP-1 Production

To clarify the specificity of LPC, the effects of other phospholipids on MCP-1 production were tested (Table 1). Neither phosphatidylcholine nor lysophosphatidylethanolamine were effective, whereas 50 nmol/L PAF significantly induced MCP-1 production.

Effects of a Protein Kinase C Inhibitor on LPC-Induced MCP-1 mRNA Expression

To investigate further the mechanism of LPC-induced MCP-1 gene expression, the effects of staurosporine, a potent inhibitor of protein kinase C (PKC) activity, were examined. Cells were incubated with 50 μmol/L LPC in the presence or absence of 0.2 nmol/L staurosporine for 6 hours followed by RNA isolation. The concentration of staurosporine (0.2 nmol/L) used in this study was similar to those previously reported to be specific for PKC inhibition. Staurosporine inhibited the 50-μmol/L LPC–induced increase in MCP-1 mRNA levels by 53% ± 7%, suggesting that LPC stimulated MCP-1 mRNA expression in part through the PKC signal transduction pathway (Fig 3).

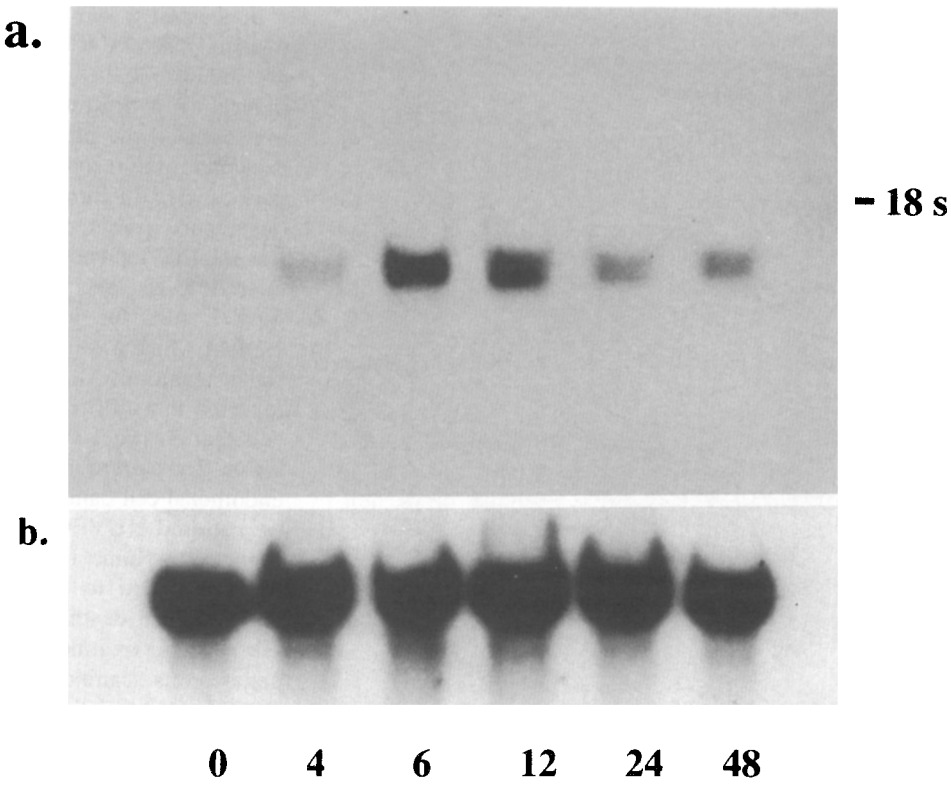

Fig 1. Time course of the increase in MCP-1 mRNA levels induced by LPC in HUVECs. HUVECs were treated with 50 μmol/L LPC for the times indicated. Total RNA was then isolated and Northern blot analysis was performed with MCP-1 (a) and glyceraldehyde-3-phosphate dehydrogenase (b) probes. A representative of 3 experiments is depicted.

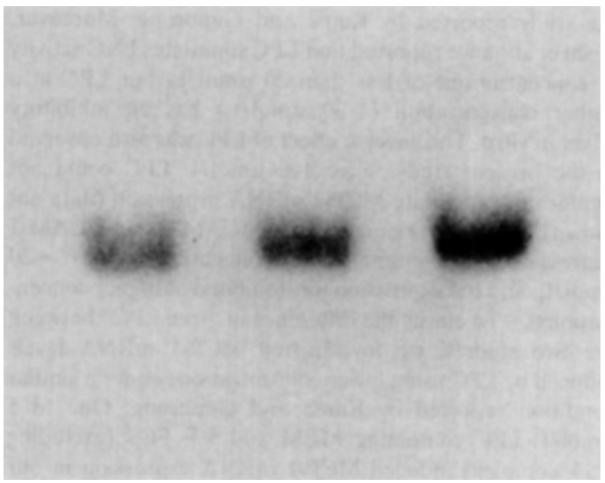

Fig 2. Dependence of MCP-1 mRNA expression on LPC concentration. HUVECs were incubated for 6 hours with the indicated concentrations of LPC. Total RNA was isolated and Northern blot analysis was performed. Mean increases in densitometric units were calculated from 8 experiments.

