Lysophosphatidylcholine Stimulates Monocyte Chemoattractant Protein-1 Gene Expression in Rat Aortic Smooth Muscle Cells

James X. Rong, Joan W. Berman, Mark B. Taubman, Edward A. Fisher

Objective—Monocyte chemoattractant protein (MCP)-1 is a proatherogenic factor that is responsible for \( \approx 60\% \) of plaque macrophages in mouse models of atherosclerosis. We investigated whether lysophosphatidylcholine (LPC), enriched in oxidized low density lipoprotein, can modulate the expression of MCP-1 in arterial wall cells.

Methods and Results—LPC induced a 3-fold increase in MCP-1 mRNA in rat vascular smooth muscle cells (VSMCs) in a time- and dose-dependent manner. Nuclear runon analysis showed that this increase was attributable to increased MCP-1 gene transcription. There was a 2-fold increase in MCP-1 protein in the conditioned media of cells treated with LPC. LPC-associated increases of MCP-1 mRNA and protein were similar to those produced by platelet-derived growth factor-BB, a known inducer of MCP-1. Analyses of the MCP-1 promoter in transiently transfected VSMCs indicated an LPC-responsive element(s) between base pairs \(-146\) and \(-261\) (relative to transcription initiation). Further studies suggested that LPC-induced MCP-1 expression partially involves mitogen-activated protein kinase/extracellular signal–regulated kinase, a tyrosine kinase, and (to a lesser extent) protein kinase C but not the activation of the platelet-derived growth factor receptor.

Conclusions—LPC stimulates MCP-1 expression at the transcriptional level in VSMCs, suggesting a molecular mechanism by which LPC contributes to the atherogenicity of oxidized low density lipoprotein. (Arterioscler Thromb Vasc Biol. 2002;22:1617-1623.)

Key Words: lysophosphatidylcholine  ■  monocyte chemoattractant protein-1  ■  smooth muscle cells  ■  tyrosine kinase  ■  mitogen-activated protein kinase/extracellular signal–regulated kinase

Oxidized LDL (oxLDL) is involved in the pathogenesis of atherosclerosis. Lysophosphatidylcholine (LPC) is a prominent component of oxLDL. During oxidation, 40% of LDL phosphatidylcholine can be converted to LPC by LDL-associated phospholipase A2, an enzyme that is an independent predictor of coronary heart disease. LPC is also an enriched atherogenic lipoprotein, \( \beta \)-VLDL.

A number of proatherothrombotic effects of these lipoprotein have been attributed to the inflammatory effects of LPC, including (1) disturbance of vascular tone, (2) induction in endothelial cells (ECs) of adhesion molecules and chemotactic, (3) stimulation of vascular smooth muscle cell (VSMC) migration and proliferation, and (4) inhibition of endothelial migration after injury. Recently, LPC has been shown to bind to G-protein–coupled receptors (GPRs) in lymphocytes and various tissues, including the aorta, to induce receptor internalization, mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK/ERK) activation, and chemotaxis. Thus, LPC can trigger signal transduction cascades involved in the initiation and development of atherosclerosis.

Another important proatherogenic molecule is monocyte chemoattractant protein (MCP)-1. MCP-1 is an immediate early gene and is induced by growth factors and inflammatory cytokines. MCP-1 recruits monocytes, precursors of foam cells, into the arterial wall and has been shown to mediate oxLDL-induced monocyte chemotaxis in cocultures of VSMCs and ECs. In mouse models of atherosclerosis, deficiency of MCP-1 or its receptor, CCR2, led to an \( \approx 50\% \) to 80% reduction in lesion size, and overexpression of MCP-1 accelerated atherosclerosis progression, thereby providing direct evidence of the pathophysiological importance of MCP-1.

