Dual Roles for Lipolysis and Oxidation in Peroxisome Proliferator-Activator Receptor Responses to Electronegative Low Density Lipoprotein*

Low density lipoprotein (LDL) exists in various forms that possess unique characteristics, including particle content and metabolism. One circulating subtraction, electronegative LDL (LDL(−)), which is increased in familial hypercholesterolemia and diabetes, is implicated in accelerated atherosclerosis. Cellular responses to LDL(−) remain poorly described. Here we demonstrate that LDL(−) increases tumor necrosis factor α (TNFα)–induced inflammatory responses through NFκB and AP-1 activation with corresponding increases in vascular cell adhesion molecule-1 (VCAM1) expression. LDL receptor overexpression increased these effects. In contrast, exposing LDL(−) to the key lipolytic enzyme lipoprotein lipase (LPL) reversed these responses, inhibiting VCAM1 below levels seen with TNFα alone. LPL is known to act on lipoproteins to generate endogenous peroxisomal proliferator-activated receptor α (PPARα) ligand, thus limiting inflammation. These responses varied according to the lipoprotein substrate triglyceride content (very low density lipoprotein >> LDL > high density lipoprotein). The PPARα activation seen with LDL, however, was disproportionately high. We show here that MUT LDL activates PPARα to an extent proportional to its LDL(−) content. As compared with LDL(−) alone, LPL-treated LDL(−) increased PPARα activation 20-fold in either cell-based transfection or radioligand displacement assays. LPL-treated LDL(−) suppressed NFκB and AP-1 activation, increasing expression of the PPARα target gene iκBα, but only in the genetic presence of PPARα and with intact LPL hydrolysis. Mass spectrometry reveals that LPL-treatment of either LDL or LDL(−) releases hydroxy-octadecadienoic acids (HODEs), potent PPARα activators. These findings suggest LPL-mediated PPARα activation as an alternative catabolic pathway that may limit inflammatory responses to LDL(−).

Extensive data links low density lipoprotein (LDL) to atherosclerosis (1, 2). This occurs in part through the induction of early atherogenic inflammatory responses, including the expression of adhesion molecules like VCAM1 by endothelial cells (ECs) (3, 4). Consistent with this, increased dietary cholesterol rapidly induces atherosclerosis in animal models, with changes in VCAM1 expression seen within 2 weeks (3). LDL, a major carrier of cholesterol, circulates in several forms in vivo (5, 6). Most LDL pathogenicity becomes manifest after LDL oxidation (1). For example, oxidized LDL (oxLDL), but not native LDL, induces endothelial VCAM1 expression in the presence of TNFα (7).

More recently, a form of native LDL containing intermediate modifications with higher electronegative charge, referred to as LDL(−), has been identified and characterized (8, 9). Several lines of evidence implicate LDL(−) as a particularly pro-atherogenic particle. Like oxLDL, LDL(−) has pro-inflammatory effects, e.g. inducing the chemokine monocyte chemoattractant protein-1 and cytokine interleukin-8 (10), both of which are NFκB- and AP-1-regulated (11). LDL(−) is a significant component of more atherogenic small dense LDL subfractions (5, 6). LDL(−) also appears to be enriched in conditions characterized by accelerated atherosclerosis, namely familial hypercholesterolemia (10), diabetes (12), and hemodialysis (13).

