Upregulation of Pentraxin-3 in Human Endothelial Cells After Lysophosphatidic Acid Exposure

Cindy Gustin, Edouard Delaive, Marc Dieu, Damien Calay, Martine Raes

Objective—The earliest event in atherogenesis appears to be endothelium dysfunction. Lysophosphatidic acid (LPA), one of the major bioactive lipid components of oxidized low-density lipoproteins (oxLDL), can cause the activation of endothelial cells (ECs), which start to secrete multiple proinflammatory polypeptides/proteins. The purpose of this study was to better document the proatherogenic properties of LPA using a subproteomic approach focused on the secretome of LPA-treated ECs.

Methods and Results—The secretome of LPA-treated ECs was analyzed using the 2D-DIGE approach. Among the 20 spots displaying significant variations of abundance compared with the control cells, we identified pentraxin-3 by mass spectrometry. Pentraxin-3 upregulation was confirmed at the mRNA and protein level, both on immortalized and primary ECs. LPA- but also oxLDL-induced pentraxin-3 upregulation was reduced in the presence of an antagonist of the LPA-receptors and largely dependent on NFκB activation. Finally, we demonstrated, for the first time, the chemotactic activity of pentraxin-3 on human THP-1 monocytes by using a chemotaxis assay.

Conclusions—Our findings favor the proatherogenic role of LPA, a bioactive lipid produced by activated platelets and present in oxLDL, because it enhances pentraxin-3 secretion that could contribute to the accumulation of monocytes in the atherosclerotic lesion. (Arterioscler Thromb Vasc Biol. 2008;28:000-000.)

Key Words: lysophosphatidic acid ■ endothelial cell ■ atherosclerosis ■ Pentraxin-3 ■ chemotactic

Cardiovascular diseases (CVDs), such as atherosclerosis, are the most common causes of death in developed countries. Atherosclerosis is a dynamic process that involves inflammation at all stages, from the early to the complex lesions. Inflammation is enhanced by all the CVD risk factors identified in epidemiologic studies, and particularly by elevated levels of low-density lipoprotein (LDL)-associated cholesterol. Oxidation of LDL is generally considered as one of the key events in atherogenesis, because oxidized LDL (oxLDL) is a major source of various bioactive modified (lyso)phospholipids. Among those oxLDL-derived lipids, lysophosphatidic acid (LPA) has been identified and largely studied to elucidate its role in atherosclerosis.

LPA is a normal constituent of serum (2 to 20 μmol/L) and plasma (80 nmol/L to 0.7 μmol/L), with palmitoyl- and oleoyl-LPA being predominant. LPA was primarily described as a growth factor, but it can provoke a large variety of different biological responses in many cell types (for a review see). LPA exerts its major biological effects by binding to specific transmembrane G protein–coupled LPA1–5 receptors, but LPA also binds with high affinity to the nuclear receptor peroxisome proliferator-activator receptor γ (PPARY). Vascular wall and blood cells express several types of LPA receptors, and there are data from many experiments indicating that LPA is a potentially athero- and thrombogenic molecule, because it stimulates platelet aggregation, promotes proliferation of vascular smooth muscle cells (VSMCs), and activates monocytes, macrophages, as well as vascular endothelial cells (ECs). Activated ECs, well known to interact with other cells such as monocytes, macrophages, or platelets, play an important role in the context of atherogenesis by secreting proteins, such as proinflammatory interleukins (interleukin [IL]-1, IL-6) and chemokines (monocyte chemotactic protein-1, IL-8). We therefore investigated the effects of LPA on the proteins secreted by ECs, using the 2D-DIGE approach to analyze the secretome. Significant variations of abundance were observed for 20 spots. One protein, found in 2 spots showing increased abundance, was identified by mass spectrometry and attracted our attention—pentraxin-3.

Pentraxin-3 is a secreted glycoprotein belonging to the pentraxin family of acute-phase proteins, such as C-reactive protein (CRP) and serum amyloid P component (SAP). Pentraxin-3, also named TSG-14 (tumor necrosis factor [TNF]α-stimulated gene-14), was originally described as a gene inducible by TNFα in human fibroblasts and, soon after, it was also identified as being induced by IL-1β in ECs. Pentraxin-3 expression is increased in several cell types including fibroblasts, ECs, chondrocytes, monocytes-macrophages, stimulated with TNFα, IL-1β, or lipopolysac-
chariade (LPS). The function currently assigned to pentraxin-3 is to regulate innate immunity at a local level, through its binding to the C1q component. The involvement of pentraxin-3 in atherosclerosis remains largely unknown, but a few recent observations suggest a possible role of pentraxin-3 in this context. First, pentraxin-3 is strongly expressed in atherosclerotic lesions compared with normal arteries. It has also been reported that pentraxin-3 is induced by oxLDL in VSMCs. Finally, Latini et al have demonstrated the higher prognostic value of pentraxin-3 in acute myocardial infarction compared with the best-known relevant biological markers. Given the inflammatory nature of atherosclerosis and our results on LPA-induced overexpression of pentraxin-3 in ECs, the data suggest that LPA as well as pentraxin-3 may participate in the early stages of atherosclerosis. The upregulation of pentraxin-3 detected by 2D-DIGE analysis was confirmed by ELISA and Western blot in both immortalized and primary ECs. Pentraxin-3 upregulation was also observed at the transcriptional level, and was largely dependent on NFκB activation. In addition, LPA - as well as oxLDL-enhanced transcription of pentraxin-3 was reduced in the presence of Ki16425, an antagonist of the LPA-1 and LPA-3 receptors. Finally, we showed that pentraxin-3 has a clear chemotactic activity on human THP-1 monocytes, which favors a proatherogenic role for pentraxin-3.

