Activation of Peroxisome Proliferator-Activated Receptor δ Inhibits Human Macrophage Foam Cell Formation and the Inflammatory Response Induced by Very Low-Density Lipoprotein

Lazar A. Bojic, Cynthia G. Sawyez, Dawn E. Telford, Jane Y. Edwards, Robert A. Hegele, Murray W. Huff

Objective—Hypertriglyceridemia is an important risk factor for cardiovascular disease. Elevated plasma very low-density lipoprotein (VLDL) puts insulin-resistant patients at risk for atherosclerosis. VLDL readily induces macrophage lipid accumulation and inflammatory responses, for which targeted therapeutic strategies remain elusive. We examined the ability of VLDL to induce macrophage foam cells and the inflammatory response and sought to define the cell signaling cascades involved. We further examined the potential of peroxisome proliferator-activated receptor (PPAR) δ activation to attenuate both VLDL-stimulated lipid accumulation and cytokine expression.

Methods and Results—THP-1 macrophages exposed to VLDL displayed significant triglyceride accumulation, which was attenuated by PPARδ activation. PPARδ agonists stimulated a transcriptional program resulting in inhibition of lipoprotein lipase activity, activation of fatty acid uptake, and enhanced β-oxidation. VLDL-treated macrophages significantly increased the expression of activator protein 1 associated cytokines interleukin-1β, macrophage inflammatory protein 1α, and intercellular adhesion molecule-1. VLDL treatment significantly increased the phosphorylation of both extracellular signal-related kinase 1 and 2 and p38. VLDL reduced AKT phosphorylation as well as its downstream effector forkhead box protein O1, concomitant with increased nuclear forkhead box protein O1. Cells treated with PPARδ agonists were completely resistant to VLDL-induced expression of inflammatory cytokines, mediated by normalization of mitogen-activated protein kinase (MAPK) and AKT/forkhead box protein O1 signaling.

Conclusion—The combined PPARδ-mediated reductions of lipid accumulation and inflammatory cytokine expression suggest a novel macrophage-targeted therapeutic option in treating atherosclerosis. (Arterioscler Thromb Vasc Biol. 2012;32:2919-2928.)

Key Words: atherosclerosis • lipids • insulin resistance • inflammation • intervention

Excessive lipid accumulation within macrophages of the arterial intima drives the synthesis and secretion of proinflammatory mediators, potentiating atherogenesis.1 Canonically, elevated plasma low-density lipoprotein (LDL) is considered the major lipoprotein contributing to accelerated atherogenesis. However, epidemiological evidence strongly suggests that hypertriglyceridemia also increases the risk of premature atherosclerosis, especially in the context of metabolic syndrome and type 2 diabetes mellitus.2,3 Plasma triacylglycerol (TG)-carrying very low-density lipoprotein (VLDL) has been localized within atherosclerotic lesions from human patients and animal models,4,5 providing rationale for examining the mechanisms by which these lipoproteins induce the development of macrophage foam cells. VLDL readily induces macrophage lipid accumulation,6,7 which in turn stimulates the synthesis of cytokines such as interleukin (IL)-1β and macrophage inflammatory protein (MIP)-1α.8-11 The mechanisms regulating these lipid-induced macrophage inflammatory responses have not been fully characterized.

In mouse macrophages, VLDL-induced expression of MIP-1α requires fatty acid liberation by lipoprotein lipase (LPL) and is dependent on the activation of extracellular signal-related kinase 1 and 2 (ERK1/2).9 Furthermore, VLDL potentiates lipopolysaccharide (LPS)-stimulated macrophage IL-1β secretion via activation of the transcription factor activator protein (AP)-1,10 which is known to be regulated by mitogen-activated protein (MAP) kinases ERK1/2 and p38.12 The involvement of p38, which is thought to act cooperatively with ERK1/2 in AP-1 associated inflammatory responses,12 has not been established.13 In addition, macrophage insulin resistance may potentiate the inflammatory response. Macrophage-specific deletion of the insulin receptor in Ldr−/− mice

Received on: June 14, 2012; final version accepted on: September 17, 2012.
The online-only Data Supplement is available with this article at http://atvb.ahajournals.orglookup/suppl/doi:10.1161/ATVBAHA.112.255208/-DC1.
Correspondence to Murray W. Huff, Robarts Research Institute, Rm 4222, The University of Western Ontario, 100 Perth Dr, London, Ontario, Canada, N6A 5K8. E-mail mhuff@uwo.ca
© 2012 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org
DOI: 10.1161/ATVBAHA.112.255208
2919
significantly increased atherosclerosis, an effect attributed to impaired macrophage AKT/forkhead box protein O1 (FoxO1) signaling. Insulin-resistant macrophages with cholesterol-induced endoplasmic reticulum-stress display impaired AKT phosphorylation, increased nuclear FoxO1 activity, and enhanced apoptosis. Furthermore, IL-1β is a FoxO1 target gene in macrophages with insulin resistance. Collectively, these studies highlight the importance of examining the role of AKT/FoxO1 signaling in the VLDL-induced inflammatory response.

The peroxisome proliferator-activated receptors (PPARs) are important regulators of metabolic and inflammatory signaling. The 3 known isoforms, namely PPARα, PPARγ, and PPARδ, each exhibit distinct tissue distribution and PPAR-specific regulation of gene transcription. In contrast to PPARα and PPARγ, expression of PPARδ is ubiquitous, with high levels in macrophages, where its biological role is unclear. Macrophage PPARδ is activated by VLDL-derived fatty acids, and conflicting reports have demonstrated that synthetic ligands promote either lipid accumulation or fatty acid catabolism. Consequently, the net effect of PPARδ activation on VLDL-induced TG accumulation is unknown.