Despite some similarities, native LDL, oxLDL, and LDL(−) have distinct characteristics that likely determine their biologic effects (14). Most fundamentally, these particles have unique compositional profiles. For example, LDL(−) contains fewer lipid peroxidation products than oxLDL, but more than native LDL (9, 14). These forms of LDL are also cleared from the circulation in different ways, potentially contributing to their unique roles in atherosclerosis. In contrast to both native LDL and LDL(−), which are taken up through the LDL receptor (LDLR) (15), oxLDL is removed after binding to scavenger

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1 The abbreviations used are: LDL, low density lipoprotein; AP-1, adaptor protein 1; BAEC, bovine aortic endothelial cell; EC, endothelial cell; ELISA, enzyme-linked immunosorbent assay; EMSA, electromobility shift assay; HBLDL, LDL modified during hemoglobin-mediated plasma oxidation; HDL, high density lipoprotein; HODE, hydroperoxy-octadecadienoic acid; HPoDE, hydroperoxy-octadecadienoic acid; HPLC, high pressure liquid chromatography; LDL, ligand binding domain; LDL(−), electronegative LDL; LDLR, LDL receptor; LPL, lipoprotein lipase; MS, mass spectrometry; NFκB, nuclear factor κB; oxLDL, oxidized LDL; PPAR, peroxisome proliferation-activated receptor; PPRE, peroxisome proliferator response element; TNFα, tumor necrosis factor α; VCAM1, vascular cell adhesion molecule-1; VLDL, very low density lipoprotein.

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or Fc receptors (1). Hydrolytic pathways for LDL for LDL particles also differ. For example, lipoprotein lipase (LPL), the predominant enzyme in triglyceride-rich lipoprotein (TRL) metabolism, can hydrolyze mildly oxidized LDL forms like LDL(−) (16), although this ability may be limited as suggested by the higher content of both triglycerides (8) and the LPL inhibitor Apo CII (17) in LDL(−) as compared with native LDL fractions.

Recently, we reported that LPL enzymatic action as a mechanism for generating endogenous peroxisome proliferator-activated receptor α (PPARα) ligands (18). This LPL/PPARα pathway replicated synthetic PPARα agonist effects, e.g., decreasing cytokine-induced VCAM1 expression (18) in vitro and in vivo. The PPARα activation through LPL varied depending on the lipoprotein substrate; it was greatest with VLDL, less with LDL, and minimal with HDL, a series corresponding to triglyceride content (18). Although this pattern and the absolute requirement for intact enzymatic catalysis for LPL-mediated PPARα activation suggests fatty acid release accounted for the responses seen, LPL-treated LDL activated PPARα to a disproportionate extent. This suggested LPL might release different PPARα mediators from LDL as compared with VLDL. If so, this indicates that the transcriptional responses to LDL might vary depending on LPL action, LPL particle composition, or its mechanism of uptake.

To pursue this hypothesis, we tested cellular responses, including PPARα activation, to different forms of native and oxidized LDL and with and without LPL treatment. In the presence of TNFα, LDL(−) uptake by the LDLR induced VCAM1 through NFκB and AP-1 activation, a previously unreported pathogenic LDL(−) effect. In contrast, LPL treatment of LDL(−) reversed this response, decreasing VCAM1 expression in a PPARα-dependent manner. Further studies reveal that LPL hydrolysis of LDL(−) generated oxidized linoleic acid (hydroxy-oleoctxadecenoic acid, or HODE) in concentrations likely to account for the PPARα activation and the subsequent anti-inflammatory effects.

EXPERIMENTAL PROCEDURES

Reagents—All reagents were purchased from Sigma-Aldrich unless otherwise indicated. All media were obtained from BioWhittaker (Walkersville, Maryland) and contained fungizone/penicillin/streptomycin. Human and murine TNFα were purchased from R&D Systems (Minneapolis, Minnesota). Fenofibric acid was a generous gift from laboratories Fournier (Dax, France).

Cell Culture—Human ECs, isolated from a saphenous vein and cultured in M199 medium (supplemented with endothelial cell growth factor (ECGF) and 5% fetal calf serum) as before (19), were all passage 3 to 5. PPARα−/− (129S3/SvImJ) mice were obtained from Jackson laboratories (Bar Harbor, Maine). PPARα−/− mice were a generous gift from T. Gonzalez (National Institutes of Health) (20). Murine ECs from 1-month-old PPARα−/− and PPARα−/− mouse hearts were isolated using double selection with intercellular adhesion molecule 2 (ICAM-2) and platelet endothelial cell adhesion molecule 1 (PECAM-1) antibodies (BD Pharamingen) bound to Dynabeads (Dynal, Lake Success, New York) as before (21).

