Abstract—The expression of tissue factor (TF), the principal initiator of coagulation, is increased during inflammation and atherosclerosis. Both conditions are promoted by lysophosphatidylcholine (lysoPC). We observed in the present study that lysoPC (1 to 10 μmol/L) dose-dependently reduced TF activity in human monocytes, as elicited by lipopolysaccharide (LPS). Lysophosphatidylethanolamine (lysoPE) and other lysophospholipids did not affect LPS-induced TF activity of human monocytes. TF antigen expression as elicited by LPS was also lowered by lysoPC. Phospholipid analyses indicated a selective increase in the lysoPC content of the monocytes after preincubation with the lysophospholipid. LysoPC inhibited the TF activity of Mono Mac-6 cells to a similar extent as in the monocytes. LPS binding to plasma membrane receptors and internalization of LPS into monocytes were not affected by lysoPC. In contrast, LPS-mediated nuclear binding of nuclear factor-κB/Rel to a TF-specific κB site was inhibited by lysoPC. Induction of TF mRNA expression by LPS tended to be partially reduced by the lysophospholipid. Preincubation with lysoPC increased monocytic cAMP levels. Inhibition of adenylyl cyclase by pretreatment with 2′-deoxy-3′-adenosine monophosphate partially reversed the inhibition of TF activity promoted by lysoPC. In conclusion, lysoPC markedly decreases LPS-mediated TF expression of human monocytes, the effect probably being mediated by both transcriptional and posttranscriptional mechanisms. LysoPC may thus attenuate activation of coagulation during inflammation and atherosclerosis. (Arterioscler Thromb Vasc Biol. 1999;19:47-53.)

Key Words: atherosclerosis ■ inflammation ■ lipopolysaccharide ■ cAMP ■ nuclear factor-κB

Tissue factor (TF) is the primary activator of the extrinsic and intrinsic pathways of coagulation (for recent reviews, see References 1 and 2). Within the extrinsic pathway of coagulation, TF forms a complex with factor VIIa that stimulates the generation of factor Xa, which in turn is necessary for the formation of thrombin from prothrombin. Additionally, the TF-factor VIIa complex activates the intrinsic coagulation pathway by stimulating the formation of factor IXa. Increased expression of TF is a feature of several pathophysiological conditions, in particular, inflammation and atherosclerosis. Agents known to induce or sustain inflammation, such as bacterial lipopolysaccharide (LPS), interleukin-1, tumor necrosis factor-α (TNF-α), or C-reactive protein, are among the most potent stimulators of TF expression. The increased activity of endothelial TF is thought to be responsible for the development of disseminated intravascular coagulation during sepsis. Furthermore, it has been proposed that TF contributes to the thrombotic complications associated with atherosclerosis. In agreement with this hypothesis, increased expression of TF has been observed in specimens taken from atherosclerotic plaques. Oxidized LDL, which is thought to be a causative factor in the pathogenesis of atherosclerosis, was shown to augment basal and LPS-induced TF activity of endothelial cells and monocytes, respectively.

Local and systemic inflammatory processes are initiated and/or promoted by activation of phospholipase A2 (PLA2), in particular, those of the group II secretory type. PLA2-catalyzed hydrolysis of phosphatidylcholine (PC), the major phospholipid of most cells, liberates arachidonic acid, which may be further processed to eicosanoids. The second product of the reaction is lysophosphatidylcholine (lysoPC). This lysophospholipid is presumed to play a role in sustaining inflammation due to transcriptional activation of genes coding for adhesion molecules, cytokines, and growth factors. LysoPC is also formed in considerable amounts by oxidation of LDL particles, probably by PLA2-dependent hydrolysis of LDL-associated PC. Several of the atherogenic effects of oxidized LDL, eg, impairment of endothelium-dependent vasorelaxation or enhanced adhesion of monocytes to the endothelium, have previously been shown to be mediated by lysoPC. Thus, lysoPC is most probably generated in substantial amounts under pathophysiological conditions, when increased expression of TF is observed. In the present study we...
therefore wished to ascertain whether lysoPC affected monocytc TF expression.