Materials and Methods

Please see the supplement data, available online at http://atvb.ahajournals.org for all details.

Cell Culture

The EAhy926 cell line is a hybridoma produced by fusing human umbilical vein endothelial cells (HUVECs) with cells of the epithelial cell line A549. Primary HUVECs were purchased from Clonetics (Cambrex). Human monocytic THP-1 cells were purchased from the American Type Culture Collection (ATCC).

Statistical Analysis

Statistical analysis was achieved by the “Sigmastat” software using Student t test and 1- or 2-way ANOVA followed with post-hoc corrections (Holm-Sidak or Tukey test).

Results

LPA Induces Pentraxin-3 Secretion in Human Endothelial Cells

Before starting the 2D-DIGE experiments, we had to optimize the LPA concentration. Therefore, we analyzed the effects of LPA over an extended range of concentrations on MCP-1 protein secretion on EAhy926 cells (unpublished data, 2007). These data revealed a concentration-dependent effect of LPA from 1 μmol/L to 50 μmol/L, with a marked effect at 25 μmol/L, followed by a plateau for the higher concentrations tested. Hence, EAhy926 cells were treated with 25 μmol/L LPA during 4 hours and the secretome of the LPA-treated cells (=LPA) was compared with nontreated cells (=CTL), using the 2D-DIGE approach and analyzed with the DeCyder software. Among the 20 spots displaying significant variations in abundance, 2 were identified by mass spectrometry as pentraxin-3 (spots 1 and 2, supplemental Figure I; NCBI entry number=4506333). Both spots showed a significant increase of abundance in LPA-treated cells (P<0.0076 and P<0.0032, respectively) with a 1.54- and 1.51-fold induction, respectively.

To confirm these data, we measured pentraxin-3 released into the supernatant of EAhy926 cells after 4 hours stimulation with LPA at 1, 10, and 25 μmol/L and after 6 and 8 hours incubation with LPA (25 μmol/L) and TNFα (10 ng/mL). The latter, known to induce pentraxin-3 expression in several cell lines, was used as positive control. As measured by ELISA, LPA induces a concentration-dependent pentraxin-3 release with a maximal effect at 25 μmol/L after 6 hours (release of about 600 ng/mL pentraxin-3 compared with 200 ng/mL for the untreated cells; Figure 1). TNFα significantly increases pentraxin-3 secretion reaching a maximum of 800 ng/mL after 8 hours incubation. We further studied pentraxin-3 expression by Western blots on LPA-stimulated EAhy926 and HUVECs. As shown in Figure 2, pentraxin-3 secretion increases after 4 and 6 hours stimulation with 25 μmol/L LPA both in EAhy926 (1.5- to 2.5-fold induction) and HUVECs (1.5- to 2-fold induction). The 2 observed bands probably correspond to the glycosylated (G-PTX3) and nonglycosylated (PTX3) forms of pentraxin-3, considering that the latter has an expected molecular mass of 40 kDa. This is also in agreement with the 2 spots identified as being pentraxin-3 in the 2D-gel analysis. Taken together, these data demonstrate an increased secretion of pentraxin-3 both in immortalized and primary ECs after LPA stimulation.

Kinetics and Concentration-Dependent Effect of LPA-Induced Pentraxin-3 Expression

We further investigated whether the LPA-induced pentraxin-3 overexpression was also controlled at the mRNA level by using real-time RT-PCR. For both EC models, kinetics of LPA (25 μmol/L) stimulation were performed. As shown in Figure 3, we observed a rapid increase in the abundance of pentraxin-3 mRNA, normalized to GAPDH mRNA, which peaked at 2 hours (±240%) for the EAhy926 cells (Figure 3A) and at 3 hours (±220%), for the HUVECs (Figure 3B), followed by a second wave of induction for both cell types. The effect of LPA on pentraxin-3 mRNA expression observed in both EC models at lower LPA concentrations (1 μmol/L and 10 μmol/L), was concentration-dependent.
with a significant effect already observed with 1 μmol/L LPA (supplemental Figure II).

LPA Is One of the Major Bioactive Phospholipids in oxLDL Inducing Pentraxin-3 Overexpression

Because LPA accumulates during LDL oxidation, EAhy926 cells were treated for 2 hours with different concentrations of oxLDL (from 50 to 200 μg/mL) and pentraxin-3 gene expression was monitored by real-time RT-PCR. As shown in Figure 4A, oxLDL induces a concentration-dependent increase of pentraxin-3 expression with approximately a 2.5-fold induction (with 200 μg/mL), almost comparable to the one observed in the presence of LPA (Figure 3A). Moreover, a preincubation with 10 μmol/L Ki16425 (Ki), a specific antagonist of LPA-1 and LPA-3 receptors,23 before the incubation with oxLDL (200 μg/mL) for 2 hours, markedly diminished this overexpression to levels that are not significantly different compared with the negative control (Figure 4B). Native LDL (LDL) (Figure 4B) and Ki16425 alone were ineffective (data not shown). To check whether LPA was the major bioactive lipid responsible for this response, we tested other lipids present within oxLDL, sphingosine-1 phosphate (S1P) and lysophosphatidylcholine (LPC). EAhy926 cells were incubated during 2, 4, 6, and 8 hours with these lipids. After real-time RT-PCR analysis, no overexpression was observed at any time or at any of the concentrations used (1 μmol/L and 20 μmol/L), as represented in Figure 4B for cells stimulated during 2 hours with 1 μmol/L S1P or LPC. We also combined S1P and LPA treatments, but S1P was unable to affect the magnitude of pentraxin-3 upregulation induced by LPA (data not shown). All together, these data demonstrate that oxLDL induces pentraxin-3 expression through LPA-1 and LPA-3 receptors in EAhy926 cells.

