High-Density Lipoprotein Hydrolysis by Endothelial Lipase Activates PPARα
A Candidate Mechanism for High-Density Lipoprotein–Mediated Repression of Leukocyte Adhesion

Waleed Ahmed,* Gabriela Orasanu,* Vedika Nehra, Liana Asatryan, Daniel J. Rader, Ouliana Ziouzenkova, Jorge Plutzky

Abstract—Although high-density lipoprotein (HDL) is known to inhibit endothelial adhesion molecule expression, the mechanism for this anti-inflammatory effect remains obscure. Surprisingly, we observed that HDL no longer decreased adhesion of U937 monocytoïd cells to tumor necrosis factor (TNF)α-stimulated human endothelial cells (EC) in the presence of the general lipase inhibitor tetrahydrolipstatin. In considering endothelial mechanisms responsible for this effect, we found that endothelial lipase (EL) overexpression in both EC and non-EL–expressing NIH cells significantly decreased TNFα-induced VCAM1 expression and promoter activity in a manner dependent on HDL concentration and intact EL activity. Given recent evidence for lipolytic activation of peroxisome proliferators-activated receptors (PPARs) nuclear receptors implicated in metabolism, atherosclerosis, and inflammation we hypothesized HDL hydrolysis by EL as an endogenous endothelial mechanism for PPAR activation. In both EL-transfected NIH cells and bovine EC, HDL significantly increased PPAR ligand binding domain activation in the order PPAR-α>>-γ>-δ. Moreover, HDL stimulation induced expression of the canonical PPARα-target gene acyl-CoA-oxidase (ACO) in a PPARα-dependent manner in ECs. Conditioned media from EL adenovirus transfected cells but not control media exposed to HDL also activated PPARα. PPARα activation by EL was most potent with HDL as a substrate, with lesser effects on LDL and VLDL. Finally, HDL inhibited leukocyte adhesion to TNFα-stimulated ECs isolated from wild-type but not PPARα-deficient mice. This data establishes HDL hydrolysis by EL as a novel, distinct natural pathway for PPARα activation and identifies a potential mechanism for HDL-mediated repression of VCAM1 expression, with significant implications for both EL and PPARs in inflammation and vascular biology. (Circ Res. 2006;98:0-0.)

Key Words: adhesion molecules • endothelial cells • HDL cholesterol • high-density lipoproteins • lipase • PPARs • transcriptional regulation

Epidemiologic studies establish a strong inverse relationship between plasma high-density lipoprotein (HDL) levels and cardiovascular events.1,2 These atheroprotective benefits of HDL are often ascribed to the role of HDL in reverse cholesterol transport and the efflux of cholesterol from foam cells within arterial plaque. Recent studies have also identified anti-inflammatory properties of HDL that may also contribute to this relationship between HDL and atherosclerosis. One important anti-inflammatory HDL effect is its well-established inhibition of adhesion molecule expression and monocyte adhesion to endothelial cells (EC), key early steps in atherosclerosis.3–5 Levels of soluble adhesion molecules are predictive of cardiovascular risk while physiologic concentrations of HDL can inhibit cytokine-induced expression of endothelial adhesion molecules like VCAM1, ICAM1, and E-selectin.4,5 The mechanisms underlying HDL’s anti-inflammatory effects have remained largely obscure. HDL may limit the effects of pro-inflammatory mediators by either physical binding or active catalysis, as suggested by lipid hydroperoxide destruction by paraoxonases present in HDL particles.6,7 HDL reportedly inhibits endothelial cell sphingosine kinase, resulting in decreased NF-κB activation.8 HDL may also increase nitric oxide levels.9 Much less is known about HDL-dependent pathways in the endothelium that may account for the ability of HDL regulate transcription and specifically repress adhesion molecule expression. In exploring this HDL/endothelial interaction, we noted that inhibition of endothelial leukocyte adhesion by HDL was blocked in the presence of the general lipase inhibitor tetrahydrolipstatin. Recently we reported evidence for spe-
pecific mechanisms through which lipoprotein metabolism can regulate distinct transcriptional responses through peroxi-
some proliferator-activated receptors (PPARs). ligand-ac-
tivated nuclear receptors involved in the transcriptional reg-
ulation of metabolism, inflammation, and atherosclerosis.  
For example, very-low-density lipoprotein (VLDL) hydroly-
sis by lipoprotein lipase (LPL) can generate peroxisome proliferator-activated receptor alpha (PPARα) ligands. In 
macrophages, LPL-treated VLDL was found to activate PPARα. These findings suggest an underappreciated role 
for lipoproteins as transcriptional regulators, with effects 
dictated by the nature of the lipoprotein particle and its 
uptake, including the biology of specific lipases. Although 
synthetic PPAR agonists, identified largely through serendip-
ity, including the biology of specific lipases. Although 
synthetic PPAR agonists, identified largely through serendip-
ous drug screen, have been variably described as 
decreasing adhesion molecule expression, endogenous 
mechanisms for PPAR activation remain poorly understood 
despite their potential molecular and clinical implications. 
We hypothesized that endothelial lipase (EL), a unique 
member of the triacylglycerol lipase family expressed in the 
endothelium, limits VCAM1 expression by activating PPARs 
via HDL hydrolysis. We present evidence here establishing 
and characterizing HDL hydrolysis by EL as a distinct, 
specific lipolytic pathway in the endothelium that preferen-
tially activates PPARα, regulates VCAM1 expression, and 
can limit leukocyte adhesion.