In addition to ECs and macrophages, VSMCs are another major source of vessel wall MCP-1. For example, MCP-1 secreted from cultured VSMCs was responsible for all of the platelet-derived growth factor (PDGF)-induced monocyte chemotactic activity. MCP-1 mRNA was found in VSMCs...
of human atherosclerotic plaques in vivo. In addition, MCP-1 secreted by VSMCs overlaid by ECs contributed to the monocyte chemotactic activity in response to oxLDL. Although LPC-induced monocyte chemotaxis could result from a direct chemotactic effect of LPC in vitro, an indirect mechanism involving MCP-1 induction is still quite important because of the central role that MCP-1 plays in atherogenesis, which may represent a final common pathway for many proatherogenic factors.

Given the foregoing and the findings that secretory phospholipase A2, LPC, and functional LPC receptors are all present in the arterial wall, we studied the regulation of MCP-1 by LPC in VSMCs, the most abundant arterial cell type. We found that LPC stimulates the production of MCP-1 by VSMCs at the level of transcription through a mechanism that involves MEK/ERK, tyrosine kinase, and (to a lesser extent) protein kinase C (PKC) activities. Therefore, a similar mechanism may contribute to the proatherogenic effects of LPC in the arterial wall.

**Methods**

The Methods section can be found online at [http://www.atvb.ahajournals.org](http://www.atvb.ahajournals.org).

**Results**

**Cytotoxicity**

High concentrations of LPC are known to be cytotoxic. To determine the maximal subtoxic concentration, rat VSMCs were treated with increasing concentrations of LPC. As assessed by a measure of mitochondrial function (Methods), rat VSMC metabolic activity was 100.0±1.9%, 102.8±1.3%, 100.8±1.3%, 78.3±2.9%, and 23.4±2.3% (relative to untreated cells, mean±SD) after treatment with 0, 20, 50, 100, and 200 µmol/L LPC, respectively. Thus, up to 50 µmol/L LPC did not affect rat VSMC metabolic activity. In addition, treatment with up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown). Therefore, concentrations up to 50 µmol/L LPC did not change cell counts, nor did we observe any changes in the cell morphology as described by others during the treatment period (data not shown).

**Induction of MCP-1 mRNA and Protein by LPC**

Figure 1A shows a representative Northern blot analysis of MCP-1 mRNA abundance in quiescent subconfluent rat VSMCs treated with 0 to 50 µmol/L LPC. The signal intensities for MCP-1 mRNA are summarized in Figure 1B. MCP-1 mRNA was detectable in untreated VSMCs, as was MCP-1 protein in the conditioned media of untreated cells (7.8 ng/mL per 10⁶ cells). There was a dose-dependent increase in MCP-1 mRNA after 4 hours of LPC treatment. At the highest LPC concentration, MCP-1 mRNA appeared to plateau at 3-fold the value in untreated cells. The induction of MCP-1 mRNA by LPC (50 µmol/L, 4 hours) was associated with a 2-fold increase in MCP-1 protein secretion (Figure 1C). The extent of LPC induction of MCP-1 mRNA (below) or protein (Figure 1C) was comparable to that by PDGF-BB (20 ng/mL for 4 hours).

**Time-Dependent Induction of MCP-1 mRNA in VSMCs by LPC**

Figure 2A shows a representative slot-blot analysis of MCP-1 mRNA abundance in quiescent subconfluent rat VSMCs treated with 50 µmol/L LPC for 0 to 24 hours. PDGF-BB treatment (20 ng/mL) served as a positive control. The signal intensities are summarized in Figure 2B. Note that in LPC-treated cells, MCP-1 mRNA abundance peaked at 4 hours and declined to baseline after 8 hours. This pattern was different from that induced by PDGF-BB treatment, which resulted in...
a more rapid induction and return to baseline, consistent with previous observations.

**Time-Dependent Induction of MCP-1 mRNA Synthesis by LPC**

To determine whether LPC induction of MCP-1 mRNA was the result of increased transcription, we performed nuclear runon assays (Figure 3). In untreated cells, there was detectable transcription of the MCP-1 gene (Figure 3A), consistent with the basal levels of MCP-1 mRNA. MCP-1 transcript levels were not changed at 15 minutes, 30 minutes (data not shown), or 1 hour after treatment but then increased to 200% of baseline 3 hours after treatment, with a return to baseline by 4 hours.

