

# Lipoprotein-X stimulates monocyte chemoattractant protein-1 expression in mesangial cells via nuclear factor- $\kappa$ B

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## Lipoprotein-X stimulates monocyte chemoattractant protein-1 expression in mesangial cells via nuclear factor- $\kappa$ B.

**Background.** Lipoprotein-X (Lp-X) is an abnormal lipoprotein found in the plasma of patients with familial lecithin:cholesterol acyltransferase (LCAT) deficiency. The majority of patients with this disorder develop progressive glomerulosclerosis. One key event in the pathogenesis of glomerulosclerosis is the infiltration of monocytes into affected glomeruli. Mesangial cells can synthesize and secrete monocyte chemoattractant protein-1 (MCP-1), an important chemoattractant for monocytes. The objective of the present study was to examine the effect of Lp-X on MCP-1 expression in mesangial cells leading to an enhanced monocyte chemotaxis and to elucidate the mechanisms involved in this process.

**Methods.** Lp-X was isolated from the plasma of a patient with familial LCAT deficiency. After rat mesangial cells were incubated with Lp-X for four or six hours, the expression of MCP-1 mRNA was determined by nuclease protection assay, and MCP-1 protein was measured by Western immunoblotting analysis. Monocyte chemotaxis was determined by using a Micro Chemotaxis Chamber.

**Results.** Lp-X (50 to 100 nmol/mL) stimulated mesangial cell MCP-1 mRNA expression (137 to 220%) and MCP-1 protein levels (233 to 375%). Conditioned media collected from Lp-X-treated mesangial cells stimulated human acute monocytic leukemia (THP-1) monocyte chemotaxis (165 to 200%). The increase in MCP-1 expression in mesangial cells was associated with an elevation of intracellular diacylglycerol levels, and activation of protein kinase C (PKC) as well as nuclear factor- $\kappa$ B (NF- $\kappa$ B).

**Conclusion.** These results suggest that Lp-X participates in the pathogenesis of glomerulosclerosis and subsequent renal failure in familial LCAT deficient patients by stimulating monocyte infiltration via a mechanism involving mesangial MCP-1 expression.

**Key words:** familial LCAT deficiency, glomerulosclerosis, protein kinase C, autosomal-recessive disorder, foam cells, unesterified cholesterol, phosphatidylcholine.

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Familial lecithin:cholesterol acyltransferase (LCAT) deficiency is a rare autosomal-recessive disorder [1]. Patients have little or no LCAT activity in their blood circulation because of mutations in the *LCAT* gene [2–4]. LCAT is an enzyme that circulates in the blood primarily bound to high-density lipoprotein and catalyzes the formation of cholesteryl esters via the hydrolysis and transfer of the sn-2 fatty acid from phosphatidylcholine to the 3-hydroxyl group of cholesterol [1]. Patients with LCAT deficiency have unusually high levels of phosphatidylcholine and unesterified cholesterol, with corresponding low levels of lysophosphatidylcholine and cholesteryl ester in the blood [1]. The lack of active LCAT, with the consequent increase in both phospholipids and cholesterol as well as a decrease in cholesteryl esters, leads to abnormalities in lipoprotein particle size and structure [5–7]. One striking feature of LCAT deficiency is the presence of an abnormal lipoprotein, lipoprotein-X (Lp-X), in patients' plasma [1]. Lp-X is believed to arise from the surface of chylomicron remnants that are not further metabolized due to the absence of active LCAT [1, 8]. The Lp-X particle is rich in phosphatidylcholine and unesterified cholesterol and is associated with a small amount of proteins (apolipoprotein E, apolipoprotein CI, CII, and CIII, albumin) [8–11].

Clinical manifestations of LCAT deficiency typically include corneal opacities, hemolytic anemia, and proteinuria leading to renal dysfunction [11–14]. A major complication of LCAT deficiency is the development of progressive glomerulosclerosis, which can lead to renal failure [1, 3, 11–14]. Renal biopsies have revealed the presence of foam cells in the glomerular tufts of affected glomeruli, thickened intimas, and narrow lumens of renal arterioles, as well as the deposition of lipids in glomerular basement membrane and mesangial regions [15, 16]. These lipid deposits in the kidney consist mostly of unesterified cholesterol and phosphatidylcholine, the primary lipids found in Lp-X [8–10]. We previously reported that the direct deposition of Lp-X lipids and Lp-X-induced foam

cell formation might contribute to subsequent renal injury in patients with LCAT deficiency [17, 18].

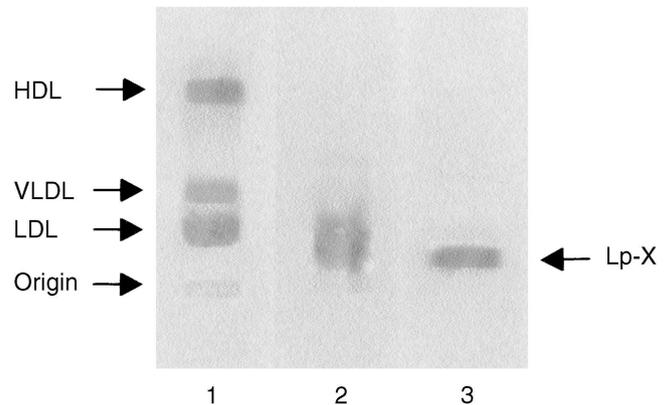
Several mechanisms by which lipoprotein abnormalities lead to glomerulosclerosis have been proposed. These include the deposition of lipids, proliferation of mesangial cells, infiltration of monocytes/macrophages, and accumulation of extracellular matrix in glomeruli [11, 19–26]. The infiltration of monocytes/macrophages is one of the key events in the development of glomerulosclerosis [11, 19–26]. Mesangial cells are able to synthesize and secrete a variety of cytokines and growth factors, including monocyte chemoattractant protein-1 (MCP-1), which is a potent chemoattractant for monocytes/macrophages [27, 28]. Similar to the development of atherosclerosis, macrophage infiltration also plays an important role in glomerulosclerosis. Once infiltrated into arterial walls or organs monocytes differentiate into macrophages, which can take up large amounts of lipoprotein-derived lipids to form foam cells [18, 19, 24, 26]. Results from our recent study suggest that very low-density lipoprotein (VLDL)-induced MCP-1 expression in mesangial cells occurred via a protein kinase C (PKC) signaling pathway and may be responsible for increased macrophage infiltration and foam cell formation in the kidney of patients with hypertriglyceridemia [29]. Furthermore, nuclear factor- $\kappa$ B (NF- $\kappa$ B), a transcription factor, also plays an important role in the up-regulation of MCP-1 expression in many cell types [30–33].

Although many mutations in the *LCAT* gene have been identified, the mechanisms by which LCAT deficiency leads to glomerulosclerosis have not been fully elucidated. The presence of foam cells in the affected kidneys of patients with LCAT deficiency suggests that the recruitment of monocytes/macrophages into glomeruli is enhanced. The objective of the present study was to investigate the effect of Lp-X on MCP-1 expression leading to enhanced monocyte chemotaxis as well as the underlying mechanisms in this process.