Materials and Methods

An expanded Materials and Methods appears in the online data 
supplement (http://circres.ahajournals.com).

Cell Culture

Bovine aortic endothelial cells (BAEC) and NIH cells were grown in 
Dulbecco modified Eagle medium (DMEM) containing 10% fetal 
bovine serum (FBS), 1% glutamine, penicillin, streptomycin, and 
fungizone as per standard procedure. Human umbilical vein endo-
thelial cells (HUVEC) (human EC throughout) were grown in 
medium M199 with similar components except for 20% FBS and 
endothelial cell growth factor (ECGF). The human hematopoietic 
medium M199 with similar components except for 20% FBS and 
thelial cells (HUVEC) were grown in 

HDL 1.063 to 1.210 g/mL. All lipoprotein concentrations (BSA 
assay; Pierce) are expressed as μg protein/mL unless otherwise 
noted. Specific triglyceride and phospholipid content in lipoproteins 
were determined using L-Type TG H and Phospholipids B kit (Wako 
Pure Chemical Industries).

Cell Transfection and PPAR-LBD Assays

Transient transfection was performed using Fugene (Roche Molecular 
Biochemicals) in 1% delipidated plasma (DLPHMEM as per 
maker’s instructions. A human EL cDNA (1.5 kb construct 
subcloned into the EcoRI site of the expression vector pcDNA3.1) 
was transfected as noted and responses compared with transfection 
of pcDNA3.1 vector alone. Catalytically inactive EL was generated 
by mutating serine 149 to alanine using reverse-transcription poly-
merase chain reaction (RT-PCR). PPAR-LBD-GAL4 assays were 
performed using standard procedures as before. A human VCAM1 
promoter construct was transfected using 1% Nutridoma SP (Roche 
Molecular Biochemicals), allowed to recover (14 hours) before 
additional stimulation.

Northern Blot Analysis

Standard Northern blot analysis was performed using VCAM1, 
acyl-CoA-oxidase (ACO) and glyceraldehyde-3-phosphate dehydro-
genase (GAPDH) (both ATCC) cDNA probes before densitometry 
quantification (ImageJ 1.33v; NIH).