**Figure 2.** LPC induction of MCP-1 mRNA is time dependent. Quiescent subconfluent rat VSMCs were treated with LPC (50 μmol/L) or PDGF-BB (20 ng/mL) in DMEM and 1% FBS for the indicated times. Total RNA was extracted and subjected to slot-blot analysis with 32P-labeled MCP-1 cDNA or β-actin DNA. A, Representative slot-blot autoradiogram. B, Densitometric analysis of MCP-1 mRNA signals after normalization to β-actin. Data are mean±SE from 2 independent experiments.

**Figure 3.** LPC induction of MCP-1 gene transcription is time dependent. Quiescent subconfluent rat VSMCs were treated with LPC (50 μmol/L), nuclei were harvested at the times indicated, and nuclear runon assays were performed. A, A representative autoradiogram is shown after hybridization of the labeled RNA to pBluescript (pBS) DNA, MCP-1 cDNA, or GAPDH cDNA. B, Summary of MCP-1 transcript abundance after normalization to the corresponding result for GAPDH is shown. Data are mean±SE from 2 independent experiments.

**LPC Responsive cis-Acting Elements of the Rat MCP-1 Promoter**

Transfection experiments were conducted to determine the LPC-responsive elements in the MCP-1 promoter. As summarized in Figure 4, serial deletions from the 5′ end of the MCP-1 promoter (−59, −146, −261, −1053, and −2565 bp from transcription start site) were ligated upstream from the firefly luciferase gene. Known cis elements for other agonists in the promoter region are also indicated in Figure 4 and include the following: AP-1/Sp1 binding sites (−54 to −39), nuclear factor (NF)-κB binding sites (−2287 to −2278 and −2261 to −2252), and PDGF-responsive elements (−146 to −128 and −84 to −59). Deletion of the region containing the NF-κB sites in the MCP-1 promoter did not decrease LPC-induced luciferase activity. After the MCP-1 promoter was truncated to <146 bp, LPC induction was lost, indicating the existence of an LPC-responsive element(s) in the region −146/−261. Further removal of a −146 to −59 region containing a PDGF-responsive element did not have an additional effect on LPC induction. The promoter was not further truncated, because we and others have shown that basal promoter activity would be lost after deletion of the AP-1/Sp1 sites.

**Involvement of MEK/ERK, PKC, Tyrosine Kinase, and PDGF Receptors in LPC-Induced MCP-1 Expression**

A functional LPC receptor, GPR4, has recently been demonstrated in the aorta and has been shown to activate MEK/ERK on LPC binding. To determine whether the GPR4-MEK/
ERK cascade is involved in LPC-induced MCP-1 expression, we treated cells with PD098059, a specific MEK/ERK inhibitor, before and during the treatment with LPC. PD098059 (at 30 μmol/L) partially inhibited LPC-induced MCP-1 mRNA accumulation (~38%, Figure 5A) and MCP-1 secretion (~50%, average of 2 determinations). At this inhibitor concentration, PDGF-BB–induced (data not shown) and angiotensin II–induced MCP-1 accumulation is completely blocked.32

The lack of complete inhibition in LPC-induced MCP-1 accumulation may reflect the involvement of other signaling pathways in addition to GPR4-MEK/ERK. PKC has been shown to be involved in LPC-induced MCP-1 expression in ECs.7 To investigate the role of PKC in VSMCs, the cells were pretreated with phorbol 12,13-dibutyrate for 24 hours to downregulate PKC before LPC treatment.33 As shown in Figure 5B, downregulation of PKC completely aborted the induction of MCP-1 by another phorbol ester, phorbol 12-myristate 13-acetate (PMA), but had only a minor effect (≈25%) on LPC-induced MCP-1 expression.