DISCUSSION

The results of the present study demonstrated that LPC can induce MCP-1 gene expression in HUVECs. The increase in MCP-1 mRNA concentration peaked at 6 hours after treatment with 50 μmol/L LPC. There appears to be a significant basal level of MCP-1 expression before LPC addition. This is somewhat in contrast to the finding that MCP-1 is suggested to be a relatively silent gene in vascular endothelial cells. However, in the present study, we cultured endothelial cells with MEM containing ECGF, heparin, and 5% FCS to protect the cytotoxic property of LPC. Levels of basal MCP-1 mRNA expression were twofold higher than in cells cultured in the absence of ECGF and heparin (data not shown). Similarly, it has also been reported that vascular endothelial cells constitutively se-

Table 1. Specificity of Various Phospholipids on MCP-1 Production

Treatment	MCP-1 Production (ng/24 h/mg cellular protein)	No.
Control	15.2 ± 0.3	10
50 μmol/L LPC	20.9 ± 0.5*	10
50 μmol/L Phosphatidylcholine	15.4 ± 0.2	4
50 μmol/L Lysophosphatidylethanolamine	15.7 ± 0.6	4
50 nmol/L PAF	23.3 ± 0.2*	4

NOTE. Data are the mean ± SE. Cells were stimulated with the various treatments for 24 hours. After incubation, culture medium was collected and MCP-1 content was assayed.

*$P < .001$ *v* control.

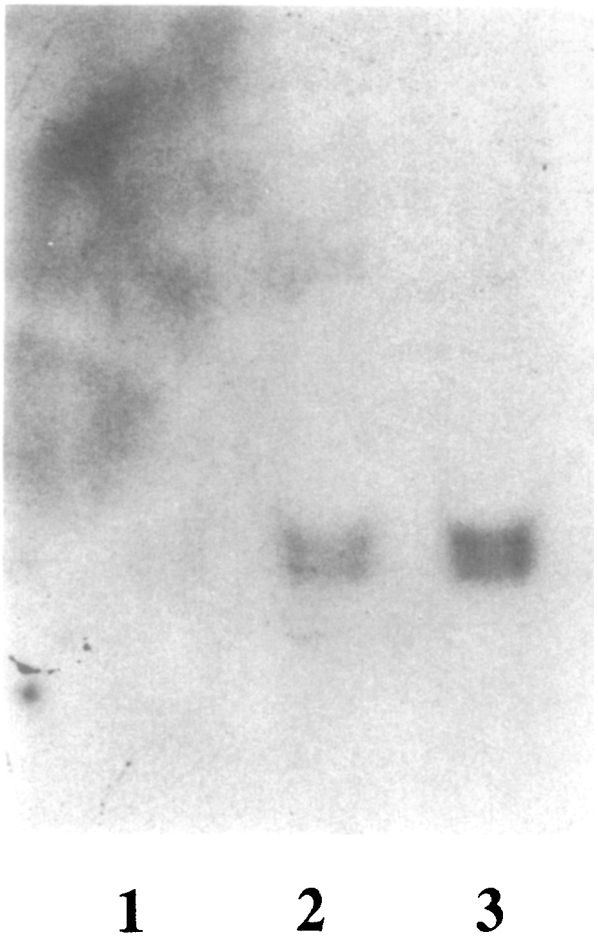

Fig 3. Effects of PKC activity on MCP-1 gene expression. Total RNA was isolated from HUVECs either untreated (lane 1) or treated with 50 μmol/L LPC (lane 3) or in combination with 50 μmol/L LPC and 0.2 nmol/L staurosporine (lane 2) for 6 hours. A representative of 3 experiments is shown.

creted MCP-1 protein in an in vitro system.[18] These findings suggest that some growth factor contained in FCS or ECGF may affect a significant basal expression of MCP-1.

LPC content in plasma obtained from healthy subjects has been reported to be 143 ± 22 μmol Pi/L.[19] LPC is generated from phosphatidylcholine by phospholipase A_2 (PLA_2) activity during oxidation of low-density lipoprotein (LDL).[3,14] Although the activation mechanism of PLA_2 activity is still unclear, LPC concentrations in both plasma and tissue are known to be affected by pathophysiologic conditions.[20,21] For example, patients with non–insulin-dependent diabetes show an increased plasma LPC concentration, although the total phospholipid content of LDL is the same between healthy subjects and such patients.[20] Furthermore, it has been demonstrated that LPC content in atherosclerotic aorta obtained from squirrel monkeys is nearly eight times that in comparable control tissue.[21] These results suggest the possibility that increased amounts of LPC in plasma or tissue can be transferred to the endothelium, where it can modulate endothelial cell gene expression in atherogenesis.