Leukocyte Adhesion Assay

Near confluent HUVECs, isolated murine PPARα+/− or PPARα−/− 
ECs monolayers (all 24-well plates) were washed (DPBS, 3 times) 
after stimulation and before adding either human mononcytid U937 
cells or mouse monocyte–macrophage-like J774A.1 previously la-
beled with 2′,7′-bis(2-carboxy)-fluorescein acetoxymethylester 
(Molecular Probes) in serum free RPMI medium (4×10⁵ labeled 
cells/well, 37°C, 30 minutes, DPBS). Nonadherent cells were 
removed by rinsing (DPBS, 3 times) before measuring fluorescence 
intensity (Cytofluor II, PerSeptive Biosystems).

Results

HDL-Mediated Inhibition of TNFα-Stimulated 
Leukocyte Adherence to Human ECs Requires 
Intact Lipase Activity

TNFα stimulation significantly increased the adhesion of 
fluorescently labeled U937 mononcytid cells to a monolayer of 
HUVECs (Figure 1A, upper left panel). As expected, 
pre-treating EC (3 hours) with the PPARα agonist WY14463 
(10 μmol/L) or HDL (90 μg/mL) significantly decreased 
leukocyte adhesion by 37% and 31%, respectively (P<0.01 
for each, Figure 1B). Surprisingly, the repression of leukocyte 
adhesion by HDL was abolished if EC were pretreated with 
the general lipase inhibitor tetrahydrolipstatin (10 μmol/L, 
Figure 1A, lower right panel). These results suggest that 
the HDL-mediated decrease in leukocyte adhesion derives at 
least in part from lipase-mediated catalysis.

HDL Suppresses TNFα-Induced VCAM1 
Expression and Promoter Responses in a 
Concentration-Dependent Manner

Because EL is considered the major lipase in EC, we 
began by considering the endothelial mechanism(s) underlying 
the observations in Figure 1 by testing the effects of HDL 
on VCAM1 expression and promoter activity in cells over-
expressing EL. Human ECs were transfected with EL, al-
lowed to recover, then stimulated with TNFα and/or HDL at 
the concentrations shown before VCAM1 Northern blot 
analysis. HDL stimulation decreased TNFα-induced VCAM1
mRNA expression in a concentration-dependent manner (Figure 2A) but not in the presence of the lipase inhibitor tetrahydrolipstatin, even at the highest HDL concentration tested (90 μg/mL, Figure 2A). One representative Northern blot of three is shown; Northern blot analysis confirmed EL overexpression in transfected cells (supplemental Figure I).

We next tested if this EL-treated HDL effect was evident at the level of the VCAM1 promoter, supporting a transcriptional mechanism at work. Previous studies indicate NIH cells lack endogenous EL,25 a finding we confirmed using RT-PCR and Northern blotting (data not shown). NIH cells were cotransfected with a human VCAM1 promoter-luciferase and either the EL-pcDNA3.1 expression construct or pcDNA3.1 alone (here and throughout) before exposure to increasing amounts of HDL. In EL-transfected cells, HDL treatment significantly reduced VCAM1 promoter activity in a concentration-dependent manner (−63% in EL-transfected cells versus −17% in pcDNA3.1-transfected cells, both at 200 μg/mL of HDL, P<0.05, Figure 2B). These results suggest that HDL repression of cytokine-induced VCAM1 involves transcriptional regulation through HDL hydrolysis by EL.

Figure 1. HDL inhibits adhesion of U937 monocytes to TNFα-stimulated ECs but not in the presence of the lipase inhibitor tetrahydrolipstatin. A, Fluorescein-labeled U937 cells were added to TNFα-stimulated (50 ng/mL) human umbilical vein EC monolayers after pre-treatment with the PPARα agonist WY14643 (10 μmol/L), HDL (90 μg/mL), or HDL (90 μg/mL) in the presence of the lipase inhibitor tetrahydrolipstatin (10 μmol/L). Fluorescent microscopy shows adherent U937 cells (green) on a near-confluent EC layer. B, Quantification of U937 adherence on EC monolayers as determined by fluorescence assay. Results are expressed as percentage of leukocytes bound to TNFα-stimulated cells. Bars represent mean±SEM (n=3); #P<0.01 TNFα-stimulated ECs vs control; *P<0.01 WY and HDL vs TNFα-stimulated ECs.