Regulation of Pentraxin-3 Expression in LPA-Stimulated EAhy926 Cells

First, we highlighted the LPA-induced NFκB transactivating activity by using a specific luciferase reporter plasmid (supplemental Figure IIIA). Then, using a colorimetric assay,24 we observed a significant increase in the DNA binding activity of NFκB (using an anti-p65 antibody) after LPA (25 μmol/L) treatment during 45 minutes (supplemental Figure IIIB). However, this increase in DNA binding activity was markedly inhibited in the presence of 10 μmol/L Ki16425 (79% inhibition) and of 500 mmol/L evodiamine (90% inhibition), a recently described NFκB inhibitor. Inter-

![Figure 2. LPA induces a concentration-dependent secretion of pentraxin-3 in ECs. Cells were stimulated during the indicated times with LPA. One immunoblot with the corresponding quantitative analysis representative of 3 independent experiments is shown. ***P<0.001; **P<0.01 vs negative control.](http://atvb.ahajournals.org/)

![Figure 3. LPA induces pentraxin-3 mRNA expression in EAhy926 (A) and HUVECs (B). Cells were stimulated with LPA during the indicated times, and real-time RT-PCR was performed with specific primers for human pentraxin-3 and GAPDH, chosen as housekeeping gene. ***P<0.001; **P<0.05 vs corresponding negative control.](http://atvb.ahajournals.org/)
Estingly, evodiamine (EVO) by itself inhibits the constitutive DNA binding activity of NFkB, as mentioned by Takada et al.\(^{25}\) on myeloma cells. Finally, we tested these inhibitors on the LPA-induced pentraxin-3 upregulation by real-time PCR. As shown in supplemental Figure IIIC, the increase in the abundance of pentraxin-3 mRNA induced by 25 \(\mu\)mol/L LPA (after 2 hours stimulation) was inhibited by approximately 77\% and 68\% after preincubation with Ki16425 (10 \(\mu\)mol/L) and evodiamine (500 nmol/L), respectively. We also demonstrated that palmitoyl-LPA (a LPA isoform with a saturated fatty acyl chain unable to activate LPA-3 receptor) induces a dose-dependent effect on pentraxin-3 mRNA expression with inductions similar to the ones obtained with oleoyl-LPA (supplemental Figure IV). This increase in pentraxin-3 expression was inhibited by approximately 80\% to 100\%, after preincubation with Ki16425. All together, these data suggest that LPA-1 receptor is mainly involved in the LPA-induced pentraxin-3 overexpression. Ki16425, but also evodiamine alone, had no effect on the abundance of pentraxin-3 mRNA (data not shown). Experiments performed with BAY 11-7082 (another well-described NFkB inhibitor), despite a higher cytotoxicity of BAY 11-7082 compared with evodiamine, confirmed the involvement of NFkB.

**Pentraxin-3 Induces the Transmigration of Monocytes In Vitro**

Because the functions of pentraxin-3 are not yet completely defined, we wondered whether pentraxin-3 could be chemotactic for monocytes. To test this hypothesis, we used recombinant human pentraxin-3 (rhPentraxin-3) at concentrations ranging from 200 ng/mL to 800 ng/mL, which is comparable to the concentrations of pentraxin-3 achieved in the supernatants of ECs after LPA stimulation (Figure 1). As shown in Figure 5A, rhPentraxin-3 used in a modified Boyden’s chamber for 4 hours induces a significant monocyte transmigration with a weak concentration-dependent effect. We then evaluated the supernatants of LPA (1 \(\mu\)mol/L)-treated cells (4 hours) using the same assay and as shown in Figure 5B, we clearly demonstrate an effect of these supernatants on monocyte transmigration (LPA compared with CTL). This effect is even more pronounced than with rhPentraxin-3 probably because of the presence of additional chemotactic factors such as MCP-1 and IL-8 (unpublished data, 2007). Moreover, as demonstrated in supplemental Figure V, by adapting the assay, we could exclude that residual LPA present in the supernatant of conditioned media (CM) was required. We thus went on to evaluate the role of secreted pentraxin-3 in this chemotactic activity by abrogating pentraxin-3 expression with specific double-stranded siRNA (small interfering RNA). Using an ELISA, we showed that anti–pentraxin-3 siRNA attenuated pentraxin-3 expression by 80\% (at the protein level) after 24 hours transfection.
(supplemental Figure VI). So, CM of EAh926 cells stimulated with LPA (1 μmol/L) and transfected or not for 24 hours with siRNA against pentraxin-3 were tested on the THP-1 transmigration. First, CM from LPA-treated cells transfected with anti-pentraxin-3 siRNA (LPA+siRNA) displayed a significantly lower chemotactic activity (Figure 5B). Moreover, this inhibition was counteracted when 300 ng/mL of exogenous rhPentraxin-3 (concentration achieved in the supernatant of ECs after LPA treatment) were added (LPA+siRNA+rhPTX3) during the transmigration assay. Secondly, we also observed a significant effect of pentraxin-3 silencing in control cells (CTL+siRNA; Figure •B). This is not surprising because nonstimulated EAh926 cells secrete detectable amounts of pentraxin-3 (Figure 1), sufficient to almost double THP-1 chemotaxis (Figure 5A). CM of cells stimulated or not with LPA and treated with control siRNA did not alter monocyte migration (CTL and LPA RF).