In contrast to our results, Kume and Gimbrone[22] have reported that LPC levels between 25 and 100 μmol/L could not induce MCP-1 mRNA expression in HUVECs. Although it is difficult to explain the differences between the previous and the present study, there are some possible explanations to reconcile the discrepancy. First, incubation periods were different between the two studies. The previous report indicated that cells treated with 25 to 100 μmol/L LPC for 4 hours did not show an increase in MCP-1 mRNA levels. We also found that treatment with 50 μmol/L LPC for 4 hours stimulated mRNA levels by 1.2-fold, which was not significant, but a 6-hour incubation could significantly stimulate them. Second, a previous study reported that LPC can exhibit detergent-like properties[23] and that 50 μmol/L or higher concentrations of LPC in serum-free culture media cause morphologically apparent endothelial cell damage within a few hours. In fact, when we cultured HUVECs under a condition similar to that reported by Kume and Gimbrone (LPC dissolved in ethanol and added to media with 5% FCS), we observed increased cell death (63% decrease in total cellular protein) with the treatment, and the release of lactate dehydrogenase was significantly increased (50-μmol/L LPC–treated *v* 50-μmol/L LPC-BSA complex–treated, 10.1 ± 0.8 *v* 2.8 ± 0.1 U/L medium above the basal level, $P < .01$). This cytotoxic effect of LPC may be explained by a difference of free LPC content between the two studies, since the amount of LPC taken up by endothelial cells depends on the concentrations of both LPC and albumin in the media.[24] Based on these observations, it is assumed that the amount of LPC actually taken up by the cells in our study using the LPC-BSA complex may be less than that in the study reported by Kume and Gimbrone. Moreover, Oishi et al[25] have reported that LPC stimulates PKC activity at concentrations of less than 20 μmol/L, but LPC at a higher concentration (>30 μmol/L) has an inhibitory effect in vitro. This inverse effect of LPC was also observed in the present study, since 100 μmol/L LPC could not significantly stimulate MCP-1 mRNA expression (data not shown), and LPC stimulated both ICAM-1 and VCAM-1 expression (the optimal LPC concentration was ~20 nmol/L, and the expression was inhibited at higher concentrations).[26] To clarify the difference in "free LPC" between the two studies, we investigated MCP-1 mRNA levels induced by LPC using lower concentrations under a similar condition reported by Kume and Gimbrone. One to 5 μmol/L LPC containing MEM and 5% FCS (excluding BSA complex) induced MCP-1 mRNA expression in our HUVECs by twofold the basal level (data not shown). Finally, we observed some variability in the inducibility of MCP-1 mRNA depending on differences in cultured cell lines. In our study, most cell lines showed maximum stimulation at 50 μmol/L LPC, but some cell lines showed maximum stimulation at 10 to 25 μmol/L LPC. A similar variability of VCAM-1 induction by LPC was also reported by Kume et al[27] (threefold to 24-fold increase, depending on cell lines). These results indicate that the difference of

the effect of LPC on the expression of MCP-1 mRNA between the two studies depended on LPC concentrations in the cells and the susceptibility of each cell line to LPC.

We also demonstrated that LPC stimulated the production of MCP-1 protein from HUVECs. These findings confirmed an essential role of LPC for MCP-1 gene expression in these cells. However, the production of MCP-1 protein induced by LPC showed only a 38% increase, whereas LPC increased MCP-1 mRNA levels by sixfold compared with basal levels. The reason for this discrepancy between levels of mRNA expression and protein production remain to be clarified in a future study.

Regarding the specificity of the effect of LPC on MCP-1 production, other phospholipids such as lysophosphatidylethanolamine, phosphatidylcholine, and PAF were examined. Lysophosphatidylethanolamine and phosphatidylcholine, could not induce an increased production of MCP-1. Thus, it is suggested that the stimulating effect was specific for LPC. However, PAF, which is an active phospholipid that causes microvascular leakage, vasodilation, or neutrophil adhesion,[28] can significantly induce MCP-1 production. Since it has been reported that PAF stimulated PKC activity in HUVECs,[29] it is postulated that PAF induces MCP-1 gene expression through the PKC signal transduction pathway. The present study also demonstrated that the PKC pathway may play a significant role in regulating MCP-1 gene expression in HUVECs. Kugiyama et al[30] have reported that LPC derived from oxidized LDL stimulates PKC activity in the membrane fraction of HUVECs. Shyy et al[31] have reported that phorbol esters induce MCP-1 mRNA expression in HUVECs. These results are comparable to our present findings of a significant effect of PKC activation on LPC-induced MCP-1 mRNA expression.

In conclusion, LPC can stimulate the expression and production of MCP-1 in cultured HUVECs. From the results of our study using staurosporine, activation of PKC by LPC may be associated with the induction of MCP-1 mRNA expression.

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