LDL Isolation—Lipoproteins were isolated using gradient ultracentrifugation of human plasma pooled from at least six healthy donors (9). Plasma of healthy donors was subjected to hemoglobin-mediated oxidation as described (13). The specific form of LDL that is formed by this plasma of healthy donors was subjected to hemoglobin-mediated oxidation as described (13). The specific form of LDL that is formed by this reagents were added to confluent human umbilical vein endothelial cells (HUVEC) monolayers (19). Treated cells were kept on ice for 10 min, washed with cold phosphate-buffered saline, incubated with human VCAM1 monoclonal antibodies (gift from Dr. M. Gimbrone), and visualized using alkaline phosphate secondary antibodies (19).

Flow Cytometry—Flow cytometry was performed using confluent mouse ECs obtained from here PPARα−/− and PPARα−/− mice. Cells were washed in phosphate-buffered saline, harvested by trypsinization, and incubated (1 h at 4 °C) with fluorescein isothyiocyanate-conjugated anti-mouse VCAM1 antibody (BD Pharamingen). The EC culture purity was examined using anti-mouse phosphatidylethanolamine-conjugated platelet endothelial cell adhesion molecule 1 (BD Pharamingen). Subsequently, washed cells were analyzed in a BD Biosciences FACScan™ flow cytometer using CELLQuest™ software. At least 20,000 viable cells per condition were analyzed.

HPLC-MS—A stock standard solution containing hydroperoxo-oleoctxadenoic acid (HpODE) and hydroxy-oleoctxadecenoic acids was prepared by diluting and combining solutions of standard mixtures obtained from Cayman Chemical (Ann Arbor, MI). Samples were extracted by the Folch method, reconstituted in isopropanol, and filtered (0.2-µm nylon). The samples and stock standard were serially diluted with 50:50 acetonirole/H2O.

The separation was performed using a Waters 2690 LC with photo diode array (Waters 996) and time-of-flight mass spectrometry (TOF/MS) detection (Micromass LC). The column used was a Luna C18 (2) 50 × 2 mm, 5 µm from Phenomenex (Torrance, CA). Mobile phases for the isocratic separation were 50% A, 4 mM ammonium acetate (Sigma- Aldrich) in H2O, and 50% B, acetonitrile (Sigma-Aldrich) flowing at 0.5 mL/min. The separation was performed at 30 °C with a total run time of 5 min. UV absorption was acquired from 200–400 nm. MS was performed using electrospray ionization operating in negative ionization mode. The ionization parameters were as follows: capillary voltage, 3200V; sample cone, 37V; extraction cone, 4V; desolvation temperature, 300 °C; source temperature, 120 °C; and ion scanning m/z range, 100–1000. Extracted ion chromatograms were constructed for HODE and HpODE using m/z values of 295 and 293, respectively. When the signal-to-noise ratio was sufficient, samples were quantified using an external calibration curve. When the signal-to-noise ratio was too low, only semi-quantitative estimates were made.