These data point out for the first time a new function of pentraxin-3 as a chemoattractant for monocytes.

Discussion

The endothelium plays a major role in atherosclerosis given its function as secretory tissue, releasing several bioactive molecules. Therefore, analyzing the secretome of activated ECs should further contribute to a better understanding of the role of these cells in the initiation of atherogenesis. To activate ECs, we chose LPA, a bioactive lipid present within oxLDL and well known because sometimes as a growth factor, but still a controversial molecule in the context of atherosclerosis, considered as either pro- or antiatherogenic.

We demonstrated, by a subproteomic approach, that LPA clearly modulates the secretome of EAh926 cells and significantly increases the abundance of pentraxin-3.

The 2D-gel data on pentraxin-3 were confirmed by ELISA and Western blot analysis on EAh926 cells. Although the EAh926 cell line is now considered as a well characterized vascular EC line, displaying various characteristics typical of human ECs, we confirmed the LPA-induced pentraxin-3 overexpression in HUVECs. We next performed a time-course analysis of pentraxin-3 mRNA expression. In both EC models, the abundance of pentraxin-3 transcripts rapidly increases, with a first peak at 2 hours and 3 hours, for the EAh926 and HUVECs, respectively, followed by a second wave of induction. These pentraxin-3 protein and mRNA patterns of expression are comparable to those described in the literature with proinflammatory agents. For example, Alles et al demonstrated that LPS induces a peak of pentraxin-3 mRNA expression between 4 hours and 8 hours in peripheral blood mononuclear cells. They also showed that the regulation of pentraxin-3 expression requires an active protein synthesis in monocytes, which supposes the production of “intermediate proteins” not yet identified. We could propose a similar hypothesis in the induction of the second wave of pentraxin-3 expression after LPA treatment. Indeed, Lin et al have demonstrated that the induction of MCP-1 and IL-8 by LPA after 12 hours is regulated by IL-1, produced by the HUVECs in response to the primary stimulus (LPA). Therefore, based on our data obtained by the 2D-DIGE analysis, the “intermediate protein” could be one of the proteins overexpressed after LPA treatment, but not yet identified, because of the very limited amounts of material available for mass spectrometric analysis. Interestingly, the levels of induction of pentraxin-3 secretion induced by LPA and TNFα are very similar. So, our data suggests that LPA may act as a true proinflammatory molecule. Secondly, we confirmed that LPA is able to modulate pentraxin-3 expression at concentrations as low as 1 μmol/L to 10 μmol/L, on both EC models. These concentrations might seem extremely high as nanomolar concentrations of LPA are sufficient to activate the LPA membrane receptors LPA1-5. But, as described in the literature, micromolar concentrations of LPA are required to induce DNA synthesis in cultured cells, and more specifically to induce transcriptional effects. Moreover, micromolar concentrations of LPA are not at all uncommon in vivo. LPA is known to be present in serum in concentrations of up to 2 to 20 μmol/L and is present in atherosclerotic lesions in concentrations 13 fold higher compared with normal arteries, although the absolute concentrations are not known. One might speculate that during the onset of inflammatory reactions, recruitment of LPA-producing cells such as monocytes-macrophages and ECs and multiplication of other potential sources for LPA production (activated platelets, higher concentrations of oxLDL, secretion of lyso-phospholipase D...), could contribute to the delivery of high levels of LPA in a localized environment. Moreover we demonstrated that oxLDL enhances pentraxin-3 expression through the activation of LPA-1 or LPA-3 receptors. To further study the specificity of the LPA-induced pentraxin-3, we also tested other bioactive lipids present within oxLDL, such as S1P and LPC, but they were unable to modulate pentraxin-3 gene expression. S1P has also been claimed to be able to counteract LPA, for instance, in human platelets. Combined with LPA, S1P (preincubated or together with LPA) was unable to affect the magnitude of pentraxin-3 upregulation induced by LPA (data not shown).

We next started a preliminary study to unravel some of the mechanisms involved in the regulation of pentraxin-3, using a inhibitor of NFκB (evodiamine) and an antagonist of LPA-receptors (KiH16425). We showed that LPA is able to induce pentraxin-3 through a mechanism involving mainly the LPA-1 receptor (because similar effects were obtained with both palmitoy- and oleoyl-LPA), as well as NFκB activation, that is in agreement with the identification of 2 kB elements in the promoter of the human pentraxin-3 gene.

Finally, we investigated what could be the pathophysiological significance of pentraxin-3 secretion by ECs. Previous studies have suggested that CRP may favor the monocyte chemotactic response to MCP-1. We thus wanted to look for a possible chemotactic activity of pentraxin-3 on monocytes, an activity not assigned up to now to pentraxin-3. Using an in vitro chemotaxis assay, we demonstrated for the first time that rhPentraxin-3 (200 to 800 ng/mL) was able to induce a significant and concentration-dependent chemotaxis of THP-1 monocytes. We next abrogated pentraxin-3 gene expression by using siRNA. Anti–pentraxin-3 siRNA reduces the chemotactic activity of the supernatants of LPA-treated cells, but this activity was recovered when using the same CM supplemented with 300 ng/mL of rhPentraxin-3. The
effect of anti–pentraxin-3 siRNA on monocyte migration was only partial (about 30% inhibition), probably because the expression of pentraxin-3 was not completely abrogated. Parenthetically, LPA induces other chemotactic agents such as MCP-1 and IL-8 (unpublished data, 2007).