Methods
Isolation of Monocytes
Human peripheral blood mononuclear cells (PBMCs) were isolated by buoyant density centrifugation with Ficoll-Hypaque essentially as described in Reference 17. Buffy coats obtained from healthy donors (anticoagulated by EDTA) were diluted with 3 vol of calcium-free PBS, and the suspension was underlayered with low-endotoxin Ficoll (d=1.077 g/mL, Pharmacia Biotech). After centrifugation for 25 minutes at 420g, the interphase was collected and washed twice with isotonic PBS containing 0.13% EDTA and 0.15% BSA (pH 7.4), which is referred to as “washing buffer.”

The cell pellet obtained after the last centrifugation was usually suspended in 10 mL plasma concomitantly obtained from the same donor. To this suspension, first 50 μL of 9% NaCl was added, after 10 minutes an additional 100 μL was added, and after a further 10 minutes another 100 μL of 9% NaCl was added. The cells suspended in the hypertonic medium were overlayered on Ficoll (d=1.077 g/mL). After centrifugation for 15 minutes at 625g, the broad interphase between the Ficoll layer and the medium was collected and washed twice with washing buffer and finally washed once with Ham’s F-10 medium supplemented with 5% FCS. Mononuclear cells were usually cultured in the latter medium. Flow cytometric analysis using FITC–labeled CD45/CD14 antibodies (Dianova-Immunotech) indicated that 54±17% of mononuclear cells were monocytes. In some experiments, monocytes were further purified by incubating the PBMC suspension for 15 minutes at 4°C with microbeads conjugated to anti-human CD14 antibodies and thereafter passed over a positive selection column (Miltenyi Biotech). Thereby a suspension consisting of 98% monocytes was obtained. The monocytic cell line Mono Mac-612 (kindly provided by Dr Lüms Ziegler-Heitbrock, University of Munich) was cultured in Ham’s F-10 medium supplemented with 5% FCS.

Endotoxin Contamination
Ham’s F-10, FCS, and NaCl-Tris buffer were routinely tested for endotoxin contamination with the Coatest kit (Chromogenix). The endotoxin concentration of the media and buffer was always <0.1 ng/mL.

Pretreatment of Cells With Lysophospholipids
Usually, 106 PBMCs in 1 mL of Ham’s F-10 medium supplemented with 5% FCS were preincubated at 37°C with different concentrations of 1-palmitoyl-2-lysoPC or either lysophosphatidylethanolamine (lysoPE), lysophosphatidylserine (lysoPS), or lysophosphatidylglycerol (lysoPG; all obtained from Sigma) dissolved in 0.5% to 95% of the cells. Some experiments, monocytes were further purified by incubating the PBMC suspension for 15 minutes at 4°C with microbeads conjugated to anti-human CD14 antibodies and thereafter passed over a positive selection column (Miltenyi Biotech). Thereby a suspension consisting of 98% monocytes was obtained. The monocytic cell line Mono Mac-612 (kindly provided by Dr Lüms Ziegler-Heitbrock, University of Munich) was cultured in Ham’s F-10 medium supplemented with 5% FCS.

Phospholipid Analysis of Monocytes
The PBMC suspension was incubated with 10 μmol/L lysoPC at 37°C or with 1% ethanol. After centrifugation and removal of the supernatant, the cell pellet was resuspended in Ham’s F-10 medium (with 5% FCS), and monocytes were isolated by using microbeads conjugated with CD14 antibodies as detailed above. Lipids from 1 mL of monocyte suspension containing 4×106 cells were extracted according to Reference 19. The lipid extract was applied to silica G60 plates (Merck), previously sprayed with potassium oxalate (1% in methanol and water, 2:3 vol/vol), and activated for 30 minutes at 80°C. The plates were developed in the first dimension with the solvent system chloromethane/methanol/ammonia/water (45:37:6:4, vol/vol/vol/vol); dried; and in the second dimension, treated with chloromethane/methanol/acetic acid/water (40:15:15:12.8, vol/vol/vol/vol/vol). For quantification of phospholipids, the phosphate contents of the spots were determined as previously detailed.20