The anti-inflammatory properties of PPARδ activation have been linked to the liberation of B cell lymphoma 6 from unliganded PPARδ, which inhibits expression of AP-1–inducible cytokines by localizing to AP-1 response elements and recruiting corepressors to these promoter regions. Additionally, in adipocytes and cardiomyocytes, PPARδ agonists inhibit LPS-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) regulated cytokine expression. The mechanism(s) underlying VLDL-induced cytokine expression in macrophages, in the absence of LPS, and the impact of PPARδ activation have not been elucidated.

In the present study, we report that synthetic ligand activation of PPARδ attenuates VLDL-stimulated TG accumulation by activating a transcriptional program resulting in attenuation of LPL activity, increased fatty acid uptake, and enhanced β-oxidation. VLDL stimulates the expression of proinflammatory cytokines IL-1β, MIP-1α, and intercellular adhesion molecule (ICAM)-1 via both ERK1/2- and AKT/FoxO1-dependent signaling mechanisms. Furthermore, macrophage treatment with synthetic PPARδ ligands inhibits proinflammatory cytokine expression, by inhibiting VLDL-stimulated ERK1/2 activation and reversing VLDL-mediated inhibition of AKT/FoxO1 phosphorylation.

Methods

An expanded Materials and Methods section is available in the online-only Data Supplement.

Lipoproteins

Subjects were recruited from the Lipid Clinic at the London Health Sciences Center University Campus (London, Ontario, Canada). The University of Western Ontario Health Science Standing Committee on Human Research approved the studies (IRB reference #15685). VLDL (Sf 20 to 400) was isolated from plasma of type IV hyperlipoproteinemic patients by differential ultracentrifugation using a Beckman 70.1 Ti rotor (16 hours, 146 682 x g 12°C) as previously described.

Cell Culture and Analyses

Human THP-1 macrophages were obtained from American Type Culture Collection (Manassas, VA) and cultured as described previously. PPARδ agonists GW0742 (Sigma) and GW501516 (Alexis Biochemicals, Plymouth, PA) were dissolved in dimethyl sulfoxide (Sigma) and incubated with cells at the indicated concentrations. THP-1 macrophages were preincubated (24 hours) in the presence or absence of PPARδ agonists. Subsequently, cells were incubated with PPARδ agonists in the presence or absence of lipoproteins or various inhibitors as indicated. Assays to determine PPARδ agonist dose–responses, cellular lipid mass, LPL activity, fatty acid uptake, fatty acid β-oxidation, TG synthesis, and luciferase activity were performed as described in the online-only Data Supplement.

Quantitative Real-Time Polymerase Chain Reaction and Immunoblotting

Total RNA was isolated from cells and specific mRNA abundances measured by quantitative real-time polymerase chain reaction (PCR) using an ABI Prism (7900HT) Sequence Detection System (Applied Biosystems, Foster City, CA). Cell lysates were fractionated into cytosolic and nuclear fractions, and proteins were separated by SDS-PAGE, transferred to polyvinyldene difluoride membranes and immunoblotted with specific antibodies as described previously.

Statistical Analyses

Data are expressed as mean±SEM. Significances of differences were determined using 1-way ANOVA followed by Bonferroni or Tukey posthoc analysis where indicated. Significance thresholds were P<0.05.

Results

PPARδ-Specific Activation Attenuates VLDL-Induced Macrophage Triacylglycerol Accumulation

THP-1 cells treated with VLDL demonstrated a dose-dependent increase in TG mass achieving a marked 5-fold increase with VLDL at 50 µg/mL (Figure 1A and 1B). Pretreatment with PPARδ agonists for 24 hours modestly but significantly reduced VLDL-induced TG mass by 25% to 30% (Figure 1A and 1B). VLDL significantly induced intracellular free fatty acid mass, which was unchanged by pretreatment with PPARδ agonists (Figure 1C). Cellular cholesteryl ester or free cholesterol concentrations were unaffected by VLDL or PPARδ agonists (Figure 1D), indicating that whole particle VLDL uptake was modest. A known PPARδ-specific target gene, adipocyte differentiation-related protein, was significantly increased by both agonists (Figure 1E), indicating PPARδ activation.

High doses of PPAR agonists can activate PPAR isoforms nonselectively. Therefore, we determined the PPARδ-specificity of the concentrations of agonists used. PPARδ-deletion results in derepression of PPARδ target genes, and transrepression of inflammatory cytokine expression, creating an experimental confounder for the present studies. We therefore assessed agonist-specificity by cotransfecting HepG2 cells with luciferase reporter constructs driven by PPAR response elements and each PPAR isoform (α, γ, δ). We determined dose–responses for each receptor in cells treated with PPARδ agonists (GW0742 and GW501516), and used agonists for PPARα (GW7647) and PPARγ (rosiglitazone) as positive controls. GW0742 at 25 nmol/L and GW501516 at 100 nmol/L were concentrations at which these ligands...
maximally activated PPARδ, without activation of either PPARα or PPARγ (Figure 1A–1D in the online-only Data Supplement). Furthermore, canonical PPARα and PPARγ target genes (ACOX and FABP4, respectively) were unaffected by either 25 nmol/L GW0742 or 100 nmol/L GW501516 (Figure 1E and IF in the online-only Data Supplement). Although unlikely, these agonists may have effects on non-PPAR targets. However, with respect to PPARs, these data demonstrate selectivity of the agonist concentrations used for PPARδ.

**Figure 1.** Peroxisome proliferator-activated receptor (PPAR) δ-specific activation attenuates very low-density lipoprotein (VLDL)-induced triacylglycerol mass accumulation. THP-1 cells were preincubated with PPARδ agonists GW0742 and GW501516 for 24 hours, followed by a 16-hour incubation with or without VLDL (50 μg-TC/mL). A and B, Triacylglycerol (TG) mass (n=5–7). C, Free fatty acid (FFA) mass (n=3–4). D, Cholesteryl ester (CE) and free cholesterol (FC) mass (n=6). E, Adipocyte differentiation-related protein (ADRP) mRNA in THP-1 cells after preincubation with PPARδ agonists for 24 hours, and after a 16-hour incubation, with or without VLDL (n=4). Data are presented as mean±SEM. Different letters indicate significant differences; ANOVA with posthoc Tukey test (P<0.05). * indicates significant difference vs dimethyl sulfoxide (DMSO) control; ANOVA with post hoc Bonferroni test (P<0.05).