In this manuscript, we show for the first time that LPA enhances pentraxin-3 expression at the mRNA as well as the protein level, in immortalized and primary ECs. Interestingly, LPA by so doing, is able to mimic oxLDL, on the contrary to S1P and LPC. We showed that this LPA-induced pentraxin-3 upregulation was reduced in the presence of an antagonist of LPA-1 and LPA-3 and was largely dependent on NFκB activation. Finally, our findings suggest, at least in vitro, a chemotactic effect of pentraxin-3, which strengthens the proatherogenic role of LPA contributing to the pathogenesis of atherosclerosis, through the secretion of pentraxin-3. Consequently, our results suggest that pentraxin-3 and LPA may be new relevant therapeutic targets to consider for the treatment of inflammatory vascular lesions.

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References


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Materials and methods

Cell culture, LPA stimulation and chemical reagents

The EAhy926 cell line is a hybridoma produced by fusing human umbilical vein endothelial cells (HUVEC) with cells of the epithelial cell line A549 (generous gift from Dr Cora-Jean Edgell, University of North Carolina, USA). These cells were maintained in Dulbecco’s modified Eagle’s high glucose (4000 mg/l) medium (DHG) containing 10 % foetal bovine serum (FBS) at 37 °C under an atmosphere of 5 % CO₂. Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Cambrex). These cells were maintained in EGM-2 medium (Cambrex) supplemented with 2 % FBS, 10 ng/ml epidermal growth factor, 12 µg/ml bovine brain extract, 1 µg/ml hydrocortisone, and 50 µg/ml gentamicin and used between passages 2 and 15.

Human monocytic THP-1 cells were purchased from the American Type Culture Collection (ATCC). THP-1 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10 mM Hepes, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L bicarbonate, 0.05 mM 2-mercaptoethanol and 10 % FBS. For all experiments, cells were cultured at about 70 %–80 % confluence and starved in serum-free medium with 0.1 % fatty acid free BSA as a LPA carrier for 1 hour before LPA stimulation. For the negative control (= CTL) in each experiment, the cells were treated exactly the same way but in the absence of LPA stimulation.

For the chemotaxis assay, THP-1 cells were prepared at 300,000 cells/500 µl/chamber in DHG + 0.1 % fatty acid free bovine serum albumin (BSA).

1-Oleoyl-lysophosphatidic acid (LPA), 1-palmitoyl-lysophosphatidic acid, sphingosine 1-phosphate (S1P), lysophosphatidylcholine (LPC), fatty acid free BSA and Ki16425 were
purchased from Sigma. A stock solution of 10 mM LPA was prepared in distilled water and stored at -70 °C. S1P and LPC were prepared in DMSO and ethanol at a final concentration of 2 mM and 10 mM, respectively. Evodiamine was purchased from Calbiochem. Each inhibitor/antagonist was pre-incubated 1 hour before LPA stimulation. LPA, as well as the inhibitors/antagonists used were evaluated for possible cytotoxicity using the MTT or ethidium/acridine orange method, at all the concentrations and for all the incubation times used in the experiments. We never observed more than 5% of cytotoxicity (data not shown).

**Isolation of secreted proteins**

Cells were grown at 80% confluence (2,500,000 cells seeded per flask of 75 cm$^2$ 36 h before the experiment). After LPA stimulation, the supernatants of non-treated (CTL = control) and stimulated cells were collected and kept on ice. BSA, present in the medium, was eliminated by passing supernatants on an affinity column with anti-BSA-IgG. The recovered secreted proteins were concentrated at 4500 rpm (Labofuge 400R, Heraus Instruments) using Centricon tubes (Millipore), to remove proteins with molecular weights lower than 5000 Da. The final step consists in a clean-up (GE Healthcare Biosciences) on each condition and was performed as recommended by the supplier. The pellet was resuspended in Dla (DIGE labeling) buffer (30 mM Tris pH 8.5 containing 7 M urea, 2 M thiourea and 4% CHAPS), which is compatible with the 2D-DIGE technique.

Protein concentration was adjusted to 5 -10 µg/µl by using the Bradford protein assay (Bio-Rad) and pH of the sample was then adjusted to pH 8.5, required for CyDye labeling. Three independent cell cultures were prepared and analyzed by 2D-gels.

**CyDye labeling of proteins**
Secreted proteins were labeled with CyDye DIGE fluorescent dyes (GE Healthcare Biosciences) according to the manufacturer’s protocol. Typically, 75 µg of protein from each condition were minimally labeled with 200 pmol of either Cy3 fluor or Cy5 fluor for comparison on the same 2D gel. Each condition for each independent culture was alternatively labeled with Cy3 and Cy5, reducing possible intensity differences between the dyes but also differential labeling by one dye. An internal standard comprising 12.5 µg of protein pooled from the 6 samples (total of 75 µg) was also minimally labeled with Cy2 fluor. The internal standard is run in all the gels and used for the spot localization, detection and normalization between all the gels. Labeling reactions were performed on ice in the dark for 30 min and then quenched with 10 mM lysine for 10 min on ice in the dark. An equal volume of lysis buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 2 % DTT, 2 % IPG buffer 4-7) was added to each sample for reduction at room temperature (RT) during 10 min. For each independent culture, 25 µg of the Cy3- and Cy5-labeled samples were then combined and mixed with the 25 µg Cy2-labeled internal standard, in order to have 3 different mixes containing each Cy3-, Cy5- and Cy2-labeled samples.