## METHODS

### Preparation of lipoprotein-X

Blood was obtained from a previously described patient of Italian and Swedish descent who was diagnosed with familial LCAT deficiency after an overnight fast [12]. The patient presented with symptoms similar to those found in other reported cases of familial LCAT deficiency [11, 13, 14]. The patient had a complete lack of active LCAT in the plasma, typical corneal opacities, mild anemia, and proteinuria [12]. Kidney biopsy revealed numerous vacuoles with lipid deposits [12]. Serum lipoprotein electrophoresis on agarose gel showed the presence of the abnormal lipoprotein, Lp-X [12]. The plasma was prepared by low-speed centrifugation ( $1200 \times g$ , 20 min) and stored at  $-70^{\circ}\text{C}$  until use. Lp-X was isolated by ultra-



**Fig. 1. Lipoprotein electrophoresis.** Samples were loaded on a 1% agarose gel and electrophoresed for 35 minutes. The lipoproteins were stained with Sudan Black. The arrows indicate the position of individual lipoproteins. Lane 1, normal plasma; lane 2, patient plasma; lane 3, isolated Lp-X.

centrifugation and column chromatography as previously described [17, 34]. Briefly, a fraction with a density ( $d$ ) of 1.019 to 1.063 g/mL was prepared by sequential ultracentrifugation. The Lp-X present in the  $1.019 < d < 1.063$  g/mL fraction was separated from low-density lipoprotein (LDL) by gel filtration column chromatography, using a matrix of Superose 6B beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Lp-X was eluted off the column as a distinct peak using a buffer of 0.15 mol/L NaCl/10 mmol/L Tris-HCl, pH 7.4. The peak was identified by measuring absorbency at 280 nm. The purity of Lp-X was determined by the electrophoretic mobility of the isolated Lp-X on 1% agarose gel (Fig. 1), as well as by the ratio ( $>95\%$ ) of unesterified cholesterol:total cholesterol [34]. Unesterified and total cholesterol in the Lp-X fraction were measured enzymatically using commercially available kits (Boehringer-Mannheim, Mannheim, Germany). The concentrations of Lp-X used in the present study were comparable to those found in the plasma of LCAT-deficient patients [35]. The concentration of Lp-X was expressed as nmol free cholesterol in Lp-X per mL (nmol/mL). The protocol of the study was approved by the university ethical review committee.

### Cell culture

Mesangial cells were isolated from the glomeruli of male Sprague-Dawley rats using a differential sieving technique as described previously [29, 36]. In brief, cortices were removed and minced before passage through a series of steel sieves with decreasing pore sizes (200, 150, and 75  $\mu\text{m}$ ). Isolated glomeruli were then digested with trypsin and collagenase. Mesangial cells were cultured in RPMI-1640 medium containing 20% fetal bovine serum (FBS) supplemented with insulin (5  $\mu\text{g}/\text{mL}$ ), transferrin (5  $\mu\text{g}/\text{mL}$ ), and selenium (95 ng/mL; Sigma Chemical Co.,

St. Louis, MO, USA). The homogeneity of the culture was verified by immunofluorescent staining and resistance to the epithelial cytotoxin puromycin aminonucleoside, as previously described [36]. Cells were subcultured at one- to two-week intervals. Cells between passages 6 to 12 were used for experiments. RPMI-1640 medium containing 0.5% lipoprotein-depleted FBS was used for experiments. Human acute monocytic leukemia (THP-1) cells, a human monocyte cell line, were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). THP-1 cells were cultured in RPMI-1640 medium containing 10% FBS. There was no lipopolysaccharide (LPS) contamination in any of our reagents or Lp-X preparations as determined by the E-TOXATE kit purchased from Sigma Chemical Co.

### Determination of MCP-1 protein

After mesangial cells were incubated with Lp-X (25 to 100 nmol/mL), the amount of MCP-1 protein in the culture medium was determined by solid-phase immunoassay [29]. Briefly, aliquots of culture media and purified MCP-1 protein (Calbiochem, La Jolla, CA, USA) were applied to a nitrocellulose membrane (0.2  $\mu$ m) in a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 2% non-fat milk in phosphate-buffered saline (PBS) for two hours and then probed with polyclonal anti-MCP-1 antibodies (Pepro Tech EC Ltd., London, UK). Blots were developed using horseradish peroxidase-conjugated secondary antibodies. Bands were visualized using enzyme chemiluminescence (ECL) reagents (Amersham, Buckinghamshire, UK) and analyzed with a gel documentation system (Bio-Rad Gel Doc1000 and Multi-Analyst<sup>®</sup> version 1.1).

### Analysis of MCP-1 mRNA by reverse transcription-polymerase chain reaction and nuclease protection assay

Total RNA from cultured cells was isolated with TRIzol Reagent as described by the manufacturer (Life Technologies, Gaithersburg, MD, USA). Total RNA was then used for reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed with 1  $\mu$ g total RNA in a total volume of 20  $\mu$ L [6 mmol/L MgCl<sub>2</sub>, 10 mmol/L dithiothreitol (DTT), 10 ng/ $\mu$ L poly dT, 1 U/ $\mu$ L M-MLV reverse transcriptase]. The nucleotide sequences of the primers used in the PCR reactions were derived from rat spleen cells [27, 29]. The MCP-1 sense primer (5'-ATC ACC AGC AGC AGG TGT CCC AAA GAA GCT-3') and antisense primer (5'-AGA AGT GCT TGA GGT GGT TGT GGA AAA GAG-3') were synthesized by Life Technologies. The reaction mixture for PCR contained 10 mmol/L Tris-HCl, 5 mmol/L MgCl<sub>2</sub>, 1 mmol/L dNTP, 0.4  $\mu$ mol/L 5' primer, 0.4  $\mu$ mol/L 3' primer, 2 U *Taq* DNA polymerase, and 2  $\mu$ L cDNA

product from the reverse transcription reaction. After an initial denaturation for five minutes at 95°C, 30 cycles of PCR amplification (95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes) were carried out followed by an additional ten-minute extension at 72°C. In preliminary experiments, we found that 30 cycles of PCR amplification provided optimum results. The PCR products were separated by 1.8% agarose gel (containing ethidium bromide) electrophoresis and were visualized under ultraviolet light using a gel documentation system (Bio-Rad Gel Doc1000). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard to verify equal PCR product loading for each experiment. After the RT-PCR reaction, the MCP-1 signal was normalized by comparison with the GAPDH signal from the same sample.

To confirm results obtained from RT-PCR, a nuclease protection assay was performed using a kit according to the manufacturer's instructions (Ambion Inc., Austin, TX, USA). Briefly, total RNA (10  $\mu$ g) was hybridized with [<sup>32</sup>P]-end-labeled MCP-1 oligonucleotide probe (Clontech Laboratory Inc., Palo Alto, CA, USA) overnight at 30°C, followed by nuclease digestion to remove the nonhybridized probe. After digestion, the protected fragments were resolved on a denaturing 12% polyacrylamide gel containing 8 mol/L urea. A positive control, 28S rRNA oligonucleotide probe (Ambion Inc.) was used as an internal control. Bands corresponding to MCP-1 mRNA and 28S rRNA were visualized using autoradiography and analyzed using a gel documentation system (Bio-Rad Gel Doc1000). The ratio of MCP-1 to 28S rRNA was calculated, and values were expressed as a percentage of control.

### Determination of chemotactic activity

THP-1 monocyte chemotaxis was measured by using a 48-well Micro Chemotaxis Chamber (Neuro Probe Inc., Gaithersburg, MD, USA) [37]. Mesangial cells were incubated for six hours in the presence or absence of various concentrations of Lp-X. After incubation, an aliquot of the medium (defined as conditioned medium) was transferred to the lower chamber of the Micro Chemotaxis Chamber. The lower and upper chambers were separated by a 5  $\mu$ m pore-size polycarbonate membrane (Neuro Probe Inc., Gaithersburg, MD, USA). An aliquot of THP-1 monocyte suspension ( $2 \times 10^6$  cells/mL) was added to the upper chamber, and the monocytes were allowed to transmigrate for two hours. After transmigration, the surface of the membrane facing the THP-1 cell suspension was scraped and washed three times according to the manufacturer's instructions. The migrated cells, toward the conditioned medium, were fixed and then stained with hematoxylin. The number of migrated monocytes was determined under light microscopy. The results are expressed as a percentage of control.

Chemotaxis assay was also performed with freshly isolated human blood monocytes. Human monocytes were isolated from peripheral blood collected from healthy volunteers using the Ficoll-Paque density gradient centrifugation procedure (Amersham Pharmacia Biotech). Mononuclear cells were separated from platelets by four washes at  $300 \times g$  for 10 minutes. Finally, monocytes were added to a culture dish containing 10% FBS and were allowed to adhere to the surface of the dish. After incubation for 45 minutes, nonadherent lymphocytes were removed from the culture dish. Adherent monocytes were recovered by incubating with PBS containing 10 mmol/L ethylenediaminetetraacetic acid (EDTA) for five minutes. After washing in PBS, monocytes were resuspended in RPMI 1640 and were used for the chemotaxis assay.