**PPARδ Agonists Regulate LPL Activity, Fatty Acid Uptake, and Fatty Acid β-Oxidation**

We examined whether PPARδ activation attenuated VLDL-induced TG mass by regulating lipoprotein hydrolysis, fatty acid uptake, or fatty acid esterification. In contrast to rosiglitazone, LPL mRNA was unchanged in response to PPARδ agonists, further demonstrating PPARδ-specificity (Figure 2A; Figure IG in the online-only Data Supplement). The PPARδ-target gene, angiopoietin-like 4, encodes a protein known to...
potently inhibit LPL activity. Angiopoietin-like 4 mRNA expression was markedly enhanced by both PPARδ agonists in the presence or absence of VLDL, which was associated with a 50% inhibition of LPL activity (Figure 2B and 2C). The PPARδ ligands significantly increased expression of the PPAR-target gene CD36 irrespective of lipid load, which was correlated with a 25% increase in palmitate uptake, but not oleate uptake (Figure 2D–2F). DGAT1 mRNA, DGAT2 mRNA, and TG synthesis were unaffected by PPARα activation (Figure II in the online-only Data Supplement). PPARδ ligands significantly upregulated CPT-1α mRNA, with or without VLDL, which was associated with a 40% increase in fatty acid oxidation (Figure 2G and 2H). Collectively, these results indicate that although PPARδ activation modestly increases palmitate uptake, attenuated LPL-mediated VLDL TG-hydrolysis and increased fatty acid β-oxidation results in a net reduction of macrophage triglyceride content.

**PPARα Agonists Inhibit VLDL- and Free Fatty Acid–Induced Cytokine Expression**

Exposure of mouse macrophages to VLDL stimulates MIP-1α expression, an AP-1–mediated inflammatory response. Here, human VLDL significantly induced macrophage expression of IL-1β, MIP-1α, and ICAM-1 mRNA. Pretreatment with PPARδ ligands significantly inhibited the VLDL-induced expression of all 3 cytokines (Figure 3A–3C), without affecting basal cytokine expression (Figure III in the online-only Data Supplement). Furthermore, media levels of VLDL-induced IL-1β were significantly decreased by both PPARδ agonists (Figure IVA in the online-only Data Supplement). Canonical NF-κB–target genes TNFα and IL-6 were unaffected by VLDL treatment (Figure IVB in the online-only Data Supplement). Moreover, parthenolide, an inhibitor of NF-κB signaling, had no effect on VLDL-stimulated expression of IL-1β, MIP-1α, and ICAM-1, however parthenolide completely inhibited cytokine stimulation by LPS, a known NFκB activator (Figure IVC in the online-only Data Supplement). Collectively, these data suggest that VLDL-induced macrophage inflammatory responses do not require NF-κB activation.

**Inhibition of VLDL-Induced Inflammation by PPARδ Activation Is Independent of Reduced Cellular Triglyceride**

We examined whether inhibition of VLDL-induced inflammation by PPARδ agonists was a consequence of reduced TG accumulation. Complete inhibition of LPL with tetradecylsodium stearate blocked cellular TG and cytokine expression. However, inhibition of TG accumulation with low-dose tetradecylsodium stearate, to the same extent as that achieved by PPARδ agonists (≈25%), decreased ICAM-1 expression by 30% but did not affect MIP-1α or IL-1β (Figure V in the online-only Data Supplement). PPARδ activation normalized VLDL-induced cytokine expression, even when β-oxidation was inhibited by etomoxir (Figure 3D), a CPT-1α inhibitor. These data suggest that increased β-oxidation only partially accounts for reduced cytokine expression by PPARδ activation.

Our results are consistent with the concept that VLDL-derived FAs are the primary mediators of the inflammatory response. Paradoxically, FAs also activate PPARδ. To reconcile this, macrophages were treated with oleic acid, which resulted in a marked induction of TG mass and expression of IL-1β and MIP-1α (Figure VIA and VIP in the online-only Data Supplement). These effects were significantly attenuated by preincubation of cells with GW501516 (Figure VIA and VIP in the online-only Data Supplement), suggesting that the ability of FAs to activate PPARδ is insufficient to prevent macrophage TG accumulation and cytokine expression.

**VLDL-Stimulated Expression of Inflammatory Cytokines Is Dependent on MAPK Activation and Repression of AKT/FoxO1 Signaling**

VLDL-induced MIP1-α expression in mouse macrophages involves activation of ERK1/2. In THP-1 cells, ERK1/2 phosphorylation increased significantly within 0.5 hour of VLDL exposure, which returned to baseline by 1 hour (Figure 4A; Figure IVC in the online-only Data Supplement). Similarly, VLDL-stimulated p38 phosphorylation, reaching peak phosphorylation by 0.5 hour, and remained elevated for 3 hours (Figure 4B). The MAP ERK kinase 1/2 inhibitor, U0126, abrogated VLDL-stimulated expression of all cytokines (Figure 4C). In contrast, the p38 inhibitor SB203580 stimulated cytokine expression under basal conditions and further increased cytokine expression in VLDL-treated cells (Figure 4D). Inhibition of p38 resulted in a 5-fold induction in ERK1/2 phosphorylation (Figure VIIA in the online-only Data Supplement), suggesting that the mitogen-activated protein kinase (MAPK)α signal stimulates cytokine expression, whereas MAPKβ represses the actions of MAPKα.