Assessment of TF Activity
After preincubation with lysophospholipids (or vehicle), PBMCs or Mono Mac-6 cells (104/well) were incubated for 6 hours at 37°C in the presence of LPS (from Escherichia coli or Salmonella minnesota) in 96-well plates with 200 μL of Ham’s F-10 medium (without phenol red) containing 5% FCS. Subsequently, the medium was removed, deoxycholate (0.15%, vol/vol) was added, and the cells were subjected to 2 cycles of freezing and thawing. TF activity was assayed essentially as described previously25 by a 1-stage amidolytic assay using the chromogenic substrate S2222. The cell lysate was incubated for 25 minutes at 30°C in the above-specified Ham’s F-10 medium containing 0.88 U/mL factor VII (final concentration) of a coagulation factor concentrate consisting of factors II, VII, IX, and X (Beriplex) and 125 μg/mL (final concentration) of the chromogenic substrate S2222 (Chromogenix). The increase in optical density at 405 nm was monitored in an ELISA reader (Dynatech MR 7000, Dynatech Laboratories). A standard curve was prepared by using dilutions of TF concentrates (Thromborel S from human placenta; Behring).

Determination of TF Antigen
After preincubation of the PBMCs with lysophospholipids and subsequent incubation in the presence of LPS, the mononuclear cells were separated from the incubation buffer by centrifugation. The supernatant was stored for determination of TF antigen released into the extracellular medium. The cells were disrupted by a 3-second sonication on ice, and cell fragments were extracted for 12 hours at 4°C with a buffer composed of 100 mmol/L NaCl, 50 mmol/L Tris (pH 7.49), and 0.1% Triton X-100. After a 10-minute centrifugation, TF antigen in the cell extracts was measured by using a commercially available kit according to the instructions of the manufacturer (Imubind TF ELISA Kit, Loxo GmbH). The method uses a monoclonal antibody against the TF antigen coupled to a biotinylated rabbit polyclonal antibody for detection.

Assessment of LPS Binding and Internalization
Subsequently to preincubation with lysoPC or vehicle, the mononuclear cell suspension was washed once with Ham’s F-10 containing 5% FCS and resuspended in the same medium. R7-LPS (from S minnesota) was added, and the suspension was incubated further for 60 minutes at 4°C. Binding of R7-LPS was detected by using a primary anti–R7-LPS monoclonal antibody (clone S3232) coupled to a secondary FITC–labeled goat anti-mouse antibody. For estimation of LPS internalization, after a 60-minute incubation of mononuclear cells at 4°C in the presence of R7-LPS, the suspension was further incubated for 360 minutes without LPS at either 4°C or 37°C. From the differences in fluorescence parameters at the 2 temperatures, the amount of LPS internalized can be estimated, because LPS is incorporated at 37°C but not at 4°C and the LPS taken up by the cells becomes inaccessible to the anti-LPS antibody. Selective LPS binding and internalization into monocytes were determined in a Cyttofluorograf (system 50H, Ortho Diagnostic Systems Inc) through gating in the forward/sideward scatter.

Determination of cAMP Content
Monocytes were isolated from the mononuclear cell suspension by using CD14 antibodies (see above) and thereafter preincubated for 60 minutes at 37°C with lysoPC. Subsequently, the cells were resuspended in a medium containing 50 mmol/L Trizma, 20 mmol/L NaCl, 5 mmol/L KCl, 9.8 mmol/L MgCl2, 2.7 mmol/L Na2EDTA, and 0.1 mmol/L 3-isobutyl-1-methylxanthine (pH 7.4) and incubated for 15 minutes at 37°C. After centrifugation for 10 minutes at 6000g and 4°C, the supernatant was analyzed for cAMP content by using a 1-stage amidolytic assay essentially as described previously21 by a 1-stage amidolytic assay using the chromogenic substrate S2222. The cell lysate was incubated for 25 minutes at 30°C in the above-specified Ham’s F-10 medium containing 0.88 U/mL factor VII (final concentration) of a coagulation factor concentrate consisting of factors II, VII, IX, and X (Beriplex) and 125 μg/mL (final concentration) of the chromogenic substrate S2222 (Chromogenix). The increase in optical density at 405 nm was monitored in an ELISA reader (Dynatech MR 7000, Dynatech Laboratories). A standard curve was prepared by using dilutions of TF concentrates (Thromborel S from human placenta; Behring).