#### Determination of protein kinase C activity

Cell extracts were prepared as described previously [29]. All steps for the preparation of cell extracts were performed at 4°C. Mesangial cells were harvested in PBS and pelleted by centrifugation. Cells were sonicated in 0.2 mL lysis buffer [50 mmol/L HEPES, pH 7.5, 0.2% (wt/vol) Triton X-100, 1 mmol/L EDTA, 1 mmol/L eg-tazic acid (EGTA), 1 mmol/L DTT, 85  $\mu$ mol/L leupeptin, 2 mmol/L benzamidine, 0.4 mmol/L phenylmethylsulfonyl fluoride] followed by centrifugation at  $100,000 \times g$  for 20 minutes (TLA 120.2; Beckman, Palo Alto, CA, USA) to obtain a detergent-soluble fraction (supernatant). A 5  $\mu$ L aliquot of this fraction was used to determine PKC activity. The PKC activity toward a synthetic peptide (Ac-FKKSFKL-NH<sub>2</sub>) was determined as described by Edwards and Newton [38]. The standard reaction mixture contained 50  $\mu$ mol/L peptide substrate in 20 mmol/L HEPES (pH 7.5), 1 mmol/L DTT, 100  $\mu$ mol/L [ $\gamma$ -<sup>32</sup>P]ATP (~1500 cpm/pmol), 5 mmol/L MgCl<sub>2</sub>, and 0.5 mmol/L CaCl<sub>2</sub>, and sonicated dispersions of brain phosphatidylserine (140  $\mu$ mol/L) and diacylglycerol (3.8  $\mu$ mol/L). In other experiments, PKC activity was determined in the absence of calcium. After an eight-minute incubation at 30°C, the reaction was terminated by spotting an aliquot of the reaction mixture onto Whatman P81 ion-exchange chromatography paper, followed by four washes with 0.4% (vol/vol) phosphoric acid and a 95% ethanol rinse. Radioactivity (<sup>32</sup>P) incorporation into the peptide substrate was determined by liquid scintillation counting [29].

#### Determination of nuclear factor- $\kappa$ B/DNA binding activity

Nuclear factor- $\kappa$ B/DNA binding activity was determined by electrophoretic mobility shift assay (EMSA) [39, 40]. Nuclear protein was isolated as previously described [41]. In brief, nuclear protein (10  $\mu$ g) was incubated with reaction buffer for 15 minutes at 25°C, fol-

lowed by incubation with [<sup>32</sup>P] end-labeled oligonucleotide containing the consensus sequence for the NF- $\kappa$ B binding site (Life Technologies). The reaction mixture was separated in a nondenaturing 6% polyacrylamide gel that was later exposed to x-ray film at -70°C. A supershift assay was also performed to identify the specific NF- $\kappa$ B subunits in the complex. Specific antibodies (2  $\mu$ g) against p50, p65, or normal rabbit IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were added to the nuclear proteins (3  $\mu$ g) prior to EMSA.

#### Determination of intracellular diacylglycerol levels by gas-liquid chromatography

Intracellular diacylglycerol levels were determined as previously described [42]. Briefly, cells were incubated in the absence or presence of Lp-X (25 to 100 nmol/mL) for 30 minutes. After incubation, cellular lipids were extracted using chloroform:methanol extraction [43]. Diacylglycerol was separated from other lipids by thin layer chromatography on silica gel 60 plates with the solvent system of toluene:diethyl ether:ethanol:triethylamine (50:40:2:1, vol/vol/vol/vol) [44]. Bands were visualized using iodine vapor. Before extraction, 5  $\mu$ g 1,2-dipalmitoyl-*sn*-glycerol (C16:0) were added as an internal standard. Diacylglycerol was extracted from the TLC plates using chloroform:methanol extraction [43]. The chloroform phase was evaporated to dryness and dissolved in 20  $\mu$ L ethyl acetate. This lipid extract (5  $\mu$ L) was injected into gas-liquid chromatography, which was performed as described previously [42] using an HP-1 Hewlett-Packard fused silica capillary column (5890 Series II Gas Chromatograph Hewlett-Packard 5971A). The oven temperature was set from 205°C to 345°C at a rate of 6°C per minute, and the carrier gas was hydrogen (0.5 bar).

#### Determination of phosphatidylcholine-specific phospholipase C activity

Phosphatidylcholine-specific phospholipase C activity was performed as previously described [45]. Briefly, cells were incubated in the absence or presence of Lp-X (25 to 100 nmol/mL) for 30 minutes. After incubation, cells were washed and collected in buffer (2 mmol/L HEPES, 154 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4). The cell suspension was sonicated for 30 seconds and then centrifuged at  $180 \times g$  for 10 minutes at 4°C. The supernatant was then centrifuged at  $100,000 \times g$  for 90 minutes at 4°C. After ultracentrifugation, the pellet was resuspended in buffer. Membrane fractions were frozen at -70°C until use. The membrane fraction (50  $\mu$ g) was incubated with 100  $\mu$ L reaction buffer (100 mmol/L HEPES, 100 mmol/L sodium acetate, pH 7.5), 100  $\mu$ L L- $\alpha$ -dipalmitoyl-[2-palmitoyl-9,10-<sup>3</sup>H(N)]-phosphatidylcholine (10  $\mu$ Ci/mL; NEN Life Science Products, Inc., Boston, MA, USA), and 100  $\mu$ L 6 mmol/L CaCl<sub>2</sub> for one hour at 37°C. Reactions were stopped by the addition of 1 mL chloroform/meth-

anol (2:1, vol/vol) containing 36 mmol/L HCl. Lipids were extracted using chloroform:methanol extraction [43]. Phosphatidylcholine and diacylglycerol were separated by thin layer chromatography as described previously in this article. Individual lipids were visualized using iodine vapor. Radioactivity was determined by liquid scintillation counting.

### Statistical analysis

The Student *t* test was used for statistical analysis between two groups. The level of statistical significance was set at  $P < 0.05$ .

## RESULTS

### Effect of Lp-X on the production of MCP-1 protein by mesangial cells

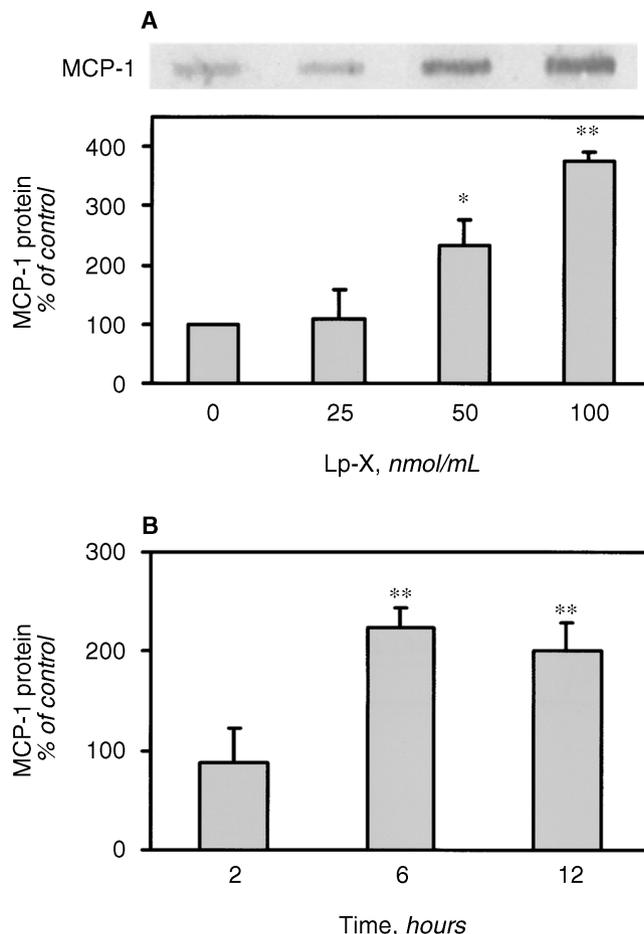
To investigate the effect of Lp-X on MCP-1 protein levels, mesangial cells were incubated with Lp-X at various concentrations. After incubation, culture media were collected and assayed for MCP-1 protein by Western immunoblotting analysis. As shown in Figure 2A, Lp-X at the concentration of 50 or 100 nmol/mL significantly increased MCP-1 protein levels in media cultured with mesangial cells (233 to 375% of control). Lp-X at low concentration (25 nmol/mL) had no significant effect on MCP-1 protein levels (108% of control). In time course experiments, the maximum stimulatory effect was observed in cells incubated with Lp-X for six hours (Fig. 2B).