The temporal disconnect between VLDL-induced MAPKα activation/deactivation (1 hour) and elevated cytokine expression (16 hours) suggests that other macrophage inflammatory signaling pathways are stimulated by VLDL. Given that IL-1β is a target of nuclear FoxO1 in macrophages in the context of insulin resistance, we examined the role of AKT/FoxO1 in VLDL-induced inflammation. Exposure of THP-1 cells to VLDL resulted in a time-dependent reduction of phospho-AKT levels for up to 6 hours (Figure 5A). Reduced phospho-AKT was correlated with attenuated phospho-FoxO1 and increased nuclear FoxO1 by 3 hours (Figure 5B and 5C), demonstrating that VLDL inhibits AKT/FoxO1 signaling. As proof-of-concept, treatment of macrophages with AKT inhibitor IV mimicked VLDL treatment, resulting in reduced phospho-AKT, reduced phospho-FoxO1 (Figure VIIB in the online-only Data Supplement) and significant elevations in the expression of IL-1β as well as MIP-1α and ICAM-1 (Figure 5D).

**VLDL-Stimulated Activation of MAPK Signaling and Repression of AKT/FoxO1 Signaling Are Corrected by PPARδ Activation**

To determine the mechanism whereby PPARδ activation attenuates VLDL-induced cytokine expression, we examined the effect of PPARδ agonists on macrophage MAPK and AKT/FoxO1 signaling. GW0742 and GW501516 significantly attenuated both VLDL-stimulated ERK1/2 and p38 activation (Figure 6A and 6B). Inhibition of β-oxidation by etomoxir had no effect on the ability of PPARδ activation to normalize VLDL-induced
MAPK signaling (Figure VIII.A and VIII.B in the online-only Data Supplement). In control cells, GW0742 and GW501516 increased both phospho-AKT and phospho-FoxO1 (Figure IX.A in the online-only Data Supplement). Importantly, both PPARδ agonists completely reversed the VLDL-induced reductions in phospho-AKT and phospho-FoxO1, and prevented the increase in nuclear FoxO1 (Figure 6.C–6.E). Etomoxir treatment had no effect on phospho-AKT and phospho-FoxO1, and prevented the increase of PPARδ agonist-induced normalization of AKT/FoxO1 signaling (Figure VII.C and VII.D in the online-only Data Supplement). Collectively, these data demonstrate that PPARδ activation inhibits VLDL-induced inflammatory cytokine expression by inhibiting MAPK signaling and restoring signaling through AKT/FoxO1. Furthermore, the modulation of both signaling pathways by PPARδ activation was independent of PPARδ agonist-induced β-oxidation.

**Discussion**

Patients with insulin-resistant syndromes such as type 2 diabetes mellitus and metabolic syndrome have significant elevations of plasma VLDL, which confers increased risk for atherosclerosis.3,31 Type 2 diabetes mellitus, metabolic syndrome, and atherosclerosis are interwoven by the commonality of chronic low-grade inflammation.32,33 However, the molecular processes that link elevated plasma VLDL, atherosclerosis, and inflammation require further elucidation. In the present study, we demonstrate that human native VLDL induces both macrophage TG accumulation and expression of proinflammatory cytokines, in the absence of exogenous LPS. Furthermore, we demonstrate that PPARδ activation attenuates both of these pathogenic macrophage foam cell processes and define the mechanisms involved. Although VLDL-derived fatty acids are the stimulus for cytokine expression, the ability of PPARδ agonists to dampen the inflammatory response is independent of agonist-induced LPL inhibition, stimulation of β-oxidation, or reduction in cellular TG.

PPARδ is expressed in abundance in macrophages,3 but its biological role in lipid homeostasis has been controversial.35-39 In this study, we demonstrate that VLDL-induced TG...
accumulation is significantly decreased by PPARδ activation. Hydrolysis of VLDL-TG by macrophage LPL is required for cellular FA uptake and TG resynthesis.6 PPARδ agonists increased angiotopietin-like 4 expression, which was coupled to a reduction in LPL activity, thereby limiting liberated FAs for macrophage uptake. These findings are consistent with recent studies demonstrating that angiotopietin-like 4 is a PPARδ target gene, which is expressed in macrophages and irreversibly inactivates LPL by converting active LPL-dimers into inactive LPL-monomers.27,28 PPARδ activation increased expression of the FA transporter CD36, which resulted in a modest increase in FA uptake, albeit from a smaller FA pool. However, we confirm that PPARδ agonists upregulate CPT-1α expression,19 which enhances FA oxidation. Furthermore, we show for the first time that PPARδ activation results in a net depletion of VLDL-induced TG accumulation. These results are consistent with the concept that one role for PPARδ activation in macrophages is to prevent lipotoxicity by limiting VLDL hydrolysis and enhancing FA catabolism18,19 and contradict the notion that PPARδ activation promotes lipid accumulation.17 Furthermore, although VLDL-derived FAs activate PPARδ,18 potent synthetic agonists of PPARδ are required to attenuate VLDL-stimulated macrophage foam cell formation as well as inhibit the inflammatory response.