Electrophoretic Mobility Shift Assay (EMSA)
Nuclear extracts from PBMCs were isolated and analyzed as previously described.25 Protein concentrations were determined by the Bradford method (Bio-Rad). Oligonucleotides with a NF-κB consensus motif or with the NF-κB-like site of the TF promoter were used as probes and labeled by annealing of complementary primers. This
process was followed by primer extension with the Klenow fragment of DNA polymerase I (Boehringer Mannheim) in the presence of [γ-32P]dCTP (>3000 Ci/mmol; DuPont) and deoxynucleoside triphosphates (Boehringer Mannheim). Nuclear extracts (5 μg protein) were incubated with radiolabeled DNA probes (~10 ng; 10^6 cpm) for 30 minutes at room temperature in 20 μL of binding buffer (20 mMol/L Tris-HCl, 50 mMol/L KCl, 1 mMol/L DTT, 0.5 mMol/L EDTA, 1 mg/mL albumin, 5% glycerol, 0.2% NP-40, and 50 ng/μL of poly[dI-dC], pH 7.9). Samples were run in 0.25× TBE buffer (10× TBE is 890 mMol/L Tris, 890 mMol/L boric acid, and 20 mMol/L EDTA, pH 8.0) on nondenaturing 4% polyacrylamide gels at 125 V. As a control, samples were treated with a 100-fold excess of nonlabeled κB oligonucleotide, which completely abolished binding of the radiolabeled oligonucleotide to the nuclear proteins. The binding of transcription factor Sp1 was analyzed by EMSA by using a specific consensus oligonucleotide labeled with [γ-32P]ATP (>5000 Ci/mmol; DuPont) and T4 polynucleotide kinase (Boehringer Mannheim). After the gels were dried, they were analyzed by autoradiography.

Northern Blot Analysis
Total RNA was extracted from PBMCs by using either the microRNA isolation kit (Stratagene) or RNA Instapure (Eurogentec). Total RNA (5 μg) was electrophoresed through a denaturing 1.2% formaldehyde gel and capillary-blotted overnight onto a nylon membrane (Hybond-N, Amersham). Hybridization was carried out overnight at 42°C by using randomly primed cDNA probes (Multiprime, Amersham). The blots were washed with increasingly stringent concentrations of SSC at 52°C and exposed to autoradiography films. The cDNA probe utilized has been described previously.23 To control for variability in sample loading, the blots were rehybridized with a 500-bp EcoRI fragment of GAPDH cDNA.

Statistical Analysis
Statistical analysis was performed by 1-way ANOVA or by Student’s paired t test where appropriate. Values of P<0.05 were considered significant.

Results
Mononuclear cells suspended in Ham’s F-10 medium supplemented with 5% FCS were incubated for different time intervals at 37°C with 0.5 to 1000 ng/mL LPS. Control experiments indicated optimal stimulation of TF activity in the presence of 10 ng/mL LPS (from either E coli or S minnesota) after 6 hours of incubation, no further increase in activity being observed at longer incubation intervals or with higher concentrations of the LPS (data not shown). In mononuclear cells treated for 6 hours with 10 ng/mL LPS, TF activity was elevated by 8.3-fold compared with monocytes incubated without LPS (Figure 1, upper panel).

Preincubation of PBMCs with 1 to 50 μmol/L 1-palmitoyl-2-lysoPC for 60 minutes dose-dependently lowered the stimulatory effect of LPS on TF activity. With 10 and 20 μmol/L lysoPC, TF activity induced by LPS was decreased by 73% and 71%, respectively, no further reduction being evident at 50 μmol/L (Figure 1, upper panel). Basal TF activity was not influenced by preincubation with 10 μmol/L lysoPC (not shown). LPS-induced TF activity was inhibited to a similar degree in monocytes preincubated for 30 minutes with 10 μmol/L lysoPC after a 6-hour stimulation with 10 ng/mL LPS from 3.1±1.2 μU/10^6 cells (without lysoPC) to 1.3±0.4 μU/10^6 cells (with lysoPC). 1-Palmitoyl-2-lysoPE, a lyso-phospholipid species differing from lyso PC by the lack of 3 methyl groups, was added to the preincubation medium in the concentration range 1 to 50 μmol/L. LPS-induced TF activity was unaffected by all concentrations of lysoPE investigated (Figure 1, upper panel). Similarly, a 60-minute preincubation with 50 μmol/L of either lysoPS or lysoPI barely altered TF activity as elicited by LPS (data not shown).