### Effect of Lp-X on the expression of MCP-1 mRNA in mesangial cells

To determine whether an increase in MCP-1 protein levels was due to changes in the expression of MCP-1 mRNA, total RNA was isolated from mesangial cells. Semiquantitative RT-PCR analysis and nuclease protection assay were performed to determine the expression of MCP-1 mRNA. Comparable results were obtained with both RT-PCR analysis (Fig. 3A) and nuclease protection assay (Fig. 3B). Lp-X (50 or 100 nmol/mL) stimulated MCP-1 mRNA expression (137 to 220% of control) in mesangial cells (Fig. 3 A, B). In time course experiments, the levels of MCP-1 mRNA in Lp-X-treated mesangial cells reached a maximum after four hours of incubation (Fig. 3C).

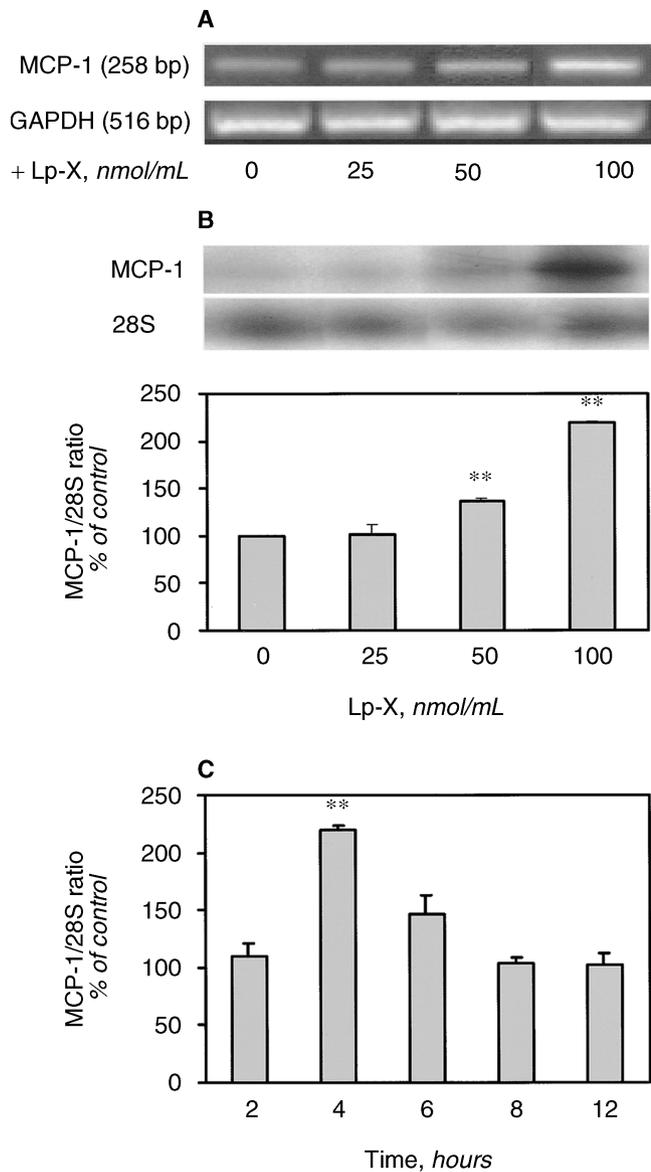
### Effect of Lp-X-induced MCP-1 expression on monocyte chemotaxis

To examine the effect of Lp-X on monocyte chemotaxis, mesangial cells were incubated with Lp-X for six hours, and the culture medium was then collected (defined as conditioned medium) and used for chemotaxis assays. As shown in Figure 4A, there was a significant increase in THP-1 monocyte chemotaxis (165 to 200% of control) toward conditioned medium collected from mes-

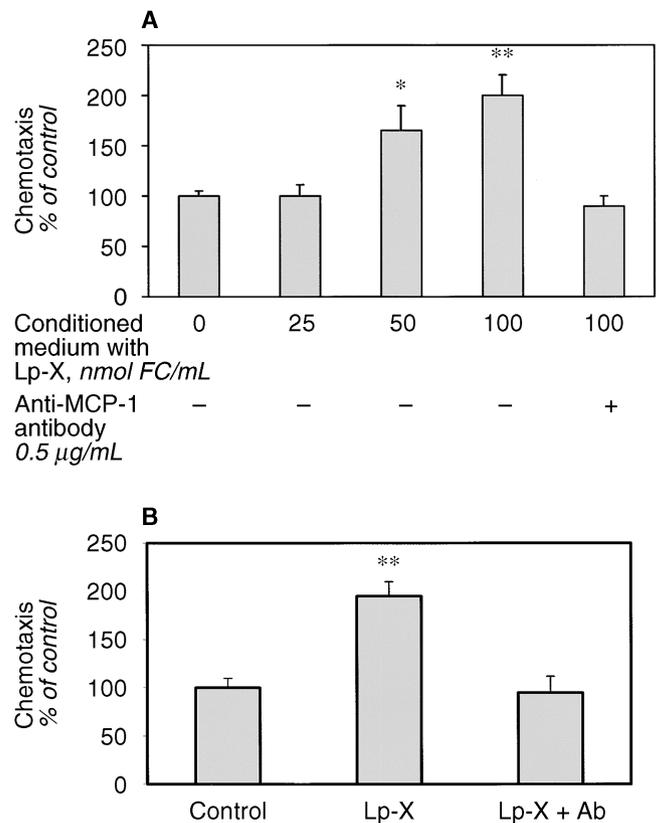


**Fig. 2. Effect of lipoprotein-X (Lp-X) on monocyte chemoattractant protein-1 (MCP-1) produced by mesangial cells.** (A) Mesangial cells were incubated for six hours in the presence or absence of different concentrations of Lp-X. Aliquots of culture media were taken, and MCP-1 protein was determined by Western immunoblotting analysis. (B) Mesangial cells were incubated with 50 nmol/mL Lp-X for various time periods, and MCP-1 protein levels in cultured media were determined. Control cells were incubated in the absence of Lp-X. Results are expressed as mean  $\pm$  SD (error bar) from three separate experiments. \* $P < 0.05$ ; \*\* $P < 0.01$  when compared with the control.

angial cells incubated with Lp-X (50 to 100 nmol/mL). Next, to determine whether MCP-1 was the major chemoattractant protein responsible for Lp-X-induced chemotaxis, antibody blocking experiments with anti-MCP-1 antibody (0.5  $\mu$ g/mL) were performed. As shown in Figure 4A, the anti-MCP-1 antibody completely abolished the enhanced monocyte chemotaxis induced by the conditioned medium, indicating that MCP-1 was the major chemoattractant protein produced by Lp-X-treated mesangial cells. Similar experiments were performed with freshly isolated human blood monocytes. As shown in Figure 4B, there was a significant increase in monocyte chemotaxis toward conditioned medium collected from mesangial cells incubated with Lp-X (100 nmol/mL). Such a stimulatory effect was abolished by anti-MCP-1 antibody (Fig. 4B).



