Upregulation of Macrophage Endothelial Lipase by Toll-Like Receptors 4 and 3 Modulates Macrophage Interleukin-10 and -12 Production

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Abstract—Limited data suggest that endothelial lipase (EL) is synthesized not only by endothelial cells but also by macrophages. Previous studies showed that proinflammatory cytokines upregulate EL in endothelial cells, but there are very few data regarding EL expression, regulation, and functional consequences in macrophages. In the present study, RAW cells and mouse peritoneal macrophages were treated with Toll-like receptor (TLR) ligands and EL expression and its consequences were assessed. We demonstrate that lipopolysaccharide, a TLR4 ligand; and polyinosinic:polycytidylic acid (poly I:C), a TLR3 ligand; but not lipoteichoic acid, a TLR2 ligand, upregulate macrophage EL expression both ex vivo and in vivo. In contrast, macrophage lipoprotein lipase expression is significantly repressed by lipopolysaccharide or poly I:C. Using C3HJ and TLR3 knockout mice, we further show that upregulation of macrophage EL expression by lipopolysaccharide or poly I:C is TLR4 or TLR3 dependent, respectively. Furthermore, we demonstrate that lipopolysaccharide induced interleukin (IL)-10 production was significantly reduced, whereas IL-12 production is significantly increased in J744 macrophages and mouse peritoneal macrophages overexpressing human EL. Conversely, significantly increased IL-10 and significantly decreased IL-12 expression were observed in mouse peritoneal macrophages isolated from EL knockout mice. Finally we show that the catalytic activity is required for EL to modulate the balance of macrophage IL-10 and IL-12 production. These results suggest that macrophage EL may play important roles in modulating the macrophage inflammatory response through local hydrolysis of HDL. (Circ Res. 2007;100:1008-1015.)

Key Words: endothelial lipase ■ macrophage ■ inflammation ■ TLR ■ cytokines

Endothelial lipase (EL) is a member of the triglyceride lipase gene family, which also includes lipoprotein lipase (LPL) and hepatic lipase (HL). Proinflammatory cytokines, such as tumor necrosis factor-α and interleukin (IL)-1β, have been shown to upregulate the expression of EL by endothelial cells. The upregulation of EL expression by these cytokines was mediated in part through the nuclear factor κB pathway. In mice, endothelial EL expression has been shown to be significantly increased by endotoxin and the upregulation of EL in the vessel wall promoted monocyte adhesion. These results suggest that endothelial EL plays a potentially important role in inflammatory responses.

Oxidized LDL was originally shown to upregulate EL expression in THP-1 macrophages. EL was shown to be expressed in atherosclerotic lesions of human coronary arteries, possibly including macrophage expression, by immunohistochemical analysis. However, there are no direct data demonstrating that EL is expressed in primary macrophages. Furthermore, the regulation and functional implications of macrophage EL expression are unknown. Macrophages express surface Toll-like receptors (TLRs), which recognize pathogen-associated molecules, such as lipopolysaccharide (LPS) and double-stranded RNA, resulting in immune responses against microbial infections. Activated macrophages secrete many different inflammatory cytokines, including IL-10 and IL-12, which have been shown to influence atherosclerosis in vivo. In addition, previous studies have further suggested that the local balance between IL-10 and IL-12 production may determine the eventual development of atherosclerotic lesions.

In the present study, we demonstrate that EL is expressed in RAW 264.7 macrophages and primary mouse peritoneal macrophages. Macrophage EL expression is upregulated by activation of TLR3 and TLR4, but not TLR2, both ex vivo and in vivo. Furthermore, expression of EL by macrophages modulates the balance between IL-10 and IL-12 secretion from macrophages.