EL Preferentially Acts on HDL to Activate PPARα

Given previous reports that synthetic PPARα agonists repress VCAM1 expression, we performed standard PPARα ligand binding domain (LBD) assays to test PPARα activation through EL interaction with different lipoprotein substrates. Because EL has predominant phospholipase activity, the phospholipid phosphotidylcholine is well-established as a model EL substrate. In EL-transfected human EC, phosphatidylcholine treatment (100 μmol/L) increased PPARα–LBD activation 16.9-fold (Figure 3A). To begin characterizing this EL pathway, we next examined EL-mediated PPARα activation in response to common circulating human lipoproteins, namely VLDL, LDL, and HDL isolated from normal subjects. Treatment of EL-transfected EC with VLDL (60 μg/mL) only negligibly activated PPARα, whereas LDL (0 to 60 μg/mL) modestly increased PPARα activation, reaching 15-fold activation at the maximum concentration of LDL tested (60 μg/mL, Figure 3A). The most potent PPARα activation occurred after HDL exposure to EL-transfected EC, with a concentration-dependent response that reached ≈30-fold at the maximal
HDL concentrations tested (60 μg/mL), as compared with 18-fold in untransfected EC (*P<0.05, Figure 3A). Of note, the PPARα activation seen with this relatively modest HDL concentration, being even less than circulating HDL levels in vivo, equaled the responses seen with the synthetic PPARα agonist WY14643 at 10 μmol/L (Figure 3A). To better define this response to HDL and in EC under basal conditions without EL overexpression, PPARα–LBD experiments were repeated in untransfected BAEC. HDL increased PPARα–LBD activation in BAEC in a concentration-dependent manner (Figure 3B). Tetrahydrolipstatin completely blocked this response (Figure 3B). EL-mediated PPARα activation by HDL, VLDL, and LDL was also analyzed as a function of the triglyceride and phospholipid content of all these lipoprotein particles. These data support HDL as the preferential substrate for PPARα activation through EL (supplemental Figure II).

Given our initial observation that HDL-mediated repression of leukocyte adhesion required intact lipase activity as well as the noncatalytic effects of lipases including EL,19,26 we next asked if EL-mediated PPARα activation required EL catalysis. Tetrahydrolipstatin inhibited the HDL effect on PPARα–LBD activation in a dose-dependent fashion in EL-transfected cells (Figure 3C). To further test this and eliminate any possible nonspecific effect of tetrahydrolipstatin, we next compared HDL-induced PPARα–LBD activation in BAEC transfected with either wild-type or a catalytically inactive EL point mutant. Although HDL treatment activated PPARα in a concentration-dependent manner in wild-type EL-transfected cells, HDL had no effect in cells expressing a catalytically inactive EL point mutant (Figure 3D). This apparent dominant negative effect of catalytically inactive EL on endogenous EL function has been previously reported and may stem from EL dimerization.27 As before, tetrahydrolipstatin completely inhibited PPARα activation in the presence of EL transfection, even at the highest HDL concentrations (Figure 3D). These observations strongly support hydrolysis of HDL as being required for HDL-mediated PPARα activation. These results also suggest that the PPARα activation seen with HDL in untransfected EC was likely attributable to the known endogenous expression of EL in these cells. To definitively address this issue, similar EL transfection experiments were next performed in NIH cells that lack endogenous EL.25

The Presence of EL Is Necessary and Sufficient to Confer HDL-Induced PPARα Activation

Standard PPARα–LBD assays were performed in NIH cells after exposure to increasing HDL concentrations (maximum 200 μg/mL) in either the absence or presence of EL transfection as before. In the absence of EL transfection, HDL stimulation had no significant effect on PPARα activation (maximum 1.7-fold activation at 200 μg/mL of HDL); in contrast, in the presence of EL transfection, HDL stimulation increased PPARα activation 7.3-fold at 200 μg/mL of HDL (P<0.005, Figure 4A).