To analyze the effect of lysoPC on TF expression at the protein level, TF antigen was determined in the mononuclear cell suspension by use of a monoclonal antibody against TF (see Methods). Column (col) 1, control (–LPS); col 2, +LPS; col 3, +1 μmol/L lysoPC+LPS; col 4, +2.5 μmol/L lysoPC+LPS; col 5, +5 μmol/L lysoPC+LPS; col 6, +10 μmol/L lysoPC+LPS; col 7, +20 μmol/L lysoPC+LPS; col 8, +50 μmol/L lysoPC+LPS; col 9, +1 μmol/L lysoPE+LPS; col 10, +2.5 μmol/L lysoPE+LPS; col 11, +10 μmol/L lysoPE+LPS; and col 12, +50 μmol/L lysoPE+LPS. Values are mean±SD of experiments on 6 through 10 different mononuclear cell preparations. *P<0.05 versus col 2 by 1-way ANOVA.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** TF activity and TF antigen expression of mononuclear cells is specifically inhibited by lysoPC. Mononuclear cells suspended in Ham’s F-10 medium supplemented with 5% FCS were preincubated for 60 minutes at 37°C with the indicated concentrations (in μmol/L) of lysoPC, lysoPE, or vehicle. Thereafter, the cells were washed once and incubated for another 6 hours with LPS (10 ng/mL) at 37°C in Ham’s F-10 medium containing 5% FCS. Subsequently, mononuclear TF activity (upper panel) was assessed by a 1-stage amidolytic assay as described in Methods. TF antigen expression (lower panel) was determined by using a monoclonal antibody against TF (see Methods). Column (col) 1, control (–LPS); col 2, +LPS; col 3, +1 μmol/L lysoPC+LPS; col 4, +2.5 μmol/L lysoPC+LPS; col 5, +5 μmol/L lysoPC+LPS; col 6, +10 μmol/L lysoPC+LPS; col 7, +20 μmol/L lysoPC+LPS; col 8, +50 μmol/L lysoPC+LPS; col 9, +1 μmol/L lysoPE+LPS; col 10, +2.5 μmol/L lysoPE+LPS; col 11, +10 μmol/L lysoPE+LPS; and col 12, +50 μmol/L lysoPE+LPS. Values are mean±SD of experiments on 6 through 10 different mononuclear cell preparations. *P<0.05 versus col 2 by 1-way ANOVA.
LysoPC Inhibits Tissue Factor Expression

TABLE 1. Selective Increase in LysoPC Contents of Human Monocytes After Preincubation With LysoPC

<table>
<thead>
<tr>
<th>LysoPC</th>
<th>Sphingomyelin</th>
<th>PC</th>
<th>PS</th>
<th>PI</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>−LysoPC</td>
<td>0.60±0.22</td>
<td>1.41±0.28</td>
<td>6.24±0.82</td>
<td>0.70±0.03</td>
<td>0.54±0.17</td>
</tr>
<tr>
<td>+LysoPC</td>
<td>1.55±0.33</td>
<td>1.49±0.27</td>
<td>6.61±0.54</td>
<td>0.73±0.14</td>
<td>0.57±0.10</td>
</tr>
</tbody>
</table>

Mononuclear cells suspended in Ham’s F-10 medium containing 5% FCS were incubated with 10 μmol/L lysoPC (dissolved in ethanol) for 60 minutes at 37°C or with 1% ethanol alone. Thereafter, the cells were washed once and monocytes were purified using CD14 antibody–coated magnetic beads (see Methods). After lipid extraction (19), phospholipids were separated by 2-dimensional Thin-Layer Chromatography (see Experimental Procedures). Mean values ±SD of experiments on monocytes from 5 different donors are shown.