Materials and Methods

Cell Culture and Reagents
RAW 264.7 cells and J774 cells were cultured in DMEM medium containing 10% FBS. Stable J774 cells lines were generated by
transducing wild-type J774 cells with lentiviral vector expressing GFP (control), full-length human EL, or mutant full-length human EL S169A. Mouse peritoneal macrophages (MPMs) were isolated from mice 3 days after intraperitoneal injection of 1 mL of 3.8% thioglycollate medium and cultured in RPMI containing 10% FBS. Lipopolysaccharide from Escherichia coli 0111:B4, lipid A from E coli, and lipoteichoic acid from Staphylococcus aureus were purchased from Sigma. Polynosinic-polycytidylic acid (poly I:C) was purchased from Amersham Biosciences. Murine interferon (IFN)-γ was from R&D Systems. For ligand treatments, cells were cultured in serum-free medium for 18 hours in the presence of absence of 100 μg/mL IFN-γ before indicated ligands were added.

RNA Isolation and Gene Expression Analysis
Total RNA was isolated using EZ1 RNA Mini kit (Qiagen) according to the instructions of the manufacturer. Then 0.5 μg of total RNA was reverse transcribed using QuantiTect Reverse Transcription kit (Qiagen). Real-time quantitative PCR was performed on an Applied Biosystems 7300 sequence detector. Primer and probe sequences are available on request.

Western Blotting
Cells were grown in DMEM + 0.2% BSA in the presence of indicated ligands for 24 hours. Medium was collected 30 minutes after the addition of heparin (10 U/mL), and protein concentration was determined using BCA protein assay kit from Pierce. Equal amount of total proteins were loaded on NuPAGE mini-gel (Invitrogen). Rabbit anti-murine EL serum was previously described.

Cytokine and Protein Assay
Mouse IL-10 and IL-12p70 ELISA kits were purchased from R&D Systems, and cultured medium was analyzed according to the instructions of the manufacturer. Total cell protein level was determined using the BCA protein assay kit from Pierce.

Animals
Age-matched TLR3 knockout mice and control C57BL/6 10SN CJ mice or 8- to 12-week female C57BL/6 and C3H/HeJ mice were purchased from The Jackson Laboratory (Bar Harbor, Me). EL knockout and hEL transgenic mice were generous gifts from Dr. Thomas Quertermous (Stanford University, Palo Alto, Calif) and have been previously described. Mice were injected intraperitoneally with either 1 mL of 3.8% thioglycollate or 20 μg of LPS in 500 μL of saline, when it was indicated. All animals were housed according to guidelines of the Institutional Animal Care and Usage Committee of the University of Pennsylvania.

Statistical Analysis
Statistical analysis was determined by 2-tailed, Student's t test with the use of GraphPad Prism Software. Results are presented as means±SD. Statistical significance was reached when P<0.05.

Results
TLR4 and TLR3 Activation Upregulates EL Expression in Macrophages Ex Vivo
To investigate whether macrophage EL expression is regulated through TLRs, we treated the murine macrophage cell line RAW 264.7 with TLR ligands: LPS, lipid A, poly I:C, and lipoteichoic acid (LTA) (Figure 1A). LPS and lipid A, both TLR4 ligands, increased EL expression by more than 2-fold. Poly I:C, a TLR3 ligand, induced EL expression by 5-fold. However, LTA, a TLR2 ligand, had no effect on EL expression. Conversely, LPS reduced LPL expression by 10-fold, and poly I:C decreased LPL expression by 4-fold in RAW 264.7 macrophages (Figure 1B). The time course of the effects of LPS and poly I:C on EL expression in RAW264.7 macrophages were very different. The maximal activation of EL by LPS occurred at 4 hours (Figure 1C). In contrast, poly I:C resulted in increasing EL mRNA through the 24-hour time point (Figure 1D). Next, we examined the dose-response curves of LPS and poly I:C on EL expression at the 4- and 24-hour time points. Treatment with LPS or poly I:C resulted in dose-dependent increase in EL mRNA levels. The maximal increase in EL expression was reached at 100 ng/mL for LPS (Figure 1E) and 5 μg/mL for poly I:C (Figure 1F). To determine whether EL protein expression was also induced by LPS or poly I:C, we treated RAW 264.7 macrophages with either LPS or poly I:C for 24 hours. Western blotting of the media after the addition of heparin showed a significant increase of EL protein in response to LPS or poly I:C (Figure 1G). Thus, both LPS and poly I:C stimulated EL mRNA and protein expression in RAW 264.7 macrophages.