VLDL markedly stimulates expression of AP-1-inducible inflammatory cytokines, which occurs in the absence of NF-κB signaling or exogenous LPS. VLDL has been demonstrated to induce MIP-1α in murine macrophages via ERK1/2 activation.9 We extend this response to human THP-1 macrophages and to include the induction of IL-1β and ICAM-1. In THP-1 macrophages, both ERK1/2 and p38 are activated in response to VLDL, and p38 phosphorylation remains elevated well beyond that of ERK1/2. Inhibition of p38 attenuates ERK1/2 phosphorylation and enhanced cytokine expression. Moreover, the combination of VLDL treatment and p38 inhibition results in additive stimulation of cytokine expression over either treatment alone, suggesting that relieving the p38-induced impediment on ERK1/2 signaling enhances cytokine expression. Although in some cells, p38 activation has no effect or amplifies ERK1/2 signaling, we and others have reported that in hepatoma cells, insulin-induced phosphorylation of p38 also acts as a negative regulator of insulin-stimulated ERK1/2 activation.35,36 The present study supports this paradigm and demonstrates for the first time that VLDL-stimulation of

**Figure 3.** Peroxisome proliferator-activated receptor (PPAR) δ agonists attenuate very low-density lipoprotein (VLDL)-stimulated expression of activator protein (AP)-1 associated inflammatory cytokines. THP-1 cells were preincubated with PPARδ agonists for 24 hours, followed by a 16-hour incubation with or without VLDL. mRNA abundance for (A) interleukin (IL)-1β, (B) macrophage inflammatory protein (MIP)-1α, and (C) intercellular adhesion molecule (ICAM)-1 was measured (n=4). D, THP-1 cells were incubated with PPARδ agonists for 24 hours, followed by an incubation with etomoxir (ETO 50 µmol/L) for 0.5 hours and a subsequent incubation with or without VLDL for 16 hours. mRNA for IL-1β, MIP-1α, and ICAM-1 mRNA was measured (n=3). Data are presented as mean±SEM. Different letters indicate significant differences; ANOVA with post hoc Tukey test (P<0.05). DMSO indicates dimethyl sulfoxide.
macrophage cytokine expression through MAPKkinase involves p38 acting as a negative regulator of ERK1/2 signaling.

VLDL-treated macrophages displayed attenuated AKT and FoxO1 phosphorylation, which coincided with increased nuclear FoxO1 and increased cytokine expression. Although the mechanism by which VLDL attenuates AKT signaling leading to activation of FoxO1 has not been defined, it is known that FoxO1 is a direct transcriptional activator of IL-1β in mouse macrophages. Some studies have suggested that free fatty acids induce macrophage inflammation through activation of toll-like receptor signaling. However, more recent reports have provided contrary evidence. The present study is consistent with the concept that VLDL-stimulated macrophage inflammatory cytokine expression results from macrophage insulin resistance, rather than elicitation of a toll-like receptor-NFκB response. This is evidenced by the following: (1) impaired AKT/FoxO1 signaling and enhanced MAPK signaling by VLDL treatment, (2) canonical NFκB target genes TNFα and IL-6 being unaffected by VLDL treatment, and (3) the inability of parthenolide (an inhibitor of NFκB signaling) to block VLDL-stimulated expression of IL-1β, MIP-1α, and ICAM-1.
It is tempting to hypothesize that VLDL-induced inflammatory responses are initially derived from rapid ERK1/2 activation followed by later and sustained AKT signaling. The ERK1/2 signal is rapidly downregulated by activated p38, whereas the AKT signal is possibly mitigated by a self-limiting feedback loop. Furthermore, with time, it is possible that incoming FAs increase the saturated lipid content of the ER membrane, thereby inducing an endoplasmic reticulum-stress response, and subsequent amplification of nuclear FoxO1. The exact relationship among time-dependent signaling events governing VLDL-stimulated inflammatory responses requires further study.

VLDL-induced expression of inflammatory cytokines was completely normalized by both PPARδ agonists, despite a lack of effect on intracellular free fatty acid levels, and even under conditions of inhibited β-oxidation. This suggests that stimulation of β-oxidation by PPARδ activation is insufficient to explain the anti-inflammatory effects. Additionally, VLDL-stimulated phosphorylation of both ERK1/2 and p38 were normalized by PPARδ activation, suggesting regulation of a common upstream MAPK factor. This concept is consistent with a previous report that GW0742 inhibited angiotensin II–induced phosphorylation of ERK1/2 and p38 in mouse macrophages, via upregulation of RGS4 and RGS5. Whether this mechanism applies to the present study remains to be determined.

Macrophages exposed to VLDL in the presence of PPARδ agonists restored levels of phospho-AKT, phospho-FoxO1, and nuclear FoxO1 to those observed in untreated cells, an effect independent of PPARδ agonist-induced enhanced β-oxidation. Although modulation of AKT activity by PPARδ activation has been observed, the mechanisms underlying this phenomenon remain unclear. Di-Poi et al demonstrated that PPARδ ligand L-165041 induced expression of integrin-like kinase and 3-phosphoinositide-dependent kinase 1, which led to phosphorylation of AKT and FoxO1. In contrast, Han

**Figure 5.** Very low-density lipoprotein (VLDL) induces human macrophage inflammation via AKT/forkhead box protein O1 (FoxO1) signaling. THP-1 cells were incubated for indicated times with or without VLDL. Representative immunoblots of (A) phosphorylated-AKT and (B) phosphorylated-FoxO1 from cytosolic fractions. C, nFoxO1 from nuclear fractions. Equal loading was confirmed by total AKT, β-Actin, and Lamin A/C, respectively (n=4). The immunoblots for Lamin A/C in (B) and for β-Actin in (C) show no detectable bands demonstrating complete separation of cytosolic and nuclear fractions, respectively. D, THP-1 cells incubated for 16 hours with or without AKT activity inhibitor (AKTIV 10 µmol/L), mRNA for interleukin (IL)-1β, macrophage inflammatory protein (MIP)-1α, and intercellular adhesion molecule (ICAM)-1 was measured by quantitative real-time polymerase chain reaction (qRT-PCR). Data are presented as mean±SEM (n=4). * indicates significant difference vs control; t test (P<0.05). A-C, Representative bands are from the same immunoblot, cut from different regions. DMSO indicates dimethyl sulfoxide.