*P<0.05 (paired t test).

be mediated by other blood cells (lymphocytes, platelets) present in the cell preparation. Therefore, experiments were conducted with the monocyctic cell line Mono Mac-6. Mono Mac-6 cells cultivated in Ham’s F-10 containing 5% FCS were preincubated with different concentrations of lysoPC and thereafter challenged with 100 ng/mL LPS for 6 hours. As can be deduced from Figure 2, increasing the lysoPC concentration from 2.5 to 20 μmol/L dose-dependently lowered the LPS-induced TF activity of the cells. A 72% and 71% inhibition was noticed at 20 and 50 μmol/L lysoPC, respectively. Preincubation with 50 μmol/L lysoPE did not alter TF activity as induced by LPS (not shown). Thus, the effect of lysoPC is most probably independent of the presence of nonmonocyctic cells.

A substantial portion of the lysoPC incorporated into the monocytes after preincubation with the lysophospholipid is expected to be localized in the plasma membrane. Therefore, lysoPC could inhibit LPS-induced TF expression by altering the interaction of LPS with LPS-binding sites on the plasma membrane of monocytes. Control experiments indicated that binding of LPS to the monocytes was saturated after 1 hour of incubation at 4°C. The amount of LPS bound to monocytes at this time point was completely unaffected by preincubation with 5, 10, or 50 μmol/L lysoPC (Table 2). This excludes the possibility that the inhibition of TF expression by lyosphospholipid was related to an effect on LPS binding. In further experiments, monocytes (pretreated for 60 minutes with either 10 μmol/L lysoPC or ethanol) were first incubated for 60 minutes at 4°C with LPS and thereafter for an additional 6 hours at either 4°C or 37°C (in the absence of LPS). The differences between the fluorescence intensities obtained at the 2 temperatures after the final 6-hour incubation period were used to estimate LPS internalization (see Methods). These differences were not affected by pretreatment with lysoPC (Table 2).

Induction of TF expression of monocytes is regulated at both the transcriptional and posttranscriptional level.22,23 Activation of p65/c-Rel complexes has been implicated in the LPS-induced expression of TF.26 Therefore, EMSAs were performed with a labeled κB consensus oligonucleotide and an oligonucleotide containing the specific κB-like site identified in the TF promoter.22,26 Treatment of mononuclear cells with LPS for 60 minutes strongly increased binding activity to both sites (Figure 3, upper and lower panels). This effect was markedly inhibited when cells had been pretreated for 60 minutes with lysoPC (10 μmol/L). No effect was seen after pretreatment with lysoPE. Binding to an Sp-1 oligonucleotide was not affected by lysoPC. Northern blot analyses of steady-state TF mRNA levels were performed after a 2-hour incubation of mononuclear cells in the presence or absence of LPS. The TF mRNA expression elicited by LPS was partially reduced after pretreatment of the cells with lysoPC (Figure 4).

In a total of 3 experiments (including the 1 shown in Figure 4), LPS-induced TF mRNA expression was lowered by 38±10% by lysoPC pretreatment, the effect not reaching statistical significance (versus LPS-mediated TF mRNA expression without lysoPC; paired t test).
Previous data indicate that induction of TF expression in different cell types is inhibited by increases in intracellular cAMP. The potential role of intracellular cAMP in the inhibition of TF activity elicited by lysoPC was therefore evaluated. In a first set of experiments, the effect of the lysophospholipid on monocytic cAMP levels was analyzed. Monocytes were isolated from mononuclear cell suspensions by using anti-CD14 antibodies (see Methods). The cells (10⁶) were incubated for 60 minutes in Ham’s F-10 medium (supplemented with 5% FCS) in the presence of either lysoPC (20 µmol/L) or ethanol (1%). Thereafter, intracellular cAMP levels were estimated by using a kit as detailed in Methods. In monocytes pretreated with lysoPC, cAMP levels were raised by 64% (10 µmol/L lysoPC) and 72% (20 µmol/L lysoPC) compared with cells pretreated with vehicle (Table 3).