Transfection—Transfection was carried out in 24-well plates at 2.3 × 104 cells per well using FuGENE (F. Hoffmann-La Roche). Cells were transfected 3 h after replating in Dulbecco’s modified Eagle’s medium containing 1% of demethylated fetal calf serum (for PPAR ligand binding domain (LBD) studies) or 1% Nutridoma SP (F. Hoffmann-La Roche) for VCAM1 promoter studies. Transfected cells were treated with the indicated treatments for at least 10 h. Constructs were generous gifts from T. Willson (GlaxoSmithKline; LBD/yeast Gal4), T. Collins (Children’s Hos- pital, Boston, MA; VCAM1 promoter), H. Hobs (University of Texas Southwestern, LDLR expression vector), and D. Rader (University of Pennsylvania; LPL and LPL mutant expression vectors). The catalytically inactive LPL mutant has a two-base pair difference (AQ/AGC) that abolishes the active site P363. The constructs were generous gifts from T. Willson (GlaxoSmithKline; LBD/yeast Gal4), T. Collins (Children’s Hos- pital, Boston, MA; VCAM1 promoter), H. Hobs (University of Texas Southwestern, LDLR expression vector), and D. Rader (University of Pennsylvania; LPL and LPL mutant expression vectors). The catalytically inactive LPL mutant has a two-base pair difference (AQ/AGC) that abolishes the active site P363. The constructs were generous gifts from T. Willson (GlaxoSmithKline; LBD/yeast Gal4), T. Collins (Children’s Hos- pital, Boston, MA; VCAM1 promoter), H. Hobs (University of Texas Southwestern, LDLR expression vector), and D. Rader (University of Pennsylvania; LPL and LPL mutant expression vectors). The catalytically inactive LPL mutant has a two-base pair difference (AQ/AGC) that abolishes the active site P363. The constructs were generous gifts from T. Willson (GlaxoSmithKline; LBD/yeast Gal4), T. Collins (Children’s Hos-
**RESULTS**

**LPL Decreases LDL(−)-mediated VCAM-1 Induction in a PPARα-dependent Manner**—We compared the effect of native LDL and LDL(−) on TNFα-induced VCAM1 expression in human ECs. As expected, TNFα induced VCAM1 protein in ECs (8-fold, set as 100% induction) on ELISA (Fig. 1A). Although concomitant treatment with native LDL led to only a modest further increase in VCAM1 (20%), LDL(−) augmented VCAM1 levels by 70% relative to TNFα alone (14-fold as compared with basal untreated levels). The presence of LPL inhibited LDL(−)-mediated VCAM1 induction in a dose-dependent manner (Fig. 1A). Similar effects were evident on Northern blotting (data not shown) and activation of the human VCAM1 promoter (Fig. 1B). Although LDL(−)-treatment induced the VCAM1 promoter 120–180% in bovine aortic EC transfections, this same stimulation in the presence of LPL repressed this response by 60–80% as compared, in both cases, with TNFα alone (Fig. 1B).

These effects of LPL/LDL(−) on TNFα-induced VCAM1 expression equaled those seen with synthetic PPARα ligands (WY14163 or fenofibric acid, Fig. 1B).

To test whether treatment of LDL(−) reduced VCAM1 expression in a PPARα-dependent manner, VCAM1 responses were examined using FACS analysis of PPARα+/− and PPARα−/− microvascular ECs (Fig. 1C). Both TNFα and TNFα/ LDL(−) stimulation of PPARα+/− ECs markedly increased VCAM1 content on the EC surface. LPL-treated LDL(−) repressed this TNFα induction in the presence, but not the genetic absence, of PPARα with effects replicating those seen with the synthetic PPARα agonist WY14643. These data suggest that LPL action on LDL(−) limits inflammation through a PPARα mechanism.