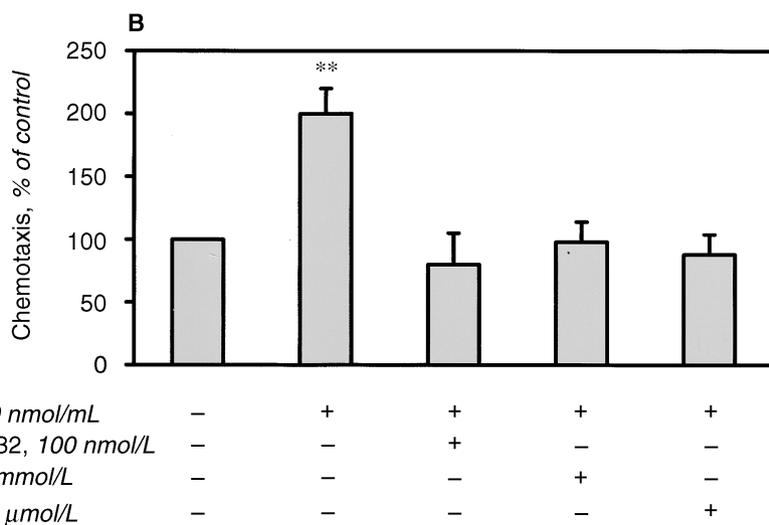
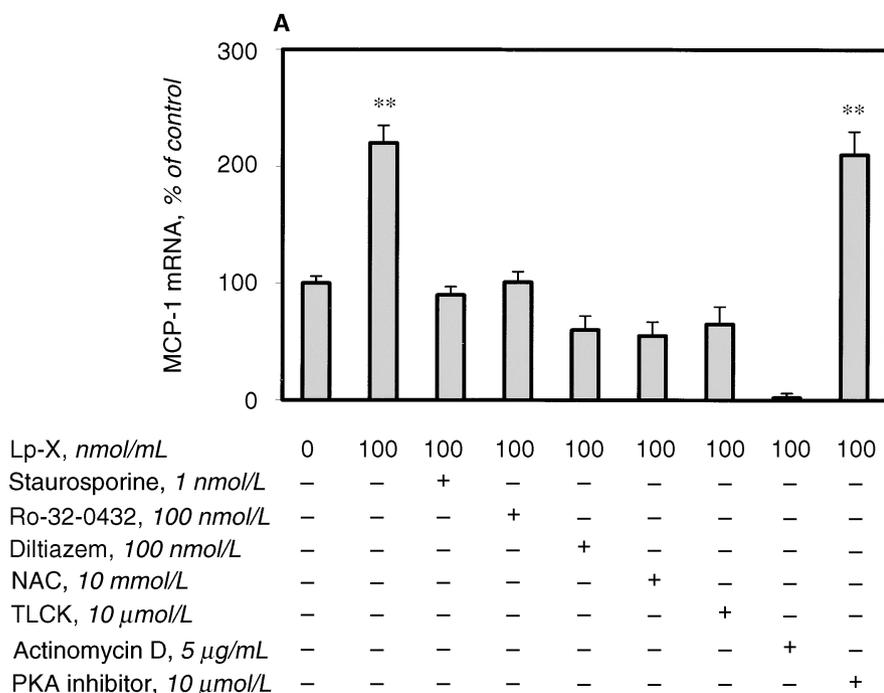
**Fig. 3. Effect of Lp-X on MCP-1 mRNA expression in mesangial cells.** Mesangial cells were incubated with different concentrations of Lp-X for four hours. (A) After incubation, MCP-1 mRNA was determined by RT-PCR analysis with GAPDH as an internal standard. The PCR-amplified cDNA derived from MCP-1 or GAPDH were separated by 1.8% agarose gel electrophoresis containing ethidium bromide and visualized under ultraviolet light using a gel documentation system. This is a typical picture representative of five separate experiments. (B) The expression of MCP-1 mRNA in mesangial cells was analyzed by nuclear protection assay with 28S rRNA as an internal standard. Bands corresponding to MCP-1 mRNA and 28S rRNA obtained from nuclear protection assay were visualized under ultraviolet light using a gel documentation system. Values were expressed as the relative expression of MCP-1 mRNA normalized to 28S rRNA levels. (C) Mesangial cells were incubated with 50 nmol/mL Lp-X for various time periods, and MCP-1 mRNA expression was determined by nuclear protection assay. Control cells were incubated in the absence of Lp-X. Results are depicted as the mean  $\pm$  SD from three separate experiments. \*\* $P < 0.01$  when compared with the control.



**Fig. 4. Effect of Lp-X-induced MCP-1 expression on monocyte chemotaxis.** Conditioned media were collected from mesangial cells preincubated for six hours in the presence or absence of Lp-X. For some experiments, 0.5  $\mu$ g/mL anti-MCP-1 antibody was added. The chemotactic activity of THP-1 monocytes (A) or fresh monocytes isolated from human blood (B) toward conditioned media was measured using a 48-well Micro Chemotaxis Chamber. The number of monocytes was counted under light microscopy. Control cells were incubated in the absence of Lp-X. The results are shown as the mean  $\pm$  SD from four independent experiments. \* $P < 0.05$  or \*\* $P < 0.01$  when compared with the control.

#### Effect of PKC inhibitors, calcium channel blocker, and NF- $\kappa$ B inhibitors on Lp-X-induced MCP-1 mRNA expression and monocyte chemotaxis

To investigate the mechanisms by which Lp-X could induce MCP-1 expression, mesangial cells were incubated with Lp-X (100 nmol/mL) in the presence or absence of PKC inhibitors, calcium channel blockers, or NF- $\kappa$ B inhibitors. As shown in Figure 5A, both staurosporine and Ro-32-0432 (bisindolylmaleimide XI, HCl; Alexis Corporation, Grünberg, Germany), two separate inhibitors of PKC, completely abolished Lp-X-induced MCP-1 mRNA expression. On the other hand, an inhibitor of cAMP-dependent protein kinase (PKI peptide, TTYADFIASGRRNAIHD) [46] did not affect the stimulatory effect of Lp-X on MCP-1 expression (Fig. 5A). Diltiazem, a calcium channel blocker, also inhibited Lp-X-induced MCP-1 expression (Fig. 5A). These results suggested that Lp-X-induced MCP-1 expression might be

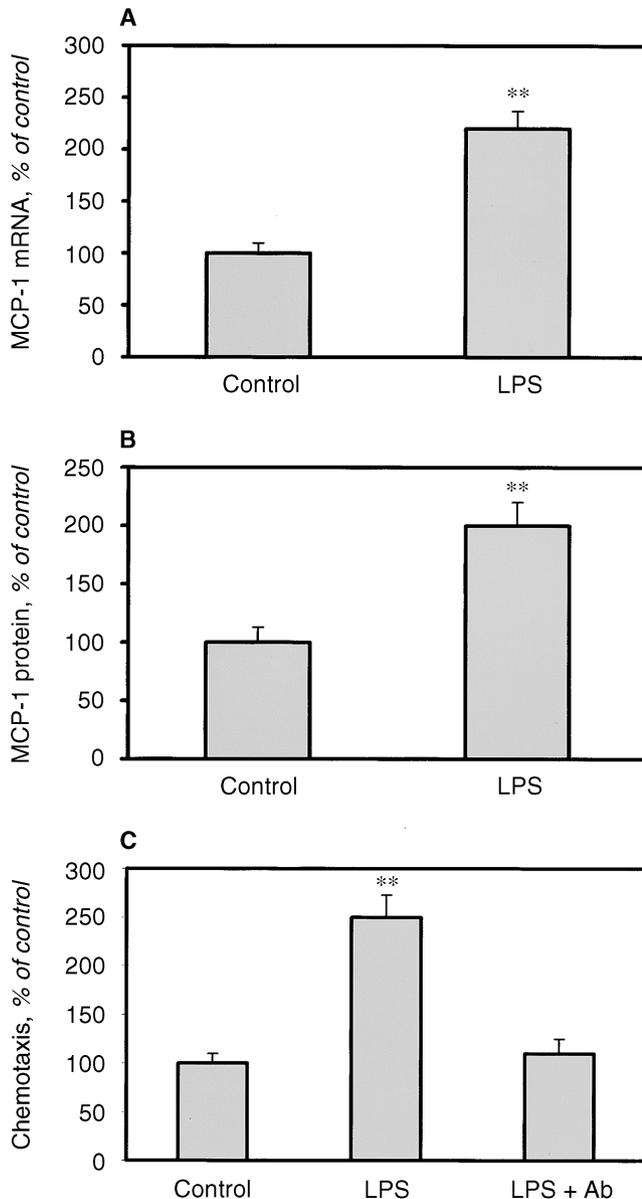


**Fig. 5. Effect of protein kinase inhibitors, calcium channel blocker, nuclear factor- $\kappa$ B/protein kinase C (NF- $\kappa$ B/PKC) inhibitors, or actinomycin D on Lp-X-induced MCP-1 mRNA expression in mesangial cells as well as on monocyte chemotaxis. (A)** Mesangial cells were incubated with 100 nmol/mL Lp-X for four hours in the presence or absence of the PKC inhibitors (staurosporine or Ro-32-0432), a calcium channel blocker (diltiazem), NF- $\kappa$ B inhibitors (NAC or TLCK), actinomycin D, or cAMP-dependent protein kinase (PKI peptide). After incubation, MCP-1 mRNA was determined and the ratio of MCP-1 mRNA to 28S rRNA was calculated. **(B)** Mesangial cells were incubated with 100 nmol/mL Lp-X for six hours in the presence or absence of the PKC inhibitor (Ro-32-0432) or the NF- $\kappa$ B inhibitors (NAC or TLCK). After incubation, culture media were collected and used for the monocyte chemotaxis assay. Control cells were incubated in the absence of Lp-X. Results are depicted as the mean  $\pm$  SD from three independent experiments. \*\*  $P < 0.01$  when compared with the control.