To further establish that EL itself can activate PPARα in the presence of HDL, PPARα–LBD experiments were repeated in NIH cells exposed to increasing volumes of conditioned medium from EL-overexpressing cells (from 50 μL/well to 500 μL/well of conditioned medium in 24-well plates). At a constant concentration of HDL (100 μg/mL), PPARα–LBD activation increased linearly as a function of the amount of EL-conditioned medium added (3.87-fold PPARα activation at 500 μL/well of EL-conditioned medium versus 1.25-fold with control medium, P<0.005, Figure 4B).
The HDL hydrolytic activity for this conditioned medium was 500 nmol FFA formed per mL after 4 hours at 37°C.

**PPAR Isotype-Specific Effects of EL/HDL**

Because EC express all PPAR isotypes to varying degrees,36 we next characterized the relative PPAR isotype selectivity through HDL hydrolysis by EL. BAEC overexpressing EL as before but now after transfection of PPARα/H9251, PPARγ/H9253, or PPARδ/H9254–LBD constructs, comparing responses to the maximal activation seen with well-characterized agonists to each PPAR isotype. In the presence of EL transfection, HDL (90 μg/mL) induced activation that was 86% of the PPARα agonist (WY14643, 10 μmol/L), 49.5% of the PPARγ agonist (BRL, 1 mmol/L), and 39% of the PPARδ agonist (bezafibrate, 10 μmol/L, Figure 5).

**EL/HDL Induces PPARα Target Gene Expression**

If HDL hydrolysis by EL activates PPARα, then it should induce expression of positively regulated, canonical PPARα target genes that contain a known PPAR response element, doing so in a PPARα-dependent manner. Acyl-CoA-oxidase (ACO) is a well-established PPARα-regulated target gene that fulfills such requirements.10 Microvascular ECs from PPARα/H11001/H11001 and PPARα/H11002/H11002 mice were transfected with EL or control plasmid and stimulated with either HDL (at 90 or 200 μg/mL) or the synthetic PPARα agonist WY14643 (10 μmol/L) before performing ACO Northern blot analysis. In the genetic presence of PPARα, HDL induced ACO expression to a similar level as the PPARα agonist WY in EL-transfected cells; this effect was absent in PPARα-deficient EC (Figure 6). Densitometry and quantification...
Hydrolysis of HDL Is a Distinct Lipolytic Mechanism for PPARα Activation

The unique biologic role of EL among lipases establishes the importance of these observations for EL/HDL-mediated PPARα activation. Nevertheless, because LPL can generate PPARα ligands, we next investigated if hydrolysis of HDL by EL is distinct from LPL in terms of PPAR activation. To address this, we asked if the presence of EL modulated the maximal PPARα activation seen with LPL after exposure to either VLDL or HDL. As previously reported, in the presence of VLDL (5 μg/mL), LPL increased PPARα–LBD activation in a concentration-dependent manner (maximum 9.9-fold, Figure 7A). This concentration of VLDL used here produces robust PPARα activation in the presence of LPL, whereas the use of even higher VLDL concentrations induces cell death. EL overexpression had no impact on the LPL/VLDL responses seen (maximum 9.6-fold, Figure 7A). In contrast, when HDL (90 μg/mL) was the substrate, the presence of EL transfection increased PPARα activation significantly (*P<0.05, Figure 8B), whereas LPL, even at maximal concentrations, had no effect on the EL/HDL/PPARα response (Figure 8B). These results support preferential hydrolysis of HDL by EL as a unique pathway for PPARα distinct from LPL/VLDL-mediated responses.