Because tyrosine kinases are important mediators of chemokine-GPR–induced signal transduction,34 we examined the effect of the general tyrosine kinase inhibitor genistein on LPC induction of MCP-1 mRNA. As shown in Figure 5A, genistein (at 30 μmol/L) completely inhibited the effect of LPC. The PDGF receptor is also a known tyrosine kinase. The characteristics of LPC induction (ie, the comparable extent to which MCP-1 expression was induced by LPC and PDGF-BB and the lag in the peak of MCP-1 transcript synthesis induced by LPC relative to the peak with PDGF-BB) were compatible with an initial stimulation of PDGF receptor (at 2E1A2, the monoclonal antibody that specifically recognizes the βPDGF receptor35,36 (the only isoform present in rat VSMCs37), abolished PDGF-BB induction of MCP-1 mRNA, but there was no effect on LPC induction. Taken with the genistein results, this indicates that a tyrosine kinase(s) other than the PDGF receptor is involved in LPC-induced MCP-1 expression.

Discussion

We have examined whether a mechanism contributing to the chemotactic activity for monocytes17 of oxLDL is the LPC–induced transcription of the MCP-1 gene in VSMCs. This would be expected to have significant atherogenic consequences because VSMCs express MCP-1 and because they are the most abundant cells in the arterial wall. We found that LPC at concentrations comparable to those found in mammalian plasma28 induces MCP-1 mRNA abundance and protein secretion in a dose- and time-dependent manner. The accumulation was mainly due to increased transcription, involving an LPC-responsive element(s) in the −261 to −146 region of the promoter. On the basis of inhibitor- and receptor-blocking experiments, the induction process appeared to partially involve MEK/ERK, PKC, and a tyrosine kinase(s) other than the PDGF receptor.

LPC has many effects that are expected to play important roles in atherogenesis.38 For example, LPC stimulates cytokine,59,60 chemokine,7 adhesion molecule,5,41 and growth factor production6 at the transcriptional level. The present study now demonstrates that 1 mechanism by which LPC may contribute to the recruitment of monocytes into the arterial wall by atherogenic lipoproteins is the transcriptional upregulation of MCP-1 in VSMCs, which is different from the direct chemotactic effect of LPC.24 Because MCP-1 is responsible for ~60% of the monocyte/macrophage area in mouse models of atherosclerosis,19,20 the indirect chemotactic effect of LPC (through MCP-1) may be quite significant in vivo.

ECs are another source of LPC-induced MCP-1 production.7 The greater cell mass of VSMCs compared with ECs and the abundance of monocytes/macrophages deep within the atherosclerotic plaque (ie, adjacent to the medial smooth muscle layer) would argue that VSMCs may be a major contributor to the concentration gradient for monocyte che-
motaxis. In fact, MCP-1 from VSMCs of the tunica media was implicated in early lesion formation in diet-induced hypercholesterolemic primates, whose lipoproteins consist mainly of HDL enriched with LPC.

We have previously identified cis-acting elements responsible for PDGF-induced MCP-1 expression in VSMCs at positions −146 to −128 and −84 to −59 of the rat MCP-1 promoter. However, these elements are not likely to be involved in the response to LPC. Instead, an LPC-responsive element(s) appears to be in the region −261 to −146 (Figure 4). Database analysis (Institute for Transcriptional Informatics, Pittsburgh, Pa, which can be accessed at http://www.ifti.org) identified a number of consensus sequences for enhancers, including a PEA3 site (also responsible for LPC-induced endothelial NO synthase expression43), a W element (in the interferon-γ-responsive region of the human class II major histocompatibility complex gene DPA44), and an element in the intron region of murine fibroblast growth factor-8 gene. Demonstration of the roles of these candidates in the transcription regulated by LPC will require additional studies.