**LPL Treatment of LDL(−) Decreases NFκB Binding**—We and others have shown that PPARα ligands decrease cytokine-mediated VCAM1 expression through effects on NFκB signaling (19, 23). Mechanisms involved reportedly include direct PPARα interaction with p65 and PPARα-dependent expression of IkBα, sequestering the inactive p65/p50 complex in the cytoplasm (24). To explore how LPL negatively regulates LDL(−)/TNFα induction of the VCAM1 promoter, we performed an EMSA of EC nuclear extracts using NFκB and PPRE binding sites. As expected, TNFα induced NFκB binding, a response augmented in the presence of LDL(−) (Fig. 2A). In the presence of LPL, however, LDL(−) significantly decreased NFκB binding; WY14163 had similar effects. This decrease in NFκB binding was paralleled by increased IkBα levels from the very same ECs (Fig. 2B). Similarly, the increase in AP-1 binding induced by LDL(−)/TNFα was decreased after LPL treatment (data not shown). In parallel with decreased NFκB binding, both LDL/ LDL(−) and WY14163 markedly increased binding to a canonical PPRE (Fig. 2C). This response was much greater than that seen with LDL(−), TNFα, or their combination (Fig. 2C). PPRE binding involved PPARα as indicated by the supershift in the
The presence of PPARs but not the PPARy antibody. These effects appear to be due to increases in PPARα activators and not PPARα itself, given the lack of significant differences in nuclear PPARα protein levels after treatment with LDL(−), LPL, or their combination (Fig. 2D). Together, these data suggest that LPL treatment of LDL(−) exerts its effects through direct interaction of LDL-derived components with NFκB and AP-1 in a manner similar to that of synthetic PPARα ligands.

**LPL Lipolysis of LDL(−) Generates PPARα Ligands—**We further examined PPARα ligand generation as a result of LPL treatment of LDL(−). The yeast Gal4/PPAR LBD hybrid assay is classically used to screen for PPARα ligand formation (25). LDL(−) stimulation led to a modest 3-fold PPARα LDL activation; in the presence of LPL and LDL(−), PPARα activation increased 30-fold (Fig. 3A). In addition to its catalytic activity, LPL can also promote the uptake of lipoproteins like LDL through non-enzymatic bridging of lipoproteins to receptors like the LDLR (26). We investigated the contribution of LPL bridging to the effects reported above. Transient transfection of the LDLR into EC did not alter PPARα LDL activation by LDL(−)/LPL. In contrast, LDL(−) treatment of LDLR-transfected ECs markedly increased VCAM1 promoter activity (Fig. 3B).

LPL/LDL(−) mediated PPARα-LBD activation required intact LPL catalysis, as evident by the concentration-dependent inhibition of PPARα responses in the presence of the synthetic lipase inhibitor tetrahydrolipstatin or the natural LPL inhibitor ApoCIII (Fig. 3C). Similar repression was seen with an antibody raised against LPL, but not an antibody to the LDLR receptor (data not shown). Furthermore, repeating these PPARα LBD experiments in bovine EC transfected with a catalytically inactive LPL point mutant failed to activate PPARα by LDL(−) above levels induced by LDL(−) alone (Fig. 3D).

The generation of direct PPARα ligands by LPL treatment of LDL(−) was tested further in cell-free PPAR radioligand displacement assays. LPL treatment of LDL(−) decreased the EC50 of PPARα activation 20-fold as compared with non-treated LDL(−) (Fig. 4A). This LPL treatment may have produced greater amounts of the ligands already present in LDL(−), converted less potent ligands to more potent forms, or both. In contrast to our prior findings with native triglyceride-rich lipoprotein (18), LPL/LDL(−) did not preferentially activate one PPAR isoform, at least in direct ligand displacement and LBD assays (Fig. 4A).

We next sought to examine how these responses varied depending on the nature of LDL particles, especially in LDL with high LDL(−) content. In the presence or absence of LPL, LDL(−) had a much more potent concentration-dependent effect on PPARα-LBD activation (Fig. 4B). One fundamental characteristic of LDL(−) is its higher relative proportion of oxidized lipids as compared with native LDL (14). To examine the contribution of oxidation on PPARα responses, we utilized standard in vitro techniques to increase the proportion of LDL(−) present in human LDL samples, employing the mild oxidative stress method in which different plasma samples are exposed to hemoglobin under anaerobic conditions (13). The LDL isolated from such plasma has a higher LDL(−) content, although TG concentrations remain unchanged (13). Using the...
same total LDL (10 μg) for all treatments, we found that a direct linear relationship existed between PPARα/LBD activation and the proportion of LDL(−) in the absence of LPL. This was evident for both unoxidized (Fig. 4B, white circles) and oxidized (Fig. 4B, black circles) plasma samples (Pearson, p < 0.001, Fig. 4C). Thus, increased LDL(−) proportions substantially affect PPARα activation in the absence of LPL. In contrast, LPL treatment of these LDL(−) samples (Fig. 4C, squares) markedly increased PPARα LBD activation regardless of the LDL(−) content. Taken together, these results suggest that LDL oxidation and the extent of LPL-mediated hydrolysis are separate variables influencing PPARα activation.