LPS Upregulates Macrophage EL Expression In Vivo
Next, we investigated whether LPS could upregulate macrophage EL expression in vivo. Three days after thioglycollate injection, LPS or saline was injected intraperitoneally into mice. At time points indicated in Figure 3A, MPMs were isolated followed immediately by total RNA extraction. The in vivo time course of LPS treatment was significantly different from what we observed with MPMs ex vivo. By 2 hours, we observed a maximal 1000-fold increase in EL mRNA level in MPMs in vivo. Even at 24 hours after LPS administration, there was still 40-fold activation of EL expression.

To investigate whether LPS-induced inflammation can upregulate macrophage EL expression in other tissues in vivo, we injected LPS and harvested macrophage-rich tissues (spleen, lung, and liver) at different time points during a 24-hour period. At baseline, liver and lung expressed 10-fold higher levels of EL compared with spleen (data not shown). As shown in Figure 3A, EL mRNA levels were dramatically increased in the spleens from LPS-injected mice at all time points collected. The maximal upregulation occurred at 8 hours with a more than 200-fold increase in EL mRNA level.
Figure 1. Ligands for TLR3 and TLR4 increase EL expression and inhibit LPL expression in RAW 264.7 macrophages. RAW 264.7 macrophages were cultured in the presence of vehicle or 100 ng/mL LPS, 50 ng/mL lipid A, 5 μg/mL poly I:C, or 5 μg/mL LTA for 24 hours. EL and LPL expression was measured by QRT-PCR. A, EL expression. B, LPL expression. RAW 264.7 macrophages were cultured in the presence of 100 ng/mL LPS or 5 μg/mL poly I:C for the indicated time periods. C, Time course of EL expression stimulated by LPS treatment. D, Time course of EL expression stimulated by poly I:C treatment. E, Dose-response curve of EL expression stimulated by LPS for 4 hours. F, Dose-response curve of EL expression stimulated by poly I:C for 24 hours. G, EL protein secretion in RAW 264.6 culture medium. RAW 264.7 macrophages were cultured in the presence of vehicle (Control), 100 ng/mL LPS, or 5 μg/mL poly I:C for 24 hours. Medium from HEK293 cells overexpressing murine EL was used as the positive control (293_mEL). Equal amounts of total proteins were subject to Western blotting with anti-mEL antiserum.
LPS also significantly increased EL mRNA expression in lung (Figure 3B) and liver (Figure 3C). When primary hepatocytes were isolated from these mice, no activation of EL by LPS was detected (data not shown). Therefore, it is likely the Kupffer cells primarily contributed to the observed increase in EL expression in mouse livers.

Expression of EL in Macrophages Modulates IL-10 and IL-12 Expression

To determine the role of EL in macrophage inflammatory responses, we overexpressed EL in J774 macrophages using a lentiviral vector encoding the full-length human EL (hEL) cDNA. Among the cytokines studied, we found that the overexpression of hEL in J774 macrophages significantly reduced IL-10 expression ($P<0.001$) and significantly increased IL-12 expression (Figure 4A, $P<0.001$). To investigate whether the catalytic activity of EL is required to modulate IL-10 and IL-12 expression, we also generated J774 macrophages stably overexpressing the catalytically inactive mutant hELS169A. Wild-type and mutant human EL were determined to express at similar levels by quantitative RT-PCR (QRT-PCR). As shown in Figure 4A, overexpression of catalytically inactive EL had no effect on IL-10 and IL-12 expression in J774 macrophages. These data suggest that the catalytic activity of EL is required for modulation of macrophage IL-10 and IL-12 expression.