It is notable that the removal of an upstream 1512-bp MCP-1 promoter segment containing the NF-κB sites, previously shown to be involved in MCP-1 expression induced by other stimuli, did not decrease the induction by LPC. In fact, there was a moderate, but not significant, increase (Figure 4). Because there could be cis elements within the deleted segment that are negative transcription factors, our results do not completely rule out the possibility of NF-κB involvement. Also of interest were cis-acting sequences for peroxisome proliferator-activated receptor (PPAR)-α, because a previous report indicated that MCP-1 expression from human ECs induced by oxLDL was mediated by PPARα. However, no PPARα consensus sequences were found in the rat MCP-1 promoter up to position −3657 bp. Thus, if there are PPARα-responsive elements, they must be further upstream.

We were interested in the signaling process by which LPC activates MCP-1 expression. LPC could stimulate PDGF expression, which then would activate its own receptor in an autocrine or paracrine fashion. Besides the kinetic evidence compatible with this scenario (summarized in Results), LPC has been shown to stimulate growth factor production in other contexts. However, the data from the antibody blockade experiment did not support this model (Figure 6).
Takahara et al. showed that in ECs, LPC-induced MCP-1 expression was significantly (53%) inhibited by the PKC inhibitor staurosporine. Our results with a highly specific PKC downregulator were less dramatic in LPC-treated VSMCs (Figure 5B). Whether this quantitative difference represents cell background effects or the known lack of specificity of staurosporine is not clear, but in any case, PKC mediation of LPC induction of MCP-1 appears to be a minor pathway in VSMCs.

Another signaling possibility is suggested by the recent demonstration of LPC-activated GPR family receptors (resulting in cell chemotaxis) in T lymphocytes and in a number of tissues, including the aorta. On ligand binding, mitogen-activated protein kinase, tyrosine kinase, and/or other mediators are activated. Our results (Figures 5A) indicate that MEK/ERK and tyrosine kinase activities are involved in the LPC induction of MCP-1. The tyrosine kinase activity appeared to be upstream from MEK/ERK because genistein was completely effective, whereas the MEK/ERK inhibitor only partially blocked the induction. The involvement of a tyrosine kinase activity has also been implicated in LPC-induced expression of intercellular adhesion molecule-1 in human umbilical ECs.

This putative tyrosine kinase activity is not likely to be the PDGF receptor, given the evidence noted above against its participation in effects of LPC. One candidate is Janus kinase, which has been shown as downstream from tyrosine kinase for a number of chemokine receptors, including CCR2 and CCR5, and has been shown to be activated by LPC in ECs. Whether this kinase or other receptor or Src-related kinases are responsible for LPC-induced MCP-1 expression requires further studies.

In summary, our results suggest that the atherogenic effects of LPC in vivo include the induction of MCP-1 expression in VSMCs at the level of transcription. It is also possible that in other cell types that express MCP-1 in the arterial wall (macrophages and ECs), a similar induction occurs, further augmenting the influence of LPC. Because LPC is a major component of oxLDL, further elucidation of the pathways by which LPC induces MCP-1 production will increase our knowledge of the molecular mechanisms by which this modified lipoprotein exerts its potent atherogenic effects.

Acknowledgments

This work was supported by National Institutes of Health grants DK-44498 (Dr. Fisher), HL-61814 (Dr. Fisher), and HL-61815 (Dr. Taubman), and American Heart Association (AHA) Grant-in-Aid (Dr. Berman). Dr. Rong was awarded an AHA Heritage Affiliate Postdoctoral Fellowship (grant 0020480T). We thank Drs. Nathalie A. Leroc, Millennium Pharmaceuticals, Inc., for kindly providing the β-PDGFR receptor monoclonal antibody 2E1A2; Dr. Kiyoshi Nose, Showa University School of Pharmaceutical Sciences, Japan, for kindly providing the MCP-1 promoter-CAT plasmid; Dr. Vladimir Y. Bogdanov, Mount Sinai, for kindly providing the MCP-1 promoter-1CAT plasmid; Dr. Vladimir Y. Bogdanov, Mount Sinai, for kindly providing the MCP-1 promoter-CAT plasmid; and Dr. Vladimir Y. Bogdanov, Mount Sinai, for kindly providing the MCP-1 promoter-CAT plasmid.