Of note, although LPL-treatment increased the PPARα activation seen with all lipoproteins tested, the greatest induction occurred with LDL(−) as a substrate. Although TG is the principal LPL substrate, the robust PPARα-activation seen with LDL treatment of LDL(−) as opposed to TG-rich VLDL suggests generation of a potent PPARα ligand.

To identify this PPARα-activating component in LDL(−), hydrolytic products of LPL-treated LDL(−) were extracted and separated using HPLC-MS. The major lipophylic compounds in LDL(−), either with or without LPL treatment, were HpODE and HODE acids (Fig. 5A). Even though HODE was present at a negligible amount in untreated LDL(−), after LPL treatment HODE amounts were ~2.5 μg per 100 μg of LDL(−). Importantly, LPL treatment of native LDL also increased HODE and HpODE, albeit to a much smaller extent. The identity of compounds was confirmed using MS (HODE and HpODE, Fig. 5, B and C, respectively).

Prior work established HODE as a PPARα activator (27, 28), although its affinity may be relatively low (29). Recently, PPARα LBD activation by HODE has been reported (30). To evaluate the extent of PPARα activation by HODE and HpODE, cell-free displacement and cell-based LBD assays were performed. Two common forms of HODE, 9- and 13-HODE, were all effective PPARα ligands. The EC50 for 13-HODE equaled 10 μM, whereas the EC50 for 9-HODE was 2.6 μM in PPARα LBD displacement assays (data not shown). These results suggest that the combined action of hydrolysis and oxidation increases the proportion of HODE to levels sufficient for PPARα activation.

**DISCUSSION**

These studies provide evidence that LPL acts on LDL(−) to generate PPARα ligands, thus countering the endothelial adhesion molecule expression induced by LDL(−) alone. These data highlight the differing transcriptional responses to LDL that depend on the mechanism of lipoprotein uptake, namely through the LDLR as opposed to hydrolysis by LPL (Fig. 6). Enhanced LDL(−) uptake through LDLR overexpression further increases VCAM-1 expression and promoter activity through TNFα-mediated NFκB and AP-1 activation. In con-
Transcriptional PPAR Responses to Electronegative LDL