Previously described human EL transgenic mice express 3.2-fold higher in mRNA and 1.9-fold higher in protein level compared with endogenous murine EL. We determined the expression of human EL in MPMs isolated from human EL transgenic mice and response to LPS. Consistently, human EL mRNA was detected in MPMs in the basal state, and treatment of LPS increased the human EL mRNA level by 2.5-fold (Figure 4B, inset). Furthermore, IL-10 expression was reduced by 40% ($P<0.001$), and IL-12 expression was increased 2-fold ($P<0.001$) in human EL-overexpressing MPMs compared with control MPMs (Figure 4B), consistent with what we observed with J774 macrophages overexpress-
We measured the protein levels of IL-10 and IL-12 secreted from hEL-overexpressing MPMs and control MPMs. The MPMs were primed with IFN-γ overnight before stimulation with LPS for 24 hours. Compared with the wild-type MPMs, hEL-overexpressing MPMs had significantly less IL-10 (P < 0.01) and significantly more IL-12 in the cell culture media (P < 0.01) (Figure 5). Thus, macrophage EL expression appears to be a determinant of macrophage IL-10 and IL-12 production.

Finally, to determine the role of endogenous EL, MPMs isolated from EL knockout and control mice were treated with LPS, and IL-10 and IL-12 mRNA levels were determined by QRT-PCR. EL knockout macrophages expressed 1.5-fold (P < 0.001) more IL-10 mRNA and 20% (P < 0.001) less IL-12 mRNA when they were stimulated with LPS (Figure 4C), a pattern opposite to that seen in EL overexpressing macrophages.

**Discussion**

In the present study, we demonstrate that the TLR4 ligand LPS and the TLR3 ligand double-stranded RNA (poly I:C) activate macrophage EL expression both ex vivo and in vivo. Within 2 hours of LPS treatment, macrophage EL mRNA was increased by 4-fold ex vivo and by more than 1000-fold in vivo. A more delayed response was observed with poly I:C treatment, which did not peak until 24 hours ex vivo. We further demonstrate that regulation of EL expression in macrophages by LPS and poly I:C is TLR4 and TLR3 dependent, respectively. The TLR2 ligand LTA had no effect on macrophage EL expression. Finally, we show that macrophage EL modulates macrophage IL-10 and IL-12 production using both overexpressing and knockout macrophages.

Understanding the molecular mechanisms and consequences of EL expression in macrophages has become increasingly important, as conflicting results have been reported for the roles of EL during the progress of atherosclerosis. Ishida et al reported that there was a ~70% decrease in atherosclerotic lesion area in EL−/− apoE−/− mice compared with apoE−/− mice. In addition, they observed significantly less macrophage content in the lesions of double knockout mice. In contrast, Ko et al reported that EL deficiency had no effect on atherosclerosis development in apoE−/− and LDLR−/− mice. Similarly, the role of LPL in atherogenesis has been controversial. It was shown that
ubiquitous overexpression of LPL protected mice from atherosclerosis progression. However, the potential proatherogenic function of LPL was demonstrated by its ability to retain low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) in the arterial wall. Furthermore, increased susceptibility to atherosclerosis in mice has been associated with high macrophage LPL expression and secretion. Consistently, macrophage specific deletion of LPL in C57BL/6 mice on high-cholesterol diet showed significantly decreased lesion formation. It is evident that the type of tissue and cell expression of LPL is critical to its contribution to atherosclerosis. Hepatic lipase HL is another member of the triglyceride lipase family that, like LPL, appears to be downregulated by LPS. Macrophage HL expression has been shown to enhance atherosclerosis in apolipoprotein E (apoE)-deficient mice. Like LPL and HL, EL is expressed in different cell types including macrophages. However, the impact of macrophage specific EL expression on atherosclerosis has not been determined. Interestingly, we show here that LPS and poly I:C oppositely regulate LPL and EL in macrophages, downregulating LPL while upregulating EL. Therefore, it appears that macrophage EL may play important roles during inflammation and potentially contribute to atherosclerosis.