mediated via PKC signaling pathway, while the cyclic adenosine monophosphate (cAMP)-dependent protein kinase signaling pathway might not be involved in such process in rat mesangial cells. Furthermore, *N*-acetylcysteine (NAC) or *N*- $\alpha$ -tosyl-L-lysine chloromethylketone (TLCK), both inhibitors of NF- $\kappa$ B, blocked Lp-X-induced MCP-1 mRNA expression (Fig. 5A), indicating that NF- $\kappa$ B activation might be involved in mediating this process. In addition, Lp-X-induced MCP-1 expression was completely inhibited by actinomycin D (Fig. 5A), indicating that Lp-X-induced MCP-1 expression was at the transcription level. As shown in Figure 5B, the conditioned medium collected from mesangial cells

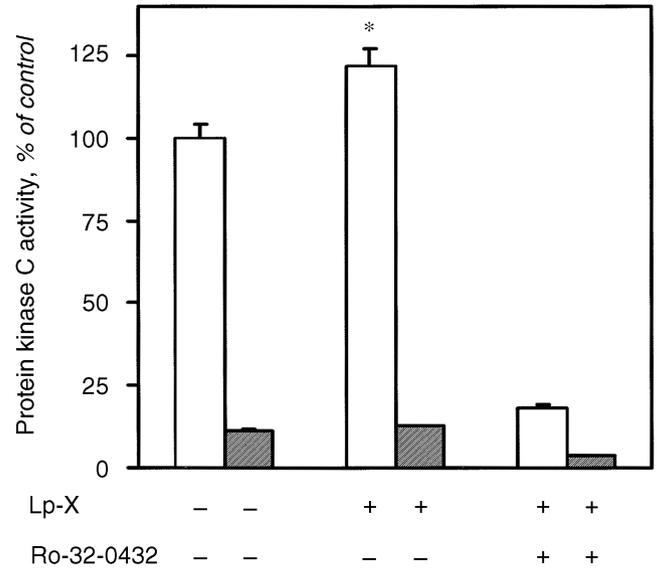
preincubated with Lp-X significantly enhanced monocyte chemotactic activity. Such a stimulatory effect was completely abolished when mesangial cells were incubated with Lp-X in the presence of PKC inhibitors or NF- $\kappa$ B inhibitors (Fig. 5B). These results suggested that the activation of PKC and NF- $\kappa$ B might be involved in Lp-X-induced MCP-1 expression in mesangial cells.

As a comparison, the effects of LPS on MCP-1 expression and monocyte chemotaxis were investigated. LPS is known to stimulate MCP-1 expression in various cell types [47, 48]. LPS (10  $\mu$ g/mL; Sigma, Chemical Co.) significantly stimulated the expression of MCP-1 mRNA (Fig. 6A) and protein (Fig. 6B). The conditioned medium



**Fig. 6. Effect of lipopolysaccharide (LPS) on monocyte chemoattractant protein-1 (MCP-1) expression and monocyte chemotaxis.** (A) Mesangial cells were incubated with LPS (10  $\mu\text{g}/\text{mL}$ ) for four hours. After incubation, MCP-1 mRNA was determined and the ratio of MCP-1 mRNA to 28S rRNA was calculated. (B) Mesangial cells were incubated for six hours with LPS (10  $\mu\text{g}/\text{mL}$ ), and MCP-1 protein levels in cultured media were determined. (C) Conditioned media were collected from mesangial cells preincubated for six hours in the presence or absence of LPS (10  $\mu\text{g}/\text{mL}$ ). For some experiments, 0.5  $\mu\text{g}/\text{mL}$  anti-MCP-1 antibody was added. The chemotactic activity of THP-1 monocyte toward conditioned media was measured using a 48-well Micro Chemotaxis Chamber. The number of monocytes was counted under light microscopy. Control cells were incubated in the absence of LPS. The results are shown as the mean  $\pm$  SD from four independent experiments. \*\* $P < 0.01$  when compared with the control.

collected from mesangial cells preincubated with LPS significantly enhanced monocyte chemotactic activity (Fig. 6C). Such stimulatory effect was completely abol-

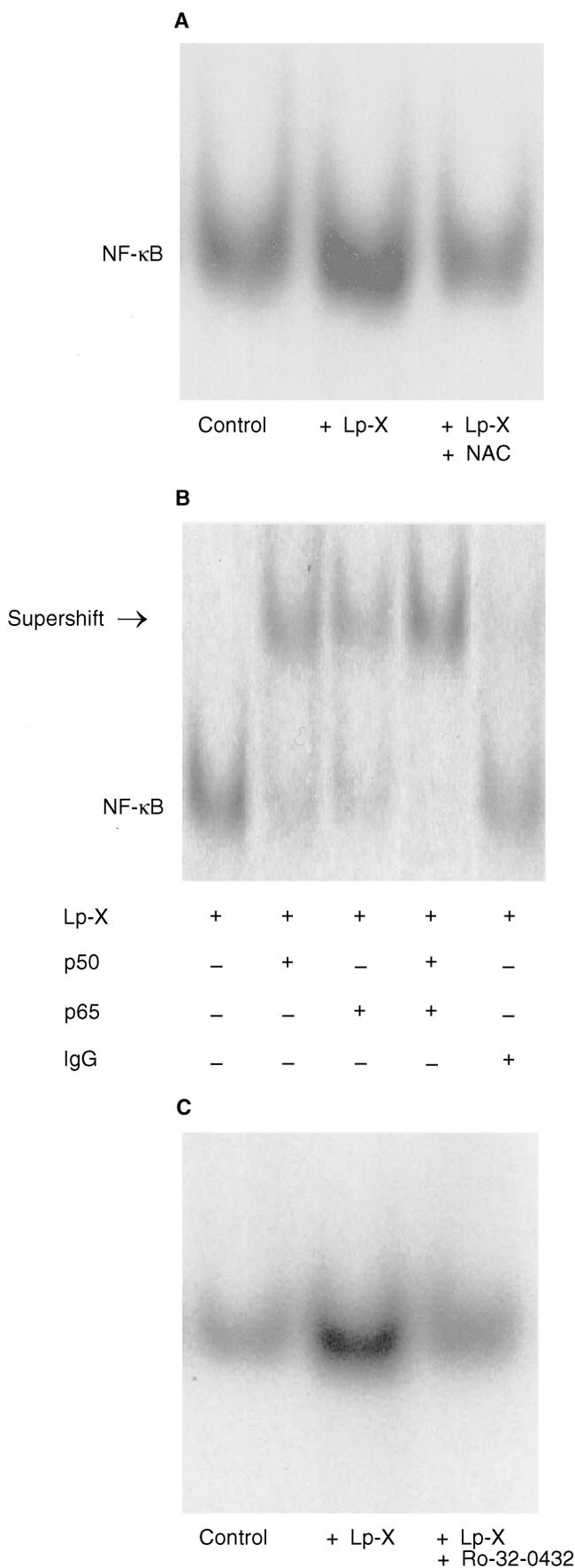


**Fig. 7. Effect of Lp-X on PKC activity.** Mesangial cells were incubated with Lp-X (100 nmol/mL) for four hours in the absence or presence of Ro-32-0432 (100 nmol/L). After incubation, PKC activities were determined in the absence (■) or presence (□) of calcium. Results are depicted as the mean  $\pm$  SD from three independent experiments. \* $P < 0.05$  when compared with the control.

ished by addition of anti-MCP-1 antibody. The effects of Lp-X at the concentration of 100 nmol/mL on the expression of MCP-1 mRNA, MCP-1 protein levels as well as on monocyte chemotaxis were similar to that of LPS at the concentration of 10  $\mu\text{g}/\text{mL}$ .