Fig. 4. LPL hydrolysis of LDL generates PPAR ligands in a manner dependent on oxidation. A, lipolysis markedly increases the generation of PPAR ligands from LDL(\(-\)) (13). The most potent response was seen with PPAR\(\alpha\) and PPAR\(\gamma\) with less displacement of PPAR\(\gamma\) ligand. Direct radiolabeled ligand displacement (scintillation proximity assay) PPAR assays were performed using LDL(\(-\)) hydrolyzed with LPL (200 units/ml, 2 h, 37 °C under argon). Competition curves were generated across a range of LDL(\(-\)) concentrations (0.003–10 μg protein/ml) by incubating the reaction mixture with specific radiolabeled PPAR activators, i.e. 5 nM nTZD3 for GST-hPPAR\(\alpha\) or GST-hPPAR\(\gamma\), or 2.5 nM nTZD4 for GST-hPPAR\(\gamma\) (22, 37). Data from duplicate determinations were pooled and \(K_s\) values (IC50) were obtained from the dose-response curves. The white bars indicate the higher concentration (\(K_s\)) of non-hydrolyzed LDL(\(-\)) required for PPAR ligand displacement; black bars reveal the more potent displacement for the same LDL(\(-\)) after LPL hydrolysis. B, concentration-dependent PPAR\(\alpha\)-LBD activation by LDL (circles) and LDL(\(-\)) (triangles) in the presence (solid line) or absence (dashed line) of LPL is shown. BAECs, transfected as in Fig. 3C, were stimulated with the concentration of lipoproteins shown after LPL (20 units/ml) treatment. C, LDL oxidation increases PPAR\(\alpha\) activation independent of LPL treatment. PPAR\(\alpha\) LBD activation was measured as in Fig. 3C. BAECs were treated with LDL (10 μg/ml; circles) or LDL/LPL (squares) obtained from four different healthy donors. The role of oxidation was assessed using native LDL (open circles) or the same LDL treated ex vivo to achieve higher LDL(\(-\)) content (black circles). The latter was produced by exposing plasma samples to hemoglobin (10 μM) under argon (12). The levels of LDL(\(-\)) in LDL obtained from native and oxidized plasma achieved were determined using HPLC and are shown on the x axis. The linear correlation between LDL(\(-\)) content and PPAR\(\alpha\)-LBD activation (\(p < 0.001\), Pearson) is shown with the drawn line. No correlation was evident after LPL treatment of LDL. D, relative PPAR\(\alpha\) LBD activation by different lipoproteins in the absence (open bars) or presence of LPL (black bars) is shown. VLDL, LDL, LDL(\(-\)) and HDL were isolated from human blood, and the plateau of maximum PPAR\(\alpha\) activation was determined from concentration-dependent curves as shown in panel A. Responses were also compared with \(\mu\)M LDL, an LDL species enriched in LDL(\(-\)) generated in vitro by exposing plasma to hemoglobin (10 μM) under anaerobic conditions before lipoprotein isolation (13). oxLDL was obtained after exposing LDL to CuSO4 (10 μM, 24 h), which completely oxidizes polyunsaturated fatty acids (32).

Contrast, LPL hydrolysis of LDL(\(-\)) limits these responses by generating PPAR\(\alpha\) ligands, thereby increasing \(\lambda\)Rα expression with subsequent NFκB inhibition. Interestingly, LPL hydrolysis of LDL(\(-\)) activates PPAR\(\alpha\) to a greater extent than either LDL or VLDL. These efficient PPAR\(\alpha\) responses were proportional to the level of LDL oxidation, particularly to the HODE content of the lipoprotein particles. Thus, both hydrolysis and oxidation appear to influence PPAR\(\alpha\) activation by LDL(\(-\)), suggesting that responses to this pro-inflammatory, proatherogenic particle may depend on its catabolism.