HDL Inhibits Leukocyte Adhesion to TNFα-Stimulated Mouse ECs in a PPARα-Dependent Manner

Given this evidence for HDL hydrolysis by EL as a mechanism for PPARα activation, we returned to our initial observation that a lipase inhibitor blocks HDL-mediated repression of leukocyte adhesion to ask if this response required the presence of PPARα. Similar adhesion assays as in Figure 1 were repeated but measuring adhesion of fluorescein-labeled murine monocyte-macrophage J774A.1 cells to microvascular EC isolated from either wild-type (PPARα+/+) or PPARα-deficient (PPARα−/−) mouse hearts. As with HUVEC, both the PPARα agonist WY144643 (10 μmol/L) or HDL (90 μg/mL) significantly decreased leukocyte adhesion by 43% and 35%, respectively, but not when tetrahydroli-
increased under both basal and TNFα-stimulated conditions ($P<0.05$).

**Discussion**

We have observed that HDL-mediated repression of leukocyte adhesion to endothelial cells is markedly decreased after inhibition of lipase activity. Subsequent studies presented here identify hydrolysis of HDL by endothelial lipase (EL) as a pathway for PPARα activation and subsequent VCAM-1 repression, as evident in LBD assays, PPARα target gene induction, and a genetic requirement for PPARα for these EL/HDL effects to be seen. EL expression was both necessary and sufficient to confer PPARα responsiveness to HDL as evident in EL-nonexpressing cells after EL transfection or exposure to EL-conditioned media. Characterization of this EL/PPAR pathway reveals selectivity as to lipoprotein sub-strate (HDL > LDL > VLDL) and the targeted PPAR isotype (PPAR-α > γ > δ). These findings reveal HDL hydrolysis by EL as a mechanism for PPAR activation and identify a plausible mechanism potentially contributing to HDL effects on limiting leukocyte adhesion to the endothelium and vascular inflammation.
LPL hydrolysis of VLDL has been shown to activate PPARs.\textsuperscript{10,14} We reported that LPL acts on triglyceride-rich lipoproteins like VLDL to most potently activate PPAR\textsubscript{\alpha}, with lesser responses to LDL and even less with HDL as LPL substrates.\textsuperscript{10} In cell–free radioligand displacement assays, LPL-treated VLDL displaced high affinity synthetic PPAR agonists from expressed PPAR proteins following the order PPAR\textsubscript{\alpha}>>PPAR\textsubscript{\delta}>>PPAR\textsubscript{\gamma}. Chawla et al concurrently reported that LPL-treated VLDL activated PPAR\textsubscript{\delta} in mouse macrophages, cells that are thought to have low if not undetectable PPAR\textsubscript{\alpha} levels, in contrast to human macrophages.\textsuperscript{14} Comparing these findings to the data presented here, EL-mediated PPAR activation appears distinct from the LPL/PPAR pathway given differences in substrate preferences and the targeted PPAR isotypes. This conclusion is further supported by the demonstration that EL-treated HDL activation of PPAR\textsubscript{\alpha} is not altered by LPL regardless of its concentration (Figure 7B). The divergence we find between LPL and EL mechanisms of PPAR activation is consistent with previous studies defining specific differences between EL, LPL, and hepatic lipase as triacylglycerol hydrolysis family members.\textsuperscript{28,29} In addition to demonstrating and characterizing this novel EL/HDL/PPAR pathway, our results also identify PPAR activation as a previously unrecognized factor to be considered in interpreting studies on endothelial responses to HDL. More broadly, these findings support our working model that different lipases act on specific lipoproteins to direct distinct transcriptional responses. The specific limbs of such a network would be dictated by the nature of the lipoprotein substrate, the unique biologic role of specific lipases, as well as the distal consequences of activating a given PPAR isotype. These variables also suggest that establishing the relative potency between EL and LPL in terms of PPAR\textsubscript{\alpha} activation may be less relevant to PPAR activation in vivo via pathways of lipid metabolism. The distinctive, nonoverlapping roles for EL and LPL in PPAR activation are particularly intriguing given very recent evidence for EL and LPL effects on fatty acid uptake in adipocytes.\textsuperscript{20}