In further experiments, 2'-deoxy-3'-adenosine monophosphate (dAMP, 0.5 mmol/L), an inhibitor of adenylyl cyclase, was added to the mononuclear cell suspension 90 minutes before the start of incubation with LPS. The stimulation of TF activity elicited by LPS was not affected by dAMP (Figure 5, columns 1 through 3). In monocytes pretreated with 10 µmol/L lysoPC, LPS-induced TF activity was inhibited by 72% (column 4). In the presence of dAMP, this inhibition was partially reversed (column 5). Furthermore, the cell suspension was incubated with dibutyryl cAMP (1 mmol/L), a membrane-permeable analogue of cAMP. Therefore, the LPS-induced TF activity was abolished both in untreated cells as well as in monocytes pretreated with lysoPC (columns 6 and 7).

**Discussion**

We observed in the present study that preincubation with lysoPC suppressed LPS-induced TF expression and activity of monocytic cells. The inhibition exerted by lysoPC was selective, because other lysophospholipids were ineffective (Figures 1 and 3 and Results). The effect of lysoPC on LPS-induced TF expression was apparently related to the presence of lysoPC within the monocytes. Indeed, preincubation with the lysophospholipid induced an increase in lysoPC contents of monocytes without inducing major changes in the levels of other phospholipids (Table 1).

**Table 3.** LysoPC Pretreatment Increases the cAMP Contents of Human Monocytes

<table>
<thead>
<tr>
<th>cAMP Contents, pmol/10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>+LysoPC</td>
</tr>
<tr>
<td>10 µmol/L</td>
</tr>
<tr>
<td>20 µmol/L</td>
</tr>
</tbody>
</table>

After isolation of monocytes from a mononuclear cell suspension by the use of anti-CD14 antibodies, the cells were incubated for 60 minutes at 37°C with lysoPC or vehicle. The contents of cAMP were determined using a kit as described in Methods. Mean values ±SD on monocytes from 5 different donors are shown.

*P<0.05 vs control (1-way ANOVA).
LysoPC Inhibits Tissue Factor Expression

Figure 5. Agents modifying intracellular cAMP levels differentially affect the TF activity of lysoPC-pretreated mononuclear cells. Mononuclear cells suspended in Ham’s F-10 medium supplemented with 5% FCS were preincubated for 60 minutes at 37°C with lysoPC (10 μmol/L) or vehicle. Thereafter, the cells were washed once and incubated for another 6 hours with LPS (10 ng/mL) at 37°C in Ham’s F-10 medium containing 5% FCS. To decrease intracellular cAMP levels, dAMP (0.5 mmol/L) was added 90 minutes before the start of the incubation with LPS. To elevate intracellular cAMP levels, dibutyryl cAMP (1 mmol/L) was added 90 minutes before the start of the incubation with LPS.

To clarify the mechanisms mediating inhibition of TF activity by lysoPC, we first evaluated whether the lysophospholipid affected the binding of LPS to the extracellular surface of the monocytes. These cells are known to possess plasma membrane-binding sites for LPS, such as CD14 receptors, for example. Preincubation with lysoPC neither influenced the interaction of LPS with its binding sites on the cell membrane of monocytes nor affected LPS internalization (Table 2). This result indicated that lysoPC interfered further downstream in the LPS-elicited signaling cascade.

According to present knowledge, LPS-mediated TF gene expression is transcriptionally regulated by members of the nuclear factor (NF)-κB/Rel family, AP-1 and Sp1. Activation of NF-κB/Rel was potently inhibited by the lysophospholipid (Figure 3). A recent report indicates that lysoPC at concentrations similar to those exerting a maximal response in the present study stimulates nuclear binding and transcriptional activity of AP-1. The stimulation of AP-1 activity by lysoPC may counteract the inhibitory effect of lysoPC on NF-κB/Rel activity. This process might explain why LPS-induced TF mRNA levels tended toward only partial reduction in the presence of lysoPC (Figure 4).