_Isoelectric focusing (IEF) and 2D gel electrophoresis_

Prior to IEF, each mix was centrifuged 10 min at 13,000 rpm (Centrifuge 5415r, Eppendorf). Immobiline TM DryStrips (pH 4-7 NL, 24 cm) were rehydrated overnight in 450 µl rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 0.5 % IPG buffer 4-7 and 2.8 mg/ml DTT) overlaid with 2.5 ml DryStrip cover fluid, in an Immobiline DryStrip reswelling tray. Samples were applied to IPG strips via cuploading near the basic end of the strips. Strips were focused using IPGphor TM cup loading strip holders on the IPGphor IEF system for 4 different steps (hold to 300 V for 1 h, ramp to 1.000 V for 8 h, ramp to 8.000 V for 3 h, hold to 8.000 V over 50.000 V/h).
Prior to SDS-PAGE, each strip was equilibrated with an equilibration buffer A (6 M urea, 50 mM Tris-HCl pH 8.8, 30 % v/v glycerol, 2 % w/v SDS) supplemented first with 10 mg/ml DTT, followed by an equilibration in the same buffer A supplemented with 25 mg/ml iodoacetamide. IPG strips were then placed on top of 10 % homogeneous polyacrylamide gels that were precast with low-fluorescence glass plates using an Ettan-DALT caster. One glass plate from each gel was presilanized (bind-silane; GE Healthcare Biosciences) according to the manufacturer’s instructions to affix the polymerized gel to only one of the glass plates, to facilitate protein excision for mass spectrometry analysis. Gels were run at 0.5 Watt per gel constant power at 15°C during 2 h followed by 1 Watt per gel during 17 h.

**Image analysis, post-staining and spot picking**

Each gel was scanned with a Typhoon 9400 scanner (GE Healthcare Biosciences) producing a Cy2, Cy3 and Cy5 image for each of the three gels at excitation/emission wavelengths of 488/520, 532/580, and 633/670 nm, respectively. Image analysis was carried out with the DeCyder 6.5 software (GE Healthcare Biosciences) using the batch processor facility, which provides a list of spots of interest with a threshold set at +/- 1.5 and a T-test confidence ≥ 95 % (P ≤ 0.05).

The batch processor automatically analyzed the scanned images using first the Differential In-gel Analysis (DIA) module where, after having estimated a number of spots (in this case 1500), the Cy2, Cy3, and Cy5 images from each gel were normalized to the “master gel” (which has the highest number of spots) and then the Cy3 and Cy5 samples were compared with the Cy2 internal standard. Finally, the Biological Variation Analysis (BVA), provides the average ratios between the different conditions with a threshold at +/- 1.5 and a t-test confidence ≥ 95 % (P ≤ 0.05), generating a list of spots of interest.
After imaging for CyDye fluorescent dyes, the non-silanized glass plate was removed, and the gels were stained with 100 nM Ruthenium Red (generous gift from Dr LePrince, University of Liege, Belgium) according to Rabilloud’s protocol.\textsuperscript{2} This post-migration stain allows visualization of about 97\% of all the proteins, ensuring accurate protein excision.

Spots of interest observed with the 2D-DIGE analyses were matched with Ruthenium protein patterns, and spots were selected for picking according to this post-stained image. Spots were excised from 2D-gels using an automated Ettan Spot Picker (GE Healthcare Biosciences) following the manufacturer’s instructions.

**Mass spectrometry**

**In-gel tryptic digestion**

Gel pieces were washed with water and acetonitrile before being proteolysed with trypsin (100 µg/ml of 50 mM acetic acid) (Promega). Peptides were then extracted from the gel in 5\% formic acid and extracted peptides were desalted on gel loader tip columns (C18 column, Proxeon) prior to mass spectrometric analysis.

**Mass spectrometric (MS) protein identification**

The proteins were identified on the basis of their “peptide mass fingerprint” obtained with a matrix-assisted laser desorption/ionisation MX (MALDI, Waters) instrument. Peptide mass maps were acquired in the reflectron mode with delayed extraction. All mass spectra were internally calibrated with trypsin autolysis peaks. This “peptide mass map” was then used to interrogate human sequences databases with the Mascot (www.matrixscience.com) software.

**ELISA**
EAhy926 cells were seeded at 180,000/well in 24-well plates the day before stimulation. The pentraxin-3 released into the culture medium was measured using an ELISA kit from Alexis, following the manufacturer’s instructions. Pentraxin-3 was quantified according to a standard curve obtained with human recombinant pentraxin-3.

**Western-Blot**

Secreted proteins were prepared from EC cells grown in T25 flasks at 80 % confluence (1,000,000 cells seeded per T25 flask 36 hours before the experiment), according to the protocol described previously for 2D-DIGE experiments. Protein concentrations were determined using the Bradford protein assay (Bio-Rad). Equal amounts of protein (10 µg) were then separated by SDS-PAGE on 10 % acrylamide gels (NuPage) and transferred to a PVDF membrane (GE Healthcare Biosciences). After blocking in TBS containing 0.1 % Tween and 2 % milk (GE Healthcare Biosciences), the blots were probed with goat polyclonal anti-Pentraxin-3 (Pentraxin-3/TSG-14) (R&D Systems) antibody (diluted 1:10,000). The secondary antibody was a donkey anti-goat IgG antibody (diluted 1:200,000) (GE Healthcare Biosciences).