EL is unique among triglyceride lipase family members given its predominant endothelial expression.\textsuperscript{18,25} The role of EL in vascular biology is not yet clearly defined. EL is known to participate in HDL metabolism,\textsuperscript{30,31} although EL-mediated nuclear receptor activation has not been previously reported. Like LPL, EL has nonenzymatic effects, including increased noncatalytic lipoprotein uptake.\textsuperscript{26} Such EL bridging effects are unlikely to contribute to PPAR\textsubscript{\alpha} activation given the absence of PPAR responses with either a catalytically inactive EL mutant or in the presence of a general lipase inhibitor (Figure 3B). Given EL’s high degree of phospholipase activity, one might predict the specific PPAR ligand released from HDL by EL to be a fatty acid derived from the SN-1 position of a phospholipid.\textsuperscript{18,25} This would also be consistent with HDL as a phospholipid-rich lipoprotein being the preferred substrate for EL-mediated PPAR activation.

EL expression is induced by inflammatory cytokines as well as physical forces such as shear stress.\textsuperscript{32–33} Repression of adhesion molecule expression as well as other reports of anti-inflammatory effects through PPAR\textsubscript{\alpha} activation raises the intriguing possibility that EL induction by these pro-inflammatory forces might foster counter-regulatory PPAR\textsubscript{\alpha} responses. Interestingly, endogenous PPAR\textsubscript{\alpha} activation has been previously implicated in terminating inflammatory responses, for example, through leukotrienes.\textsuperscript{37} Alternatively, because previous work suggests EL can increase lipid uptake and promote atherosclerosis,\textsuperscript{34} EL-mediated PPAR activation might contribute to these pro-atherogenic processes as well. Regardless of its net effect, the expression of EL in EC and atherosclerotic plaque\textsuperscript{35} makes its activation of PPAR\textsubscript{\alpha} of obvious physiologic and pathologic relevance.

In summary, these data establish hydrolysis of HDL by EL as a mechanism for PPAR\textsubscript{\alpha} activation and PPAR\textsubscript{\alpha} regulation of VCAM1 expression. This EL-mediated PPAR activation identifies a specific mechanism through which HDL can exert...
transcriptional effects in the endothelium, thus representing an appealing mechanism contributing to the anti-inflammatory effects of HDL.

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**Materials and Methods**

**Reagents:**

The EL constructs used have been previously described \(^1\). Briefly, a human EL cDNA (1.5 kb) was subcloned into the EcoRV site of the expression vector pcDNA3.1. LPL (200 units/ml) was obtained from Sigma (St.Louis, MO). Synthetic PPAR agonists, used at the indicated concentrations, were obtained as follows: WY14643 (10\(\mu\)mol/l), Biomol (Plymouth Meeting, MA); bezafibrate (10\(\mu\)mol/l), Sigma; and BRL49653/rosiglitazone (1\(\mu\)mol/l), GlaxoSmithKline, (Research Triangle Park, NC). Tetrahydrolipstatin/Orlistat (THL,10\(\mu\)mol/l), was obtained from Roche Laboratories, (Indianapolis, IN). VCAM1 was induced using human TNF\(\alpha\) (20ng/ml, R&D, Minneapolis, MN). L-Phosphatidylcholine (100\(\mu\)mol/l) was obtained from Sigma. Dulbecco’s phosphate buffered saline (DPBS) was obtained from BioWhittaker (Walkersville, MD). All media used was from BioWhittaker.

**Generation of EL-conditioned media**

EL is a highly secreted protein \(^{18,19}\). The EL-conditioned media was added in increasing amounts (50\(\mu\)l - 500\(\mu\)l/well) to a constant concentration of HDL (100\(\mu\)g/ml) and the combination used in PPAR\(\alpha\)-LBD assays performed in NIH cells.