We explored potential functional consequences of macrophage EL expression in acute inflammatory responses stimulated by LPS. We found that overexpression of macrophage EL reduced IL-10 while increasing IL-12 expression. In
contrast, deficiency in macrophage EL expression resulted in increased IL-10 and decreasing IL-12 expression. By overexpressing catalytically inactive hEL in J774 macrophages, we demonstrated that the cross-regulation of IL-10 and IL-12 by EL in macrophages requires EL enzymatic activity.

The balance of IL-10 and IL-12 has been suggested to influence atherogenesis. Deficiency of IL-10 in mice promoted early atherosclerosis, characterized by particularly activated T cells and increased levels of proinflammatory cytokines. Moreover, macrophage-specific deletion of IL-10 in LDL receptor knockout mice induced a 2-fold increase in lesion development in the thoracic aorta. Splenocytes from the same mice also had a marked increase in IFN-γ production, suggesting the protective role of IL-10 is in part mediated by modulating immune responses. Conversely, overexpression of IL-10 in mice decreased formation of early fatty-streak lesions and inhibited atherosclerosis. Deficiency of IL-12 in mice was associated with reduced atherosclerotic development. Selective deficiency of IL-12 in macrophages also reduced lesion formation. Consistently, daily administration of IL-12 led to an accelerated atherosclerosis in apoE knockout mice. In humans, elevated serum IL-10 levels were associated with a more favorable prognosis in patients with acute coronary syndromes. Our results demonstrate that macrophage EL, when upregulated by inflammatory cytokines, regulates IL-10 and IL-12 production, which could in turn influence atherosclerosis development. Indeed, EL expression is increased in the atherosclerotic aortas of the apoE knockout mice.

The molecular mechanism by which macrophage EL modulates IL-10 and IL-12 expression is unclear. The members of triglyceride lipase family have been suggested to link lipid metabolism with inflammation through the activation of nuclear receptor peroxisome proliferator-activated receptors (PPARs). For example, hydrolysis of VLDL by LPL provides ligands for PPARα, resulting in the repression of VLDL/tumor necrosis factor-α–induced vascular cell adhesion molecule 1 expression in endothelial cells. Recently, EL hydrolysis of HDL has also been shown to activate endothelial PPARα and inhibit leukocyte adhesion to tumor necrosis factor-α–stimulated endothelial cells. Catalytic activity is required for both lipases to activate PPARs. Our results demonstrate that TLR4 and TLR3 activation significantly upregulate EL expression in macrophages, whereas they substantially repress LPL expression, potentially making EL the more important of the 2 macrophage lipases under inflammatory conditions. EL might modulate the macrophage inflammatory response by generating ligands for PPARs or potentially other nuclear receptors. Further experiments in PPAR knockout macrophages are needed to determine whether the ability of EL to modulate IL-10 and IL-12 expression is PPAR dependent. Recently, free fatty acids (FFAs) have been shown to activate TLR4 signaling in macrophages. Therefore, it is also feasible that FFAs that are generated from EL by hydrolysis of lipoproteins would result in a sustained activation of TLR4. It could also explain why we observed more significant changes in IL-10 and IL-12 expressions in hEL overexpression macrophages (Figure 4A and 4B) as more TLR4 ligands were formed. In any cases, it is likely, under different circumstances, the type of FFAs generated from EL hydrolysis and subsequent activation of the receptors during inflammation will influence the different aspect of the physiological phenotypes.

In summary, in contrast to LPL, macrophage EL expression is positively regulated by LPS and double-stranded RNA through TLR4 and TLR3. Macrophage EL negatively modulates IL-10 and positively modulates IL-12 production. Thus, induction of macrophage EL by proinflammatory stimuli can modulate further macrophage inflammatory responses, potentially influencing atherosclerosis.

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Disclosures
None.

References


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