Chemiluminescent detection was performed using horseradish peroxidase conjugated to the secondary antibody and membranes were revealed with ECL (GE Healthcare Biosciences).

**Quantitative reverse transcription Polymerase Chain Reaction**

Cells were grown at 80 % confluence as described in the “Western-Blot” section. Preparation of total RNA was performed using the “RNAgent total RNA isolation system” kit (Promega) according to the manufacturer’s instructions. cDNA was obtained from total RNA with the Superscript II transcriptase kit (Invitrogen) according to the manufacturer’s instructions. mRNA abundance was estimated by Real-Time RT-PCR using specific primers for human
pentraxin-3 and GAPDH and the SYBR Green PCR Master Mix (Applied Biosystems). A standard curve from several dilutions of a sample of total RNA was established to calculate the amplification efficiency for each gene. Pentraxin-3 mRNA abundance was quantified using the Ct method and after normalization to the relative amounts of the mRNA of the housekeeping gene GAPDH.

**Lipoprotein isolation and preparation of oxidized LDL (oxLDL)**

Native low density lipoprotein (LDL) were isolated by sequential density gradient ultracentrifugation from healthy blood donor plasma. Oxidized LDL were obtained by incubating native LDL with 10 μM CuSO₄ for 24 hours at 37°C. Oxidation was then stopped by addition of BHT (25 μM) on ice for 1 h. Native and oxidized LDL were used after dialysis against phosphate-buffered saline (PBS) through a PD-10 desalting column (GE Healthcare Biosciences) and sterilized by filtration through a 0.22 μm filter. Native and oxidized LDL concentration was assayed by using the Folin method.

**Transfection experiments and reporter gene assay**

EAhy926 cells were seeded at 120,000 cells/well in 24-well plates the day before transfection. Transfections were performed with the Superfect transfection reagent (Quiagen) according to the manufacturer’s protocol. After 3 hours transfection, cells were rinsed once with DHG medium alone before being stimulated during 18 hours with LPA. To assay the transcriptional activity of NFκB, we used a specific firefly luciferase reporter vector containing 5 κB cis-elements, upstream of the firefly luciferase gene. A plasmid, which contains the renilla firefly gene downstream of the CMV promoter, was also used to normalize the firefly luciferase activity. Luciferase assays were performed using the Dual-
Luciferase Assay System (Promega) according to the manufacturer’s protocol. Measurements were obtained using a luminometer (Luminoskan Ascent).

**Colorimetric DNA binding assay**

DNA binding was assayed for NFκB using a colorimetric assay (Trans-AM) developed in our laboratory and commercialized by Active Motif. The assay was performed as recommended by the supplier.

**Gene silencing experiments**

Transfection experiments with small interfering RNA (siRNA) against pentraxin-3 were performed using a smart-pool of four specific siRNA (Thermo Fisher Scientific, Lafayette, CO; Dharmacon Catalog # M-017765-00-0010) targeting human pentraxin-3 (NM_002852). Sense (S) et antisense (AS) sequences for the four siRNA against pentraxin-3: (1) S = GCACAAAGAGGAAUCCAUAUU, AS = 5′-PUAUGGAUCCUCUUUGUGCUU; (2) S = CAAUGAGGCUUGAGUCUUUUU, AS = 5′-PAAAGACUCAAGCCCUAUGGUU; (3) S = UGGCUUUGAGAAACAUAUUU, AS = 5′-PUAAUGGCUUAUCAAAGCCAUU; (4) S = CCAUUGCUGUCCAAGAAAGAUU, AS = 5′-PUCUUUCUGGAACGCACUUGGUU.

A control siRNA, which is chemically modified to impair its uptake by RISC (RNA-induced silencing complex) (“RISC-free” siControl synthesized by Dharmacon), was also tested. Cells were transfected with Dharmafect 1 (Dharmacon) according to the manufacturer’s instructions. Efficiency of RNA interference on pentraxin-3 expression was determined by Real-Time RT-PCR (90 % silencing) with specific primers and by ELISA (80 % silencing). Cells were plated in 25 cm² flasks at 50 % confluence (1,500,000 cells/T25) the day before transfection with 20 nM siRNA or incubated with Dharmaject alone. After 24 hours
transfection, the cells were stimulated during 4 hours with LPA and supernatants were collected for ELISA or chemotactic activity analyses.

**Chemotaxis bioassay**

The chemotactic activity of recombinant human pentraxin-3 (rhPentraxin-3) diluted in DHG + 0.1 % fatty acid free BSA (R&D Systems) and endothelial cell conditioned media was tested using a Boyden’s chamber, by introducing them into the bottom wells of 24-wells plates covered by a 8-µm pore polycarbonate membrane (Nunc). THP-1 monocytes (300,000 monocytes/500 µl/well) were added to the upper compartment and incubated at 37 °C. After 4 hours incubation, the cells having migrated into the lower part of the Boyden’s chamber were stained with Calcein-AM (Molecular Probes) and the number of migrating monocytes is estimated by measuring the corresponding fluorescence relatively to the fluorescence emitted by 300,000 THP-1 cells. Fluorescence was determined (excitation at 485 nm – emission at 520 nm) after 30 minutes (Fluoroskan Ascent).