#### Effect of Lp-X on PKC activity and NF- $\kappa$ B activation

To determine the effect of Lp-X on PKC activity, mesangial cells were incubated for four hours in the absence or presence of Lp-X (100 nmol/mL). After incubation, cell extracts were prepared, and PKC activity was determined in the presence or absence of calcium. As shown in Figure 7, Lp-X significantly stimulated the calcium-dependent PKC activity but had no effect on the calcium-independent PKC activity. Such effect was abolished in the presence of a specific PKC inhibitor, Ro-32-0432. These results indicated that Lp-X treatment increased PKC activity in mesangial cells. Next, to determine the effect of Lp-X on NF- $\kappa$ B activation in mesangial cells, EMSA was performed using a specific oligonucleotide probe containing a NF- $\kappa$ B/DNA binding site. An increase in NF- $\kappa$ B/DNA binding activity was observed in mesangial cells treated with Lp-X (100 nmol/mL; Fig. 8A). This stimulatory effect was abolished by the addition of NF- $\kappa$ B inhibitor (Fig. 8A). To determine which NF- $\kappa$ B subunits were activated, a supershift assay using antibodies against individual NF- $\kappa$ B subunits was performed. As shown in Figure 8B, the addition of either p50 or p60 antibodies resulted in supershifts, indicating



that these subunits were activated in Lp-X-treated cells. Nonspecific IgG did not result in any shift of the NF- $\kappa$ B/DNA band (Fig. 8B). These results suggested that Lp-X-induced MCP-1 expression might be mediated by NF- $\kappa$ B activation in mesangial cells. Next, the effect of a PKC inhibitor (Ro-32-0432) on Lp-X-induced NF- $\kappa$ B activation was examined. As shown in Figure 8C, the presence of Ro-32-0432 (100 nmol/L) in the culture medium blocked Lp-X-induced NF- $\kappa$ B activation in mesangial cells.

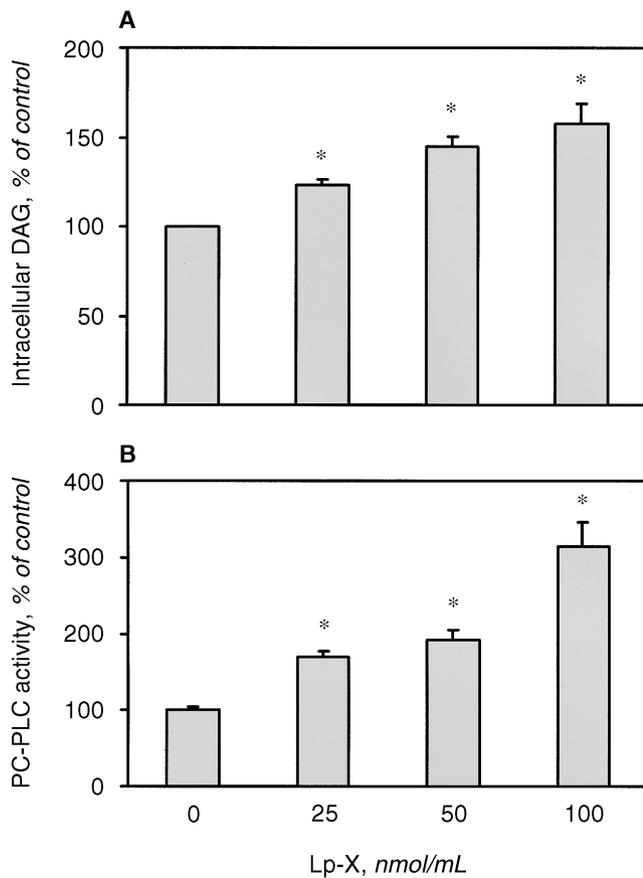
#### Effect of Lp-X on intracellular diacylglycerol levels and phosphatidylcholine-specific phospholipase C activity

Lipoprotein-X is a lipid bilayer particle enriched with phosphatidylcholine and unesterified cholesterol [1, 8–10]. To examine whether Lp-X could modulate effectors upstream of PKC (that is, diacylglycerol), mesangial cells were incubated in the absence or presence of Lp-X for 30 minutes. After incubation, intracellular diacylglycerol level and phosphatidylcholine-specific phospholipase C activity were determined. Lp-X (25 to 100 nmol/mL) significantly increased intracellular diacylglycerol levels (123 to 158% of control; Fig. 9A). Elevated diacylglycerol levels were accompanied by a significant increase (169 to 315% of control) in phospholipase activity (Fig. 9B) following treatment with Lp-X (25 to 100 nmol/mL).

#### DISCUSSION

Similar to atherosclerosis, the findings that macrophages and foam cells accumulate in affected glomeruli suggest that the recruitment of monocytes is increased in glomerulosclerosis [13–17, 19–22, 37, 49–51]. An increased expression of MCP-1 mRNA has been detected in kidneys of patients with several types of renal diseases [47, 52–56]. However, the involvement of MCP-1 in familial LCAT deficiency has yet to be investigated. The novel findings of the present study are as follows: (1) Lp-X-stimulated MCP-1 expression in mesangial cells leading to an enhanced monocyte chemotaxis; (2) that stimulatory effect was abolished by inhibitors of PKC or NF- $\kappa$ B; and (3) Lp-X increased the activity of phos-

**Fig. 8. Effect of Lp-X on NF- $\kappa$ B/DNA binding activity.** (A) Mesangial cells were incubated with Lp-X (100 nmol/mL) for four hours in the absence or presence of NF- $\kappa$ B inhibitor (10 mmol/L NAC). EMSA was performed to determine the NF- $\kappa$ B/DNA binding activity. (B) Supershift assay was performed in the presence of antibodies against p50, p65, or nonspecific rabbit IgG. Antibodies against p50 or p65 caused a shift of the NF- $\kappa$ B/DNA band. (C) Mesangial cells were incubated with Lp-X (100 nmol/mL) for four hours in the absence or presence of a specific PKC inhibitor (Ro-32-0432, 100 nmol/L). EMSA was performed to determine the NF- $\kappa$ B/DNA binding activity. The autoradiograph is a representative EMSA from three separate experiments.



**Fig. 9. Effect of Lp-X on intracellular diacylglycerol levels and phosphatidylcholine-specific phospholipase C activity in mesangial cells.** Mesangial cells were incubated in the absence or presence of Lp-X (25 to 100 nmol/mL) for 30 minutes. (A) After incubation, cellular lipids were extracted, and diacylglycerol (DAG) was measured by gas-liquid chromatography. (B) The membrane fraction of cells was used for the determination of phosphatidylcholine-specific phospholipase C activity (PC-PLC). Control cells were incubated in the absence of Lp-X. Results are depicted as the mean  $\pm$  SD from three separate experiments. \* $P < 0.05$  when compared with the control.