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>IL-1β</th>
<th>MIP-1α</th>
<th>ICAM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fold Change</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>AKTIV 10µmol/L</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* indicates significant difference vs control; t test (P<0.05).
et al\textsuperscript{45} reported that GW501516-treated endothelial progenitor cells displayed marked elevations in phospho-AKT, without increased integrin-like kinase expression. In our experiments, PPAR\(\delta\) agonists increased phospho-AKT and phospho-FoxO1, without effecting integrin-like kinase or 3-phosphoinositide-dependent kinase 1 expression (Figure IX in the online-only Data Supplement). This suggests that the anti-inflammatory effect of PPAR\(\delta\) activation is, in part, attributable to direct stimulation of AKT/FoxO1 phosphorylation, thereby preventing VLDL from dysregulating this signaling cascade.

In summary, VLDL-induced macrophage lipid accumulation and proinflammatory cytokine synthesis are attenuated by PPAR\(\delta\) activation, effects which involve ERK1/2- and AKT-dependent signaling mechanisms. These combined reductions of lipid accumulation and inflammatory cytokine expression by PPAR\(\delta\) ligands reveal a novel mechanism for preventing the deleterious consequences of macrophage foam cell formation.

Sources of Funding

This work was supported by grants from the Canadian Institutes of Health Research (FRN-3014 to M.W. Huff) and the Heart and Stroke Foundation of Canada (PRG-5967). L.A. Bojic held an Ontario Graduate Scholarship.

References

40. Borradaile NM, Han X, Harp JD, Gale SE, Ory DS, Schaffer JE. Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death. J Lipid Res. 2006;47:2726–2737.
Activation of Peroxisome Proliferator-Activated Receptor δ Inhibits Human Macrophage Foam Cell Formation and the Inflammatory Response Induced by Very Low-Density Lipoprotein

Lazar A. Bojic, Cynthia G. Sawyez, Dawn E. Telford, Jane Y. Edwards, Robert A. Hegele and Murray W. Huff

*Arterioscler Thromb Vasc Biol.* 2012;32:2919-2928; originally published online September 27, 2012;

doi: 10.1161/ATVBAHA.112.255208

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2012 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/32/12/2919

Data Supplement (unedited) at:

http://atvb.ahajournals.org/content/suppl/2012/09/27/ATVBAHA.112.255208.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:

http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:

http://atvb.ahajournals.org/subscriptions/
Supplemental Material

Detailed Methods

Cell Culture
The human THP-1 macrophage-like cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). For experiments, cells were cultured at 4.0x10^6 cells/35-mm plate (Falcon Scientific, BD Biosciences) in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma), β-mercaptoethanol (5x10^-5 mol/L), 100 units/mL penicillin, and 100 μg/ml streptomycin and differentiated with 300 nmol/L phorbol 12,13-dibutyrate (PDB, Sigma) for 1 week prior to use in experiments as described. HepG2 cells were obtained from ATCC and grown as described previously. For experiments, HepG2 cells were plated in either 100-mm or six-well (35-mm) culture plates (Falcon, Mississauga, ON) and maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), but switched to MEM containing 5% human lipoprotein-deficient serum (LPDS) for experimental incubations.

Luciferase Reporter Assays
Luciferase reporter assays were performed as previously described. Briefly, HepG2 cells were transfected with 0.01µg/mL human PPARα,γ or δ.SG5 expression vectors and reporter gene plasmids, 0.5µg/mL of pTK-PPRE(x3)-Luc and 0.05µg/mL of the TK promoter-Renilla luciferase construct, (tk.pRL) (provided by Dr. John Capone, McMaster University, Hamilton ON). Cells were incubated for 24 hr with DMSO or the appropriate PPAR agonists: PPARα (10nmol/L, GW7647, Sigma); PPARγ (3µmol/L, rosiglitazone, Alexis Biochemicals, Cedarlane Laboratories, Burlington, ON), or PPARδ (GW0742 and GW501516) at a range of concentrations. Cell lysates were prepared and the Luciferase activity (relative light units (RLU)) was measured and normalized to Renilla activity, as previously described.

Cellular Lipid Mass
THP-1 macrophages were preincubated in the presence of PPARδ agonists or equal volume of DMSO (not to exceed 0.5% of total medium) for 24 hr in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 300nmol/L PDB. Cells were incubated for a further 16 hr with fresh media containing 5% LPDS and compounds in the absence or presence of HTG-VLDL (50 μg of lipoprotein total cholesterol (TC)/mL medium). Cellular CE, TC, FC, TG, FFA (NEFA) and protein mass were determined using enzymatic colorimetric assays for NEFA, TC and FC (Wako Diagnostics, Richmond, VA) as well as TG (Boehringer Mannheim, Laval, QC) as previously described. Cellular CE was determined as the difference between TC and FC.

Enzyme-linked immunosorbent assay
THP-1 macrophages were preincubated in the presence of PPARδ agonists or equal volume of DMSO (not to exceed 0.5% of total medium) for 24 hr in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 300nmol/L PDB. Cells were incubated for a further 16 hr with fresh media containing 5% LPDS and compounds in the absence or presence of HTG-VLDL (50 μg of lipoprotein total cholesterol (TC)/mL medium). Media was collected and analyzed for IL-1β secretion via enzyme-linked immunosorbent assay (ELISA) using the BD OptEIA human IL-1β ELISA kit II (BD Biosciences, Mississauga, ON) as per manufacturer’s protocol.