**Statistical analysis**

All experiments were performed at least 3 times independently and data are expressed as mean ± standard deviation (SD). Statistical analysis was achieved by the “Sigmastat” software using Student’s *t*-test and one- or two-way ANOVA followed with post-hoc corrections (Holm-Sidak or Tukey test). For the 2D-DIGE experiments, statistical analysis was achieved by the DeCyder Software using Student’s *t*-test. The differences were considered as significant if *P* values were lower than 0.05. All data are expressed as a mean of 3 independent experiments ± SD

**References used in the supplement**

Supplemental Figures

Figure 1

A

Gel 1: IPG pH 4-7

CTL - Cy5 scan

LPA - Cy3 scan

Pooled CTL and LPA treated cells (internal standard) - Cy2 scan

B

Spot 1

Spot 2

C

Graphs showing comparisons between CTL and LPA conditions.
**Figure 1:** LPA induces pentraxin-3 secretion in EAhy926 cells. (A) 2D-DIGE gels of the proteins secreted by EAhy926 cells stimulated or not with 25 µM LPA (n = 3). Secreted proteins were separated on a pH 4-7 IPG-strip in the first dimension and on a 10 % SDS-polyacrylamide gel in the second dimension. A typical CyDye-stained gel was scanned at 3 different wavelengths specific for the emission for the 3 CyDyes. (B) Enlarged area of the 2D-DIGE scans with 2 spots showing significant variation of abundance and identified by mass spectrometry as being pentraxin-3 (arrows). (C) 3D and graphical view of the normalized log abundance relative to the internal standard for each of the triplicate gels. (+ : Mean of the triplicates for each condition. The red line represents the variation of abundance between the treated and non-treated cells after normalization with the internal standard).
Figure II

LPA induced pentraxin-3 expression at the mRNA level in EAhy926 (A) and HUVEC (B) cells. Cells were stimulated during 2 hours (A) or 3 hours (B) with different LPA concentrations and Real-Time RT-PCR was performed as previously described. Results are expressed in fold induction relative to the appropriate negative control (= 1) at each time. 

***P<0.001 ; *P<0.05 vs corresponding negative control.
Figure III. NFκB is activated by LPA and is involved in the induction of pentraxin-3 by LPA. (A) LPA induces the expression of a NFκB-driven luciferase gene. EAhy926 cells were transfected with a firefly luciferase reporter plasmid containing 5 κB cis-elements and with a renilla luciferase reporter plasmid for normalization. The firefly luciferase activity was assayed and normalized by the renilla luciferase activity. Results are given as a mean of 3 independent experiments. ***P<0.001 vs CTL. (B) Effects of evodiamine and Ki16425 on
LPA-induced NFκB DNA binding activity. Nuclear protein extracts were tested for DNA binding activity to a probe containing one NFκB consensus sequence. The presence of the DNA-bound transcription factor is then detected with an anti-p65 antibody and revealed by colorimetry. **P<0.01 ; ***P<0.001 ; ‡‡P<0.01 vs negative control (= 1). (C) Effects of evodiamine and Ki16425 on LPA-induced pentraxin-3 upregulation at the mRNA level evaluated by using Real-Time RT-PCR. Results are expressed in fold induction relative to the appropriate control and given as a mean of 3 independent experiments ± SD. *P<0.05 vs LPA
**Figure IV**: Effect of oleoyl-LPA and palmitoyl-LPA on pentraxin-3 expression at the mRNA level in EAhy926 cells. Cells were stimulated with the two LPA isoforms during 2 hours at 1 or 25 μM. Before LPA stimulation, cells were pre-incubated or not with Ki16425 (10 μM) during 1 hour and Real-Time RT-PCR was performed with specific primers for human pentraxin-3 and GAPDH, chosen as housekeeping gene. Results are expressed in fold induction relative to the negative control (= 1) and given as a mean of 3 independent experiments ± SD. ***P<0.001; **P<0.01 vs the same condition without pre-incubation with Ki. (Ol = oleoyl-LPA ; Pal = palmitoyl-LPA ; Ki = Ki16425).
Figure V : Chemotactic activity on THP-1 cells of conditioned media obtained from EAhy926 cells stimulated with LPA. EAhy926 cells were incubated or not in the presence of 1 μM LPA for 4 hours (CTL 4h and LPA 4h) or for 2 hours followed by a “chase” (cells are stimulated during 2 hours with LPA and after removing the supernatant, cells are incubated the 2 remaining hours with fresh medium in the absence of LPA (= CTL 2h+2h and LPA 2h+2h fresh medium). The supernatants were then evaluated for their chemotactic activity using the chemotaxis assay described in the Materials and Methods. Results are expressed as fluorescence intensity (excitation at 485 nm – emission at 520 nm) and are given as a mean of 3 independent experiments ± SD. ***P<0.001 vs corresponding CTL.
Figure VI: Effect of siRNA against pentraxin-3 on LPA-induced pentraxin-3 secretion. EAhy926 cells were transfected or not with anti-pentraxin-3 or control siRNA (= RF siRNA) and treated or not 24 hours post-transfection for 4 hours with LPA (1 µM). The corresponding conditioned media were collected and tested using a pentraxin-3 ELISA. Results are expressed as optical density at 450 nm and are given as a mean of 3 independent experiments ± SD. ***P<0.001. (Ctl + siRNA = cells incubated with the anti-pentraxin-3 siRNA without stimulation with LPA; LPA + siRNA = same as previous but with stimulation with LPA; Ctl + RF siRNA = cells incubated with the control siRNA without LPA treatment; LPA RF = cells incubated with the control siRNA before stimulation with LPA. (RF = Risc-free control siRNA which is chemically modified to impair its uptake by RISC).