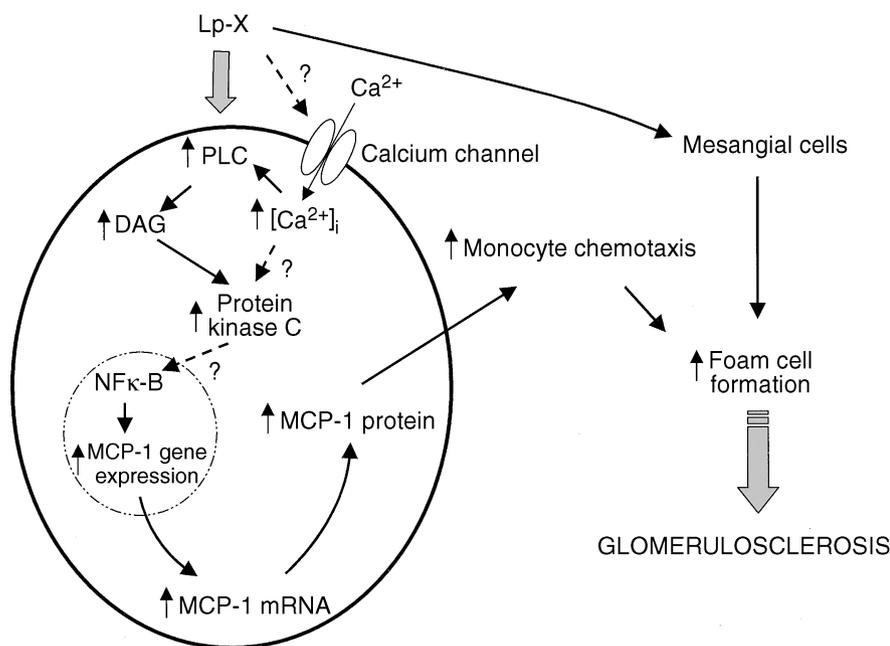
phatidylcholine-specific phospholipase C, causing an elevation in the level of diacylglycerol in mesangial cells. In view that the stimulation of monocyte chemotaxis was completely abolished by anti-MCP-1 antibodies, Lp-X-induced MCP-1 might be the major chemoattractant protein responsible for enhanced monocyte chemotaxis in LCAT-deficient patients. Such a stimulatory effect in mesangial cells might be mediated by NF- $\kappa$ B activation. The monocyte recruitment via MCP-1 expression may be one of the important mechanisms contributing to the development of glomerulosclerosis in patients with familial LCAT deficiency.

In the present study, we investigated the possible mechanisms by which Lp-X stimulated MCP-1 expression, and hence monocyte chemotaxis, in mesangial cells. It has been shown that VLDL or glucose-induced MCP-1 expression was mediated by PKC in rat or human mesan-

gial cells [29, 51]. Calphostin C, a PKC inhibitor, was also shown to inhibit the stretch-induced elevation of MCP-1 mRNA levels in human endothelial cells [57]. In our study, Lp-X significantly stimulated PKC activity in mesangial cells. Furthermore, the PKC inhibitors staurosporine and Ro-32-0432 completely abolished Lp-X-induced MCP-1 expression. Taken together, these results indicate that Lp-X-induced MCP-1 expression in mesangial cells is mediated via the PKC signaling pathway.

Analyses of the promoter region of the *MCP-1* gene have revealed several putative binding sites for transcription activator factors, including NF- $\kappa$ B [30–33]. The activated NF- $\kappa$ B can bind to the promoter region of the *MCP-1* gene and stimulate the expression of this chemoattractant protein [30–33]. In the present study, several lines of evidence point toward a role for NF- $\kappa$ B in MCP-1 expression. First, the NF- $\kappa$ B inhibitors NAC and TLCK completely blocked Lp-X-induced stimulation of MCP-1 mRNA levels and subsequent monocyte chemotaxis. Second, the NF- $\kappa$ B/DNA binding activity was significantly elevated in Lp-X-treated cells. Third, a PKC inhibitor (Ro-32-0432) abolished Lp-X-stimulated NF- $\kappa$ B activation in mesangial cells. Moreover, Lp-X-induced stimulation of MCP-1 expression appeared to occur at the transcriptional level, as evidenced by the inhibitory effect of actinomycin D. These results suggest that Lp-X-induced PKC activity and subsequent NF- $\kappa$ B activation can stimulate the transcription of MCP-1.

The effect of Lp-X on diacylglycerol levels was also investigated. Diacylglycerol is formed via the action of phospholipase C on either phosphatidylinositol or phosphatidylcholine [45, 58]. Because Lp-X contains a high level of phosphatidylcholine [8–10], it is plausible that treatment of cells with Lp-X may stimulate phosphatidylcholine-specific phospholipase C activity to produce diacylglycerol, which in turn activates PKC. We previously reported that mesangial cells were able to accumulate phosphatidylcholine and lysophosphatidylcholine when Lp-X was added to the culture medium [36]. In the present study, Lp-X treatment increased phosphatidylcholine-specific phospholipase C activity and intracellular diacylglycerol levels in mesangial cells. While Lp-X at the concentration of 25 nmol/mL elicited a significant response in both phospholipase C activity and diacylglycerol levels, Lp-X at this concentration had no significant effect on the expression of MCP-1 mRNA and protein levels. Such a discrepancy might be due to the requirement of a threshold level of intracellular calcium before a significant effect on activation of calcium-dependent PKC and subsequent MCP-1 expression was evoked by Lp-X. The minimum amount of Lp-X required for activation of phospholipase C leading to an elevation in the levels of intracellular diacylglycerol, a well-established allosteric activator of PKC [59, 60], might be different



**Fig. 10. Proposed mechanism for Lp-X-induced monocyte chemotaxis.** Lp-X stimulates phosphatidylcholine-specific phospholipase C (PLC) activity in mesangial cells, producing increased levels of diacylglycerol (DAG) that consequently activate PKC. Increased PKC activity in turn stimulates the binding of NF- $\kappa$ B to the MCP-1 gene, which subsequently leads to an increase in MCP-1 mRNA expression and protein secretion. Increased monocyte chemotaxis due to Lp-X-induced MCP-1 secretion contributes to monocyte infiltration, lipid accumulation, and foam cell formation, which are key events in the development of glomerulosclerosis.

from that required for elevation of intracellular calcium for the activation of PKC.

In addition to the infiltration of circulating monocytes into affected glomeruli, another major event in the pathogenesis of glomerulosclerosis is the accumulation of lipids in the kidneys of patients with LCAT deficiency [1, 8–10]. Unesterified cholesterol from Lp-X, once taken up into the cell, was esterified to cholesteryl ester by mesangial cells [36]. We have reported previously that Lp-X caused the direct deposition of lipids in perfused rat kidney and induced foam cell formation in rat peritoneal macrophages [17, 18]. Hence, Lp-X may contribute to the progression of glomerulosclerosis in familial LCAT deficiency by first stimulating lipid accumulation in the kidney [19], followed by Lp-X-induced monocyte infiltration into affected glomeruli via a mechanism involving MCP-1 expression. Once having been recruited into the kidney, these infiltrated monocytes/macrophages could take up excessive lipoprotein lipids and become foam cells [18, 24, 26]. In turn, these foam cells may then secrete a wide variety of cytokines and growth factors that could exacerbate and further contribute to the pathogenesis of glomerulosclerosis [50].

Based on the results obtained from the present study, as well as from previous reports [17, 18, 29, 36], we propose the following mechanism by which Lp-X stimulates monocyte chemotaxis (Fig. 10). Lp-X has a high content of phospholipids, especially phosphatidylcholine. Uptake of Lp-X by mesangial cells may stimulate phosphatidylcholine-specific phospholipase C activity that acts on the phosphatidylcholine present in Lp-X to produce phosphocholine and diacylglycerol. Lp-X treatment may

also elicit calcium influx leading to a rise in intracellular calcium. Such an increase in calcium, in combination with the elevation in diacylglycerol levels, can activate calcium-dependent PKC. Increased PKC activity subsequently may activate NF- $\kappa$ B, which in turn leads to an elevation in the expression of MCP-1 mRNA and MCP-1 protein. In the kidney, high concentrations of MCP-1 would enhance monocyte chemotaxis and infiltration from the circulation into affected glomeruli. Infiltrated macrophages as well as mesangial cells can continue to take up excess lipids, ultimately forming foam cells. Monocyte infiltration into affected glomeruli, the presence of foam cells, and the deposition of lipids are all key events in the development of glomerulosclerosis.

In conclusion, the present study has clearly demonstrated that Lp-X, at pathophysiological concentrations, stimulates monocyte chemotaxis via a mechanism involving MCP-1 expression in mesangial cells. Lp-X-induced activation of PKC may mediate this effect by activation of NF- $\kappa$ B. These findings add to our understanding of the mechanisms by which patients with familial LCAT deficiency develop glomerulosclerosis.

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