References

response to PDGF is due predominantly to the induction of JE/MCP-1.


Lysophosphatidylcholine Stimulates Monocyte Chemoattractant Protein-1 Gene Expression in Rat Aortic Smooth Muscle Cells
James X. Rong, Joan W. Berman, Mark B. Taubman and Edward A. Fisher

Arterioscler Thromb Vasc Biol. 2002;22:1617-1623; originally published online August 29, 2002;
doi: 10.1161/01.ATV.000035408.93749.71
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/22/10/1617

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2002/10/09/22.10.1617.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/
Methods (Rong et al)

Cell Culture & Treatments—Vascular SMCs (VSMC) were isolated from thoracic aortas of Sprague-Dawley rats, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and used between passages 12 and 27. For cytotoxicity assays, VSMC (~8x10^4 cells) were plated in 24-well Corning plates in 10% FBS in DMEM, grown to subconfluence (80-90%, ~48 hours after plating), and switched to 1% FBS in DMEM for 24 hours. Cells were then treated with 0, 20, 50, 100, or 200 μM LPC (Avanti Polar Lipids, Inc., Alabaster, AL; stock solution of 80 mM in ethanol) for 4 hours. To assess cytotoxic effects, CellTiter 96® Aqueous One Solution Reagent (Promega Corp., Madison, WI; 75 μl/well) was then added and incubated with the cells for 1 hour. The reagent contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt), which is converted by metabolically active cells into a water-soluble, colored formazan product measurable at 490 nm. Cytotoxicity is inversely related to the relative metabolic activity, which for LPC can be determined by the following formula:

Relative Metabolic Activity (%) = (Absorbance in LPC-treated well – Background) / (Absorbance in untreated well – Background) x 100.

The background was determined by the absorbance of CellTiter 96® Aqueous One Solution Reagent in 1% FBS in DMEM.

For Northern blot, slot/dot blot, and enzyme-linked immunosorbant assays (ELISA), subconfluent VSMC in 75-cm^2 T-flasks were serum-starved (1% FBS in DMEM, 24 hours) to become quiescent, and treated with LPC for the intervals and
concentrations indicated in Results. Medium was removed for ELISA, and total RNA was extracted from cells for Northern/slot/dot blot (see below). Treatment with human PDGF BB (Roche Diagnostics; 20 ng/ml, 4 hours) served as the positive control for MCP-1 protein secretion or mRNA accumulation in some experiments.

To examine the potential role of tyrosine kinases, MEK/ERK, protein kinase C (PKC), or the PDGF receptor in mediating LPC’s effects on MCP-1 mRNA abundance, before LPC or PDGF treatment, cells were pretreated for 1 hour with either the tyrosine kinase inhibitor genistein (Calbiochem, La Jolla, CA; 30 μM in 1% FBS in DMEM, diluted from 30 mM stock in dimethylsulfoxide—DMSO) or MEK/ERK inhibitor PD098059 (Calbiochem; 30 μM in 1% FBS in DMEM, diluted from 30 mM stock in DMSO), or for 24 hours with phorbol 12,13 dibutyrate¹ (PDBu; Calbiochem; 1 μM in 1% FBS in DMEM, diluted from 2 mM stock in DMSO) to down-regulate PKC, or for 15 minutes with 2A1E2 (20 nM) a monoclonal antibody to α-PDGF receptor²,³ (kindly provided by Dr. Nathalie A Lokker, COR Therapeutics, South San Francisco, CA). Cells were then treated with LPC or phorbol 12-myristate 13-acetate (250 nM) for 4 hours in the presence of the above inhibitors/antibody, but in the absence of PDBu.