Quantitative Real-Time PCR Gene Abundance Analysis
THP-1 cells were incubated for 24 hr in RPMI 1640 with 5% LPDS, 300nmol/L PDB, in the presence or absence of PPARδ agonists, and subsequently total RNA was isolated using TriZol reagent (Invitrogen, Burlington, ON) according to manufacturer’s instructions. In experiments examining VLDL-induced inflammatory cytokine expression, cells were preincubated for 24 hr in RPMI 1640 with 10% FBS, 300nmol/L PDB, in the presence or absence of PPARδ agonists.
Cells were then incubated with fresh 5%-LPDS media and compounds with or without the CPT-1α inhibitor etomoxir (Sigma, 50μmol/L for 0.5 hr, followed by a further 16 hr in the absence or presence of HTG-VLDL (50μg-TC/mL) prior to TriZol RNA extraction. Abundance of total RNA (2μg) was reverse transcribed using the Applied Biosystems High Capacity cDNA reverse transcription kit according to the manufacturer’s protocol. Subsequently, cDNA (10ng) was analyzed in triplicate by quantitative real time RT-PCR (qRT-PCR) on an ABI Prism (model 7900HT) Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions and as previously described.² Primer-probe sets for each gene were obtained from Applied Biosystems (Carlsbad, CA). Abundance of target genes was normalized to GAPDH abundance.

**LPL Activity, TG synthesis, FA β-oxidation, and FA uptake.**

LPL activity of THP-1 cells (cell surface and medium) was determined following a 24 hr incubation in RPMI 1640 with 5% human LPDS and 300nmol/L PDB in the presence or absence of selected concentrations of PPARδ agonists, as the release of free fatty acids from intralipid (an exogenous lipid source) as previously described.⁷ The synthesis of triacylglycerol was measured in THP-1 cells following a preincubation in 5% LPDS-containing medium for 19 hr in the presence or absence of selected concentrations of PPARδ agonists. For a subsequent 5 hr incubation, 0.08nCi/mL [1⁴C]oleic acid (Amersham Biosciences) complexed with fatty acid-free bovine serum albumin in a molar ratio of 5.36:1 (Sigma) was added as described previously.⁸ Cellular lipid extracts were separated by thin layer chromatography.⁸ Fatty acid β-oxidation experiments were performed as described previously.⁴ Briefly, THP-1 cells were preincubated in the presence or absence of PPARδ agonists in 10% FBS-containing medium for 24 hr, followed by the addition of 2.0μCi/mL [3H]-palmitate in 100μmol/L palmitate per well for 0.5 hr. The media was removed and 10% trichloroacetic acid was added. Unreacted FAs were extracted from the supernatant with n-hexane and the remaining counts determined by scintillation counting. Data was determined as nmol palmitate oxidized/min/mg cell protein and was corrected for differences in fatty acid uptake in the presence of PPARδ agonists. Fatty acid uptake experiments were performed as described.⁸ Briefly, THP-1 cells were preincubated in the presence or absence of PPARδ agonists in 5% LPDS-containing medium for 24 hr, followed by the addition of either [1⁴C]oleic acid or [3H]-palmitate (both prepared as described above) for 1min. Ice cold stop solution (200μmol/L phloretin in phosphate buffered saline) was added directly to the culture medium. Cells were washed five times with ice cold stop solution. Cells were lysed in 1mL of 0.1N NaOH, and aliquots were used to determine protein concentrations and the amount of unprocessed radiolabelled fatty acid.

**Immunoblot Analysis and Densitometry**

Total cell lysates were fractionated into cytosolic and nuclear fractions as previously described.¹⁰ Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and immunoblotted as described previously.⁶ Cellular cytosolic fractions were probed using antibodies against human phospo (p)-FoxO1, pERK1/2, p-p38, pAKT, FoxO1, ERK1/2, p38, AKT, β-actin (Cell Signaling, Danvers, MA) and nuclear fractions were probed using antibodies against human total-FoxO1 and Lamin A/C (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Quantification analysis of the developed films was performed using an imaging densitometer (Bio-Rad Quantity One Software). Phospho-proteins from cytosolic fractions were normalized to their respective total proteins or β-Actin, whereas nuclear fractions were normalized to lamin A/C. Additionally, cytosolic and nuclear fractions were immunoblotted for Lamin A/C and β-Actin, respectively, in order to demonstrate complete separation of these fractions.
Statistical Analyses
Data are expressed as means +/- standard error of the mean (SEM). The Shapiro-Wilk normality test was used to test for parametric distributions in each data set. \( P \) values for observed differences between treatment and control groups were calculated by one-way ANOVA followed by Bonferroni post hoc test or paired student’s \( t \)-test where indicated. \( P \) values for observed pair-wise comparisons were calculated by one-way ANOVA followed by Tukey’s post hoc test. Significance thresholds were \( P \) values less than 0.05. Statistical analyses were performed with SigmaPlot 11.0 software (Systat, Inc, San Jose, CA).
References


Supplemental Figure I. GW0742 and GW501516 are PPARδ-specific agonists.

Human hepatoma (HepG2) cells were co-transfected with plasmids encoding a PPRE-luciferase construct, a Renilla luciferase construct (transfection control) and vectors encoding each individual PPAR isoform as indicated. Cells were incubated with (A) PPARα agonist GW7647, (B) PPARγ agonist rosiglitazone or PPARδ agonists (C) GW0742 or (D) GW501516 for 24hr. Cell lysates were isolated and luciferase relative light units (RLU) were measured (n=2-4). In separate experiments, THP-1 cells were incubated with PPAR agonists for 24hr. Total RNA was isolated and (E) ACOX, (F) FABP4 and (G) LPL mRNA abundance was measured by qRT-PCR (n=3-6). Data is presented as mean +/- SEM. * indicates significant difference versus DMSO control; ANOVA with post hoc Bonferroni test (P<0.05)
Supplemental Figure II. Effect of PPARδ activation on FFA re-esterification mechanisms.