LDL(\(-\)) is a unique lipoprotein (14). It is the only mildly oxidized LDL subfraction in the circulation that undergoes both LPL hydrolysis and LDLR uptake. One recent report suggests that mild oxidation increases LDL susceptibility to LPL-mediated hydrolysis (16), potentially amplifying the responses seen here with LDL(\(-\)). In contrast, oxLDL, which is more extensively oxidized, does not bind to LPL and is taken up via scavenger and Fc receptors in an LPL-independent manner (1, 16). Even though oxLDL contains several reported PPAR\(\alpha\) activators, including oxidized phospholipids (30, 31) and non-esterified fatty acids (such as HODe) (30), PPAR\(\alpha\) activation in vitro by oxLDL does not decrease inflammation as is characteristic seen with synthetic PPAR\(\alpha\) agonists. OxLDL reportedly increases VCAM1 expression in the presence of TNF\(\alpha\) (7). This discrepancy may be due to high levels of other lipid peroxidation and decomposition products that may have potent inflammatory and cytotoxic effects, e.g., oxysterols, hydroxynonenal, or lysophosphatidylethanolamine (32). This last product is known potent NFκB activator (33) that may overcome PPAR\(\alpha\) activation. Alternatively, responses may vary depending on the pathway of lipoprotein uptake. The effects of oxLDL on PPAR\(\alpha\) may depend on cytoplasmic phospholipase A\(\alpha\) action (30); this is not likely relevant to the results presented here, because LPL neither acts on nor binds to oxLDL (16). In our studies, LDLR overexpression significantly increased LDL(\(-\)) activation of the VCAM1 promoter but not PPAR\(\alpha\) LBD activation. In contrast, LDL(\(-\)) hydrolysis significantly decreased VCAM1 expression in a PPAR\(\alpha\)-dependent manner, with responses equivalent to synthetic PPAR\(\alpha\) agonists (Fig. 1). Inhibiting LPL hydrolytic activity through expression of a catalytically inactive LPL mutant, the presence of either natural (ApoCIII) or synthetic (tetrahydrodipractin) LPL inhibitors, or co-stimulation with an LPL antibody prevented LDL(\(-\))/LPL-mediated PPAR\(\alpha\) activation (Fig. 3). Together, these findings imply that intact LPL hydrolysis is re-
Several prior studies have found that oxidation plays a role in PPAR ligand generation, with certain oxidized molecules, e.g. oxidized phospholipids and certain fatty acids, having greater effects than their native forms (27, 29–31). To examine this issue, the amount of LDL (HDL) in a given LDL sample was increased in a controlled fashion using hemoglobin plasma oxidation in vitro (HBLDL). Greater PPAR activation occurred with HBLDL stimulation than with native LDL, suggesting that oxidation changes the ligands present in these lipoproteins. In fact, the proportion of LDL (HDL) in these samples correlated with PPAR activation in a linear fashion (Fig. 4C).

The oxidized molecules likely responsible for these effects have been identified as 9- and 13-HODE, both established PPAR (27) and PPAR activators (30). Such HODEs are released during LDL hydrolysis of LDL (HDL) (Fig. 5A). In fact, the PPAR activation seen with LPL-treated LDL (HDL) equaled that seen after direct HODE stimulation. The larger proportion of HODE in LDL (HDL) as compared with native LDL may account for the greater PPAR activation seen either before or after LPL treatment. Moreover, hydrolysis of native LDL also increases its HODE content, potentially contributing to its LPL-mediated PPAR activation. The possibility that the HODEs liberated from LDL and LDL (HDL) undergo further changes intracellularly cannot be excluded. The greater PPAR activation seen after HODE treatment of cell-based LBD assays as compared with cell-free direct displacement assays supports such a notion. Although HODEs seem the most likely candidate for explaining the LDL (HDL) responses seen, PPAR activators other than HODEs may be generated by LPL or augmented in its presence.

Previously, the formation of the natural PPAR ligands...
HETE and LTB₄ during acute inflammatory events was suggested as a feedback loop terminating inflammation and decreasing pro-inflammatory eicosanoids via β-oxidation (34). The data presented here suggest another endogenous mechanism through PPARαs that may limit inflammatory responses, namely LPL catalysis of LDL(−). Increased concentrations of LDL(−) have been associated with pro-inflammatory conditions such as familial hyperlipidemia, diabetes, and hemodialysis, with levels up to 20% of total LDL found in some subjects (10, 13). Interestingly, many of these clinical conditions are also characterized by dysfunctional LPL (35, 36). One recent study found that only the LDL content of the LPL inhibitor apolipoprotein CIII independently predicted cardiovascular events, whereas total LDL concentration did not (35). Our results suggest that the combination of increased circulating pro-inflammatory LDL(−) and ineffective LPL action may be a particularly deleterious combination, with the responses to LDL(−) compounded by decreased PPARα ligand generation. Our prior observations suggested that LPL mediates PPARα ligand generation under physiologic conditions (18); these findings extend lipolytic PPAR activation to limiting pathologic responses through LDL(−). Such data further support the concept of circulating lipoproteins as a reservoir for PPAR ligands and lipolysis as a means of accessing them.

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