**Lipoprotein Isolation**

After a 12hr fast, venous blood was collected into ethylenediamine tetraacetic acid (EDTA; 1 mg/ml blood) containing Vacutainers followed by immediate plasma isolation by centrifugation (1500g, 10 min, 4\(^\circ\)C).
**Cell Transfection and PPAR-LBD assays**

Briefly, cells were co-transfected with constructs for PPAR-LBD GAL4 fusion, the GAL4-responsive luciferase reporter pUASX4TK-luc, and \( \beta \)-galactosidase. Where indicated, cells were also transfected with a vector (pcDNA3.1) containing wild-type EL, catalytically-inactive point mutant form of human EL, or the vector alone. Luciferase counts, normalized to \( \beta \)-galactosidase activity, were obtained using luciferase substrates (BD PharMingen); CPRG was used for \( \beta \)-galactosidase activity assays (Roche Diagnostics), all as before 10.

**Leukocyte Adhesion Assay:** Near confluent HUVECs, isolated murine PPAR\( \alpha \)^{+/+} or \( \Delta ^{-} \)ECs monolayers (24-well plates) were pretreated with WY, HDL, or both HDL and tetrahydrolipstatin (3 hrs, concentrations shown) before TNF\( \alpha \) stimulation (4 hrs) and subsequent removal of the medium.

**Statistical Analysis**

All results, performed in triplicate, are reported as mean ± SD. Differences were analyzed by the Wilcoxon test except for Figure 8, in which the student’s t test was employed. A value of P<0.05 or less was regarded as significant.
Supplemental Data Figure Legends

Supplemental Data Figure 1. Confirmation of transfected and endogenous EL expression in cell lines and types

A. The wildtype EL (1.5 kB) construct was transfected (EL +) into either bovine aortic EC or NIH cells, which are known to lack native EL expression, using the protocols described in the manuscript. The appropriately sized EL band is seen in transfected BAEC and NIH cells after 6 hrs of exposure. B. Longer exposure (24 hrs) of this same Northern blot reveals the native EL band. mRNA markers are shown.

Supplemental Data Figure 2. HDL is the preferred substrate for EL-mediated PPARα activation

Standard PPARα-LBD-GAL4 assays were performed in NIH cells, which lack endogenous EL expression, either with or without co-transfection of human EL before stimulation (16h) with different amounts of human lipoprotein classes (HDL, VLDL, LDL) normalized for their total protein, triglyceride (TG) and phospholipid content (determined as described in methods). Lipoproteins were isolated from normal human plasma as before and the amount of each lipoprotein class used calculated based upon the amount of protein, triglyceride or phospholipid contained in HDL60µg/ml, given its most potent EL-mediated PPARα activation (Fig. 3A). (A) Based upon a total protein concentration of 60µg/mL, HDL significantly activates PPARα in the presence of EL expression,
whereas no difference is seen with VLDL or LDL at a similar concentration of total protein in either the presence or absence of EL. (B) In these samples, HDL 60\(\mu\)g protein/ml (0.45\(\mu\)l by volume/well) contains 4.08\(\mu\)g/ml of TG. PPAR\(\alpha\) activation was tested using VLDL and LDL containing this same total TG content (4.08\(\mu\)g/ml/well), which involved addition of 0.15 \(\mu\)l VLDL and 1.05 \(\mu\)l LDL by volume/well (brought to a total volume of 300 \(\mu\)l/well using lipid-free serum). Despite similar TG content, neither VLDL nor LDL significantly activated PPAR\(\alpha\) in the presence or absence of EL. (C) PPAR\(\alpha\)-LBD-assays were repeated using a similar phospholipid content as HDL 60\(\mu\)g protein/ml/well, namely 5.06\(\mu\)g/ml (by volume/well). In the presence of EL, HDL activates PPAR\(\alpha\) to a significantly greater extent than VLDL or LDL containing a similar total mass of phospholipid (2.58\(\mu\)l or 1.57\(\mu\)l respectively by volume/well; * \(p<0.05\), comparing HDL in the presence versus absence of EL; for VLDL and LDL versus HDL, both in the presence of EL).
Supplemental data, Figure 1
Equal protein concentration (60 µg/ml)

Equal TG concentration (4.08 µg/ml)

Equal phospholipid concentration (5.06 µg/ml)

Supplemental data, Figure 2