THP-1 cells were incubated with PPARδ-specific agonists for 24hr. Total RNA was isolated and (A) *DGAT1* and (B) *DGAT2* mRNA was measured by qRT-PCR (n=4). C, Following a 19hr incubation with PPARδ agonists at indicated concentrations, THP-1 cells were incubated for a further 5hr with PPARδ agonists and 1-[14C]oleic acid to measure oleate incorporation into cellular triacylglycerol (n=3). Data is presented as mean +/- SEM.
Supplemental Figure III. PPARδ activation does not affect basal cytokine expression in THP-1 human macrophages
THP-1 cells were incubated with PPARδ-specific agonists at the indicated concentrations for 24hr. Total RNA was isolated and IL-1β, MIP-1α and ICAM-1 mRNA was measured by qRT-PCR (n=4). Data is presented as mean +/- SEM (n=4).
Supplemental Figure IV

Supplemental Figure IV. The effect of PPARδ agonists on IL-1β; the effect of VLDL on TNFα or IL-6 expression and the VLDL-induced inflammatory response in the presence of an inhibitor of NFκB. THP-1 cells were pre-incubated with PPARδ agonists for 24hr, followed by a 16hr incubation with or without VLDL (50μg-TC/mL). A, Media was collected and analyzed for IL-1β protein levels by enzyme-linked immunosorbent assay. In separate experiments, THP-1 cells were incubated with or without VLDL (50μg-TC/mL) for 16hr. Total RNA was isolated and (B) TNFα and IL-6 mRNA was measured by qRT-PCR (n=4). C, THP-1 cells were incubated with iκB kinase inhibitor (Parthenolide 10μmol/L) for 0.5hr followed by 16hr incubation with or without VLDL or LPS at the indicated concentrations. Total RNA was isolated and IL-1β, MIP-1α and ICAM-1 mRNA was measured by qRT-PCR (n=3-4). Data is presented as mean +/- SEM. Different letters indicate significant differences; ANOVA with post-hoc Tukey’s test (P<0.05).
Supplemental Figure V. Prevention of VLDL-induced TG accumulation and cytokine expression are independent effects of PPARδ activation.

THP-1 cells were incubated with lipolysis inhibitor (THL) at the indicated concentrations for 0.5hr followed by 16hr incubation with or without VLDL (50μg-TC/mL). A, TG mass (n=2). B, TG mass (n=4). THP-1 cells were incubated with THL at the indicated concentrations for 0.5hr followed by 16hr incubation with or without VLDL (50μg-TC/mL) or LPS (0.1ng/mL). Total RNA was isolated and (C) IL-1β, MIP-1α and ICAM-1 mRNA was measured by qRT-PCR (n=3). Data is presented as mean +/- SEM. Different letters indicate significant differences; ANOVA with post-hoc Tukey’s test (P<0.05).
Supplemental Figure VI. Free fatty acids are the primary effectors of the VLDL-induced inflammatory response in human macrophages. THP-1 cells were pre-incubated with PPARδ agonist GW501516 for 24hr, followed by a 16hr incubation with or without oleic acid (OA, 200μmol/L). A, TG accumulation (n=4). B, Total RNA was isolated and IL-1β and MIP-1α mRNA was measured by qRT-PCR (n=4). C, In a separate set of experiments, THP-1 cells were pre-incubated with the PPARδ agonist at the indicated concentration for 24hr, followed by a 0.5hr incubation with or without 200μmol/L OA. Cytosolic fractions were isolated and immunoblotted for ERK1/2. Equal loading was confirmed by total ERK. Representative immunoblots for phosphorylated and total ERK1/2 with quantitation shown (n=4). Data is presented as mean +/- SEM. Different letters indicate significant differences; ANOVA with post-hoc Tukey’s test (P<0.05).
Supplemental Figure VII. Effects of inhibitors on cell signalling cascades. THP-1 cells were incubated with or without p38 inhibitor SB203580 (10μmol/L) for 0.5hr. Cytosolic fractions were isolated and immunoblotted for (A) pERK1/2 and p-p38. Equal loading was confirmed by total ERK and total p38, respectively (n=3). In a separate experiment, THP-1 cells were incubated for indicated times with or without AKT inhibitor (AKTi) IV (10μmol/L). Levels of (B) pAKT and pFoxO1 were determined by immunoblotting of cytosolic fractions. Quantitation was determined relative to total AKT and β-Actin, respectively (n=3). Relative intensities represent the mean ratio of phospho:total protein relative to the respective DMSO control of 3 independent experiments.
Supplemental Figure VIII. Inhibition of β-oxidation had no effect on the ability of PPARδ activation to correct VLDL-induced inflammatory signalling. THP-1 cells were pre-incubated with PPARδ agonists for 24hr, followed by a 0.5hr incubation with the CPT-1α inhibitor etomoxir (ETO). For a subsequent 0.5hr, cells were incubated with or without VLDL. Representative immunoblots of phosphorylated (A) pERK1/2 and (B) p-p38 from cytosolic fractions (n=3). C,D, THP-1 cells were pre-incubated with PPARδ agonists for 24hr, followed by a 0.5hr incubation with ETO. For a subsequent 6hr, cells were incubated with or without VLDL. Representative immunoblots of phosphorylated (C) pAKT and (D) pFoxO1 from cytosolic fractions (n=3). Data is presented as mean +/- SEM. Different letters indicate significant differences; ANOVA with post-hoc Tukey’s test (P<0.05).
Supplemental Figure IX. Effects of PPARδ agonists on phosphorylation of AKT/FoxO1, and expression of ILK and PDK1.

THP-1 cells were incubated for 6hr with or without PPARδ agonists. Levels of (A) pAKT and pFoxO1 were determined by immunoblotting cytosolic fractions. Equal loading was confirmed by total AKT and β-Actin, respectively (n=2). Relative intensities represent ratio of phospho:total protein relative to the respective DMSO control of the representative immunoblot. In separate experiments, THP-1 cells were incubated with PPARδ-specific agonists at the indicated concentrations for 24hr. Total RNA was isolated and (B) ILK and PDK1 mRNA was measured by qRT-PCR (n=4). Data is presented as mean +/- SEM. A, Representative bands are from the same immunoblot, cut from different regions due to loading of multiple replicate lanes.