Several previous investigations reported inhibition of TF expression by agents known to increase intracellular cAMP levels. Furthermore, intracellular cAMP contents were shown to be increased by lysoPC in platelets and THP-1 cells. We therefore investigated whether intracellular cAMP was involved in the effect of lysoPC on the TF activity of monocytes. Preincubation of the mononuclear cell suspension with dAMP, an inhibitor of adenylyl cyclase, partially reversed the inhibition of LPS-induced TF activity elicited by lysoPC (Figure 5). LysoPC pretreatment led to an increase in monocytic cAMP levels (Table 3). These results argue in favor of the hypothesis that inhibition of LPS-induced TF activity induced by lysoPC is in part mediated by an increase in intracellular cAMP. Inhibition of NF-κB/Rel activity in lysoPC-pretreated monocytes (Figure 3) is in agreement with this hypothesis, as increases in intracellular cAMP were shown to block the activities of these transcription factors in several investigations. A recent study indicates that although elevations in intracellular cAMP levels resulted in inhibition of NF-κB/Rel-mediated transcription of several genes (including TF), nuclear binding activities of these factors were unchanged. The basis for these differences is unknown at present.

LPS-promoted monocytic TF expression was clearly diminished by lysoPC (Figures 1 and 2). TF mRNA expression tended to be partially lowered (Figure 4). Together, these results could indicate that posttranscriptional mechanisms are also involved in the inhibitory effect of lysoPC on TF expression. Two recent reports underline the importance of posttranscriptional processes for the inhibition of TF expression. In 1 of those studies, pyrrolidine dithiocarbamate, a strong inhibitor of NF-κB activation, was found to inhibit TF expression without inducing considerable alterations in TF mRNA levels. A somewhat similar situation was encountered in the present study in human monocytes preincubated with lysoPC.

In vitro oxidized LDL has been shown to alter activation of NF-κB/Rel complexes, the effect depending on the degree of oxidation. Whereas mildly oxidized LDL particles stimulated the activities of NF-κB/Rel, strongly oxidized LDL particles were shown to inhibit activation of the transcription factors. The lysoPC contents of LDL increase with increasing strength of oxidation. Accordingly, the inhibitory action of heavily oxidized LDL on activation of NF-κB/Rel could probably be caused by elevations in LDL-associated lysoPC.

Stimulation of TF synthesis has been proposed to be responsible for the thromboembolic complications associated with inflammation and atherosclerosis. As outlined in the introduction, under these conditions increased levels of lysoPC are frequently observed as a result of activation of PLA₂. LysoPC appears to promote a variety of inflammatory and atherogenic responses in cellular components of the vascular bed, such as increased adhesion of monocytes to the endothelium or activation of distinct growth factors contributing to proliferation of endothelial cells, for example. However, the influence of lysoPC on the expression of several endothelial proteins involved in inflammation and atherogenesis clearly differed from the effects exerted by inflammatory cytokines such as tumor necrosis factor-α.

Furthermore, lysoPC has recently been shown to increase the synthesis of endothelial prostacyclin, a potent inhibitor of platelet aggregation. This lysophospholipid was also shown to directly block platelet aggregation and the synthesis of platelet thromboxane A₂ as elicited by platelet agonists. According to the results of the present study, LPS-induced expression of TF is markedly diminished in the presence of lysoPC. When monocytes are activated during inflammation, TF expression is increased. Under the same conditions, lysoPC is generated by several cells present in the blood and the vascular wall. This event, in turn, might contribute to mitigate initiation of coagulation of the inflammatory process. Some recent data point to a decisive role for macrophage TF expression in the thrombogenicity of the atherosclerotic plaque. The high levels of lysoPC in the plaques are expected to reduce TF expression of macrophages. Taken
together, the results of the present and the above-mentioned investigations indicate that lysoPC, while promoting several inflammatory and atherogenic cellular responses, may concomitantly attenuate activation of coagulation.

**Acknowledgments**

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**References**

Tissue Factor Expression of Human Monocytes Is Suppressed by Lysophosphatidylcholine
Bernd Engelmann, Susanne Zieseniss, Korbinian Brand, Sharon Page, Arnd Lentschat, Artur J. Ulmer and Eckehart Gerlach

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