For nuclear run-on experiments, after serum-starvation (as above), VSMC were treated with LPC (50 μM) for 0, 1, 2, 3, and 4 hours. Cells were then lysed by NP-40 as described⁴, and nuclei were immediately isolated for the analysis of new transcript synthesis (see below).

*Northern & Slot/Dot Blot*—Total RNA was extracted using the QIAGEN RNeasy kit. For Northern blot analysis, the RNA was subjected to denaturing agarose gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH).
For dot/slot blot analysis, the RNA was blotted to the membrane directly using a Bio-Rad (Hercules, CA) Bio-dot microfiltration apparatus. As previously described\(^5\), the membranes were hybridized to \(^{32}\)P-labeled rat MCP-1 cDNA or \(\beta\)-actin DNA (loading control). The intensities of the MCP-1 signals on the autoradiograms were determined by the Bio-Rad Gel Doc 1000 System equipped with Quantity One software (Hercules, CA). After normalization to the corresponding data for \(\beta\)-actin, the results were expressed relative to those in the untreated cells.

**Nuclear Run-On Assays**—Synthesis of nascent RNA transcripts in nuclei and the isolation of the transcripts were performed as previously described\(^4,6\). The resulting \(^{32}\)P-UTP-labeled transcripts were hybridized to linearized and denatured rat MCP-1 cDNA immobilized on nitrocellulose filters. Immobilized glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used as the control for the general level of transcriptional activity, and the pBluescript vector used as a negative control for non-specific binding of labeled transcripts. Densitometric analysis of MCP-1 was performed as above. The data were normalized to the results for GAPDH, and are expressed relative to those in the untreated cells.

**MCP-1 ELISA**—MCP-1 protein in the medium was determined by an anti-rat ELISA kit (BD PharMingen, San Diego, CA) according to manufacturer's instructions, and the results were expressed relative to those in the untreated cells.

**Plasmid Construction**—Luciferase reporter plasmids containing the DNA segments -1053, -261, -146, or -59-bp upstream of the transcription start site of the rat MCP-1 gene were constructed previously\(^6\). The -2565-luciferase plasmid, which contains the NF\(\kappa\)B sites, was constructed by replacing a 229-bp fragment at the 5’- end of the -1053
promoter with a 1741-bp fragment between the BsrD I and NcoI site from the -2,621-CAT plasmid, kindly provided by Dr. K. Nose, Showa University, Japan.

**Transient Transfections**—Subconfluent VSMC in 6-well plates were transfected with the MCP-1 reporter plasmid constructs described above using LipofectAmine Plus Reagent (Invitrogen Corporation, Carlsbad, CA) following the kit protocol. A human growth hormone (hGH) plasmid expression vector (pXGH5; Promega, Madison, WI) was co-transfected (at ~1/3 molar ratio of the MCP-1 plasmids) to serve as the control for transfection efficiency. Total DNA in each well was 1.5 μg.

**Luciferase Assays**—VSMC were washed twice at 25°C with phosphate-buffered saline, treated with luciferase cell culture lysis reagent (Promega) and assayed for luciferase activity in a BioOrbit 1251 luminometer (Wallac, Gaithersburg, MD) using luciferase assay reagent (Promega). Levels of luciferase activity were normalized to the levels of hGH in the conditioned medium from the corresponding plates. The mean normalized luciferase activity detected in VSMC was expressed relative to the normalized luciferase activity in untreated VSMC.

**hGH Assays**—Conditioned media from the transfected cells were collected and the secreted hGH was determined using an hGH radioimmunoassay kit (Nichols Institute Diagnostics, San Juan Capistrano, CA) as described.

**Statistics**—Within an experiment, duplicate or triplicate wells were used for each condition or treatment. All experiments were repeated at least once. Numerical data in cytotoxicity, ELISA, Northern/slot/dot blot, and luciferase assays were averages of duplicates from two to three independent experiments and expressed as average±S.E. For
ELISA, differences between samples were analyzed by two sample student t-test, and P values < 0.05 were considered significant.

References:


