Differential Effects of Peroxisome Proliferator-Activated Receptor Activators on the mRNA Levels of Genes Involved in Lipid Metabolism in Primary Human Monocyte-Derived Macrophages

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Peroxisome proliferator-activated receptors (PPARs) are key regulators of macrophage lipid metabolism. We compared the effects of three PPAR activators (bezafibrate, fenofibrate, and troglitazone) on the mRNA levels of genes involved in lipid metabolism in primary human macrophages and macrophage-derived foam cells. Treatment of human macrophages for 24 hours with 100 μmol/L bezafibrate, a nonselective drug that activates the 3 PPAR subtypes (PPARα, PPARβ/δ, and PPARγ), caused an 87% ($P < .01$) and a 230% rise in CD36 and adipocyte fatty acid–binding protein (aP2) mRNA levels, respectively, whereas the expressions of PPARγ, PPARα, acyl-CoA oxidase, carnitine palmitoyltransferase I (CPT-I), adenosine triphosphate (ATP)-binding cassette transporter 1 (ABCA1), neutral cholesteryl ester hydrolase, and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) were not modified. However, treatment with selective PPARα (fenofibrate at 100 μmol/L) and PPARγ (troglitazone at 5 μmol/L) activators had different effects. Fenofibrate increased PPARα (62%, $P < .05$) and LOX-1 (180%, $P < .05$) mRNA levels; and troglitazone upregulated CPT-I expression (75%, $P < .05$). When the effects of these drugs were assessed in macrophage-derived foam cells, we found that troglitazone caused a 134% ($P < .05$) and a 66% ($P < .01$) rise in ABCA1 and CPT-I mRNA levels, respectively, whereas the 3 drugs significantly increased aP2 transcripts (about 100% induction). Given that troglitazone treatment resulted in the upregulation of genes involved in the mitochondrial β-oxidation of fatty acids (CPT-I) and in the reverse-cholesterol-transport pathway (ABCA1), we subsequently determined whether these changes affected intracellular cholesterol ester accumulation. In macrophage-derived foam cells a significant reduction (32%, $P < .01$) was observed in intracellular cholesterol accumulation after troglitazone, but not after bezafibrate or fenofibrate treatment. Since CPT-I inhibition promotes cholesterol incorporation into cholesteryl esters in macrophages, study is now needed on whether CPT-I induction by troglitazone may reduce the availability of fatty acids for synthesizing cholesterol esters, leading to less foam cell formation.

MACROPHAGE-DERIVED foam cell formation is a hallmark of the atherosclerotic process.[1] Foam cell formation occurs as a result of unregulated uptake of modified lipoproteins by scavenger-receptors and leads to the deposition of cholesterol esters in the cytoplasm. Stored cholesterol esters are in a dynamic equilibrium with free cholesterol, undergoing continuous hydrolysis and re-esterification. Free cholesterol moves to the plasma membrane and is then transferred by ABCA1 to a cholesterol acceptor (eg, apolipoprotein [apo]AI), resulting in net cellular cholesterol efflux. In addition, macrophages themselves may facilitate cholesterol efflux by secreting apoE that may act as an acceptor for free cholesterol in human macrophages.[2]

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate lipid and glucose metabolism[3] and are involved in the development of atherosclerosis in macrophages. PPARα (NR1C1) is expressed in cells of the arterial wall (such as monocyte-derived macrophages, smooth muscle cells, and endothelial cells), liver, heart, muscle, and kidney, and is activated by fibrates (fenofibrate, bezafibrate, gemfibrozil), fatty acids, and eicosanoids. PPARδ/β (NR1C2) is ubiquitously expressed in many tissues and there is no class of drugs able to act as selective ligands for this receptor. PPARγ (NR1C3), in addition to being expressed in adipose tissue, is present in monocyte-derived macrophages and is activated by thiazolidinediones (troglitazone, rosiglitazone, and pioglitazone) and fatty acids. PPARα and PPARγ activation in macrophages results in transcriptional regulation of genes involved in macrophage lipid metabolism and may influence development and evolution of atherosclerosis. Thus, PPARγ activation results in the transcriptional induction of CD36 expression in macrophages,[4] although this effect does not lead to intracellular cholesterol accumulation.[5] Moreover, PPARα and PPARγ activation induce the expression of the gene encoding adenosine triphosphate (ATP)-binding cassette transporter 1 (ABCA1), which is involved in the reverse-cholesterol-transport pathway and in the control of the plasma levels of high-density lipoprotein (HDL), a major protective factor against atherosclerosis.[5] Regulation of ABCA1 by PPARs is consistent with the protective effects against atherosclerosis afforded by both PPARα (fibrates) and PPARγ (thiazolidinediones) activators.[6,7] In contrast, the role of PPARβ in foam cell formation is controversial, since there are studies showing that agonists of this receptor may promote[8] or reduce[9] lipid accumulation in macrophages.

To further characterize the role of PPARs in lipid metabolism in macrophages, we compared the effects of 3 PPAR ligands (bezafibrate, fenofibrate, and troglitazone) in primary human macrophages and macrophage-derived foam cells on the

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mRNA levels of several genes involved in fatty acid and cholesterol metabolism, the 2 components of cholesterol esters. We focused on the effects of these drugs on the mRNA levels of genes involved in mitochondrial (carnitine palmitoyltransferase [CPT-I]) and peroxisomal (acyl-CoA oxidase [ACO]) fatty acid β-oxidation and on those involved in cholesterol homeostasis (neutral cholesteryl ester hydrolase [nCEH] and ABCA1). We report that PPAR activators differ in their modifications of the expression of the genes studied. Troglitazone was the only one of the drugs that significantly increased the expression of CPT-I and ABCA1 and reduced intracellular ester accumulation in foam cells in the absence of a cholesterol acceptor in the culture media. Increased expression of CPT-I after troglitazone treatment may contribute to the reduction of cholesterol ester levels, since it may reduce the availability of fatty acids for cholesterol esterification. The reduction in cholesteryl ester formation may then facilitate the efflux of free cholesterol by an apoE-mediated mechanism.

MATERIALS AND METHODS

Cell Culture

Human monocytes were isolated from buffy coats obtained from the peripheral blood of healthy human donors (Hospital Vall d'Hebron, Barcelona, Spain). A 15-mL quantity of buffy coat was diluted in 20 mL of phosphate-buffered saline (PBS; without Ca^{2+}, Mg^{2+} and bicarbonate, and with 0.2 μmol/L EDTA), and layered over 12 mL of Ficoll-Hypaque (Amersham Pharmacia, Uppsala, Sweden). After centrifugation (1,600 × *g* for 20 minutes at room temperature), the mixed mononuclear cell band was removed by aspiration and washed 3 times in PBS. The mononuclear cells were suspended in medium A (RPMI 1640 with L-glutamine and 25 mmol/L Hepes, supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 1% glutamine, 1% nonessential amino acids and 2% sodium pyruvate; Life Technologies, Paisley, Scotland). Cells (5.5×10^6) cells were then plated in 60-mm dishes and allowed to adhere for 2 hours at 37°C. Nonadherent cells were removed by washing with PBS (with Ca^{2+} and Mg^{2+}, without bicarbonate) and medium A with 20% (vol/vol) heat-inactivated human AB serum (Sigma, Madrid, Spain) was added. After 5 days, the proportion of human serum was reduced to 10%. On day 10, completely differentiated macrophages were lipid-loaded during a 48-hour incubation with 150 μg/mL acetyl–low-density lipoprotein (LDL) in medium A containing 1% (vol/vol) human AB serum.

Macrophages and lipid-loaded macrophages were treated with either 100 μmol/L bezafibrate, 100 μmol/L fenofibrate, or 5 μmol/L troglitazone for 24 hours. After incubation, RNA was extracted as described below.

Lipoproteins

LDLs were obtained by ultracentrifugation from human serum, as described elsewhere,[10] and acetylated according to the method of Basu et al.[11] In brief, equal volumes of LDL suspension and a saturated solution of sodium acetate were mixed by continuous stirring in an ice-water bath. Acetic anhydride (total mass equal to 1.5-times the mass of protein of the LDL suspension) was added in small aliquots over a 1-hour period. The mixture was stirred for another 30 minutes, without further addition, and dialyzed for 48 hours against 200 vol of PBS, with changes of buffer every 12 hours. Acetyl-LDL were concentrated using Centriplus devices (Millipore, Madrid, Spain), and final protein concentration was assessed by the Bradford method. Adequate acetylation of LDL was confirmed by an at least 2.5-fold increase from the native LDL level in relative electrophoretic mobility, on a 1% agarose gel in Tris-barbitone buffer at 90 V for 1 hour.

RNA Preparation and Analysis

Total RNA was isolated using the Ultraspec reagent (Biotecx, Houston, TX). Relative levels of specific mRNAs were assessed by the reverse-transcription polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from RNA samples by mixing 0.5 μg of total RNA, 125 ng of random hexamers as primers in the presence of 50 mmol/L Tris-HCl buffer (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies), 20 U RNAsin (Life Technologies) and 0.5 mmol/L of each dNTP (Sigma) in a total volume of 20 μL. Samples were incubated at 37°C for 60 minutes. A 5 μL aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 25-μL PCR reaction contained 5 μL of the RT reaction, 1.2 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 1.25 μCi [^{32}P]-dATP (3,000 Ci/mmol; Amersham), 1 U of Taq polymerase (Ecogen, Barcelona, Spain), 0.5 μg of each primer, and 20 mmol/L Tris-HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at 60°C). The sequences of the sense and antisense primers used for amplification were: PPARγ, 5′-CATTCTGGCCCACCAACTTTGG-3′ and 5′-TGGAGATGCAGGCTCCACTTTG-3′; PPARα, 5′-GGAAAGCCCACTCTGCCCCCT-3′ and 5′-AGTCACCGAGGAGGGGCTCGA-3′, ACO, 5′-GCCCAGGTGAAGCCTGATGGA-3′ and 5′-GACTGGTGCCTCACAGCGCTG-3′; CPT-I, 5′-CAGGCCGTGGCCTTCCAGTTC-3′ and 5′-CCATGCTGAGAAGTGCCCGGG; adipocyte fatty acid–binding protein (aP2), 5′-TCCAGTGAAAACTTTGATGATTAT-3′ and 5′-ACGCATTCCACCACCAGTTTATCA-3′; CD36, 5′-CTGTGACCGGAACTGTGGGCT-3′ and 5′-GAAGATGGCACCATTGGGCTG-3′; LOX-1, 5′-ACTCTCCATGGTGGTGCCTGG-3′ and 5′-CATTCAGCTTCCGAGCAAGGG-3′; nCEH, 5′-CACAGCCTGTGGCCATTTTCC-3′ and 5′-CCTCCGTGGATCCACACCATC-3′; ABCA1, 5′-GGAGGCAATGGCACTGAGGAA-3′ and 5′-CCTGCCTTGTGGCTGCAGTGT-3′; and β-actin, 5′-TTGTAACCAACTGGGACGATATGG-3′ and 5′-GATCTTGATCTTCATGGTGCTAGG-3'. PCR was performed in an MJ Research Thermocycler equipped with a Peltier system (Waltham, MA) and temperature probe. After initial denaturation for 1 minute at 94°C, PCR was performed for 18 (CD36 in macrophages), 21 (ABCA1 in foam cells), 23 (LOX-1, nCEH, and ABCA1 in macrophages and CD36 in foam cells), 25 (PPARγ), and 27 (CPT-I and PPARα) cycles. Each cycle consisted of denaturation at 92°C for 1 minute, primer annealing at 60°C, and primer extension at 72°C for 1 minute, 50 seconds. A final 5-minute extension step at 72°C was performed. Five microliters of each PCR sample was electrophoresed on a 1-mm thick 5% polyacrylamide gel. The gels were dried and subjected to autoradiography using Kodak X-ray films (Eastman Kodak, Barcelona, Spain) to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (PPARγ, 229 bp; PPARα, 235 bp; ACO, 161 bp; CPT-I, 295 bp; aP2, 319 bp; CD36, 361 bp; LOX-1, 251 bp; nCEH, 295 bp; ABCA1, 181 bp; β-actin, 764 bp). Preliminary experiments were performed with various amounts of cDNA to determine nonsaturating conditions of PCR amplification for all the genes studied. In these conditions, relative quantification of mRNA was assessed by the RT-PCR method.[12] Radioactive bands were quantified by video-densitometric scanning (Vilber Lourmat Imaging, Marne-de-Vallee, France). The results given for the expression of specific mRNAs were always relative to the expression of the control gene (*β-actin*).

Preparation of Macrophage Cell Extracts

Cells were washed 3 times, collected in PBS to a final volume of 1 mL, and sonicated with a Branson Sonifier (Danbury, CT; 3 × 10 seconds at 25 W). A 0.2-mL quantity of the lysate was used for protein

determination, and the rest was divided into 2 aliquots. A standard solution of cholestanol was added, and lipids were extracted with methanol/chloroform 1:2 (vol/vol).

Analysis of Cellular Cholesterol

The extract from one of the aliquots was saponified, following Klansek et al,[13] and used to determine total cholesterol, while the nonsaponified extract was used for free cholesterol quantification. Both extracts were dried under N_2 and redissolved in 25 μL of anhydrous pyridine (Merck, Barcelona, Spain). Sylon BTZ (Sulpeco, Bellefonte, PA), 25 μL, was added, and the mixture was kept at room temperature for 20 minutes to complete the silanization reaction.[14] Samples were injected in a Hewlett-Packard (Palo Alto, CA) 5890 gas chromatograph equipped with a flame-ionization detector and a fused-silica capillary column (25 m $\times$ 0.25 mm i.d.) with stationary phase of dimethylpolysiloxane (CP-Sil 5 CB, Chrompack, Middelburg, The Netherlands). From each sample, the cholesterol/cholestanol area ratio was determined, and the amount of cholesterol was quantified from a calibration curve constructed with samples with different ratios. The results were then expressed in micrograms of cholesterol per milligram of cellular protein, and cholesteryl ester content was calculated from the difference between total and free cholesterol.

Statistical Analyses

Results are expressed as means $\pm$ SD of 3 experiments. Significant differences were established by analysis of variance (ANOVA). When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. All statistical analyses were performed using the computer program GraphPad Instat (GraphPad Software, San Diego, CA).

RESULTS

The main goal of this study was to find the effects of three different PPAR activators (bezafibrate, fenofibrate, and troglitazone) on the mRNA levels of genes involved in fatty acid and cholesterol metabolism in primary human monocyte-derived macrophages and macrophage-derived foam cells. These drugs activate different PPAR subtypes. Thus, at the concentrations used, bezafibrate activates the 3 PPAR subtypes with comparable EC_{50} values,[15] whereas fenofibrate and troglitazone selectively activate PPARα and PPARγ, respectively. Figure 1 shows the effects of a treatment with these drugs for 24 hours on the mRNA levels of the genes analyzed in primary human monocyte-derived macrophages. We first examined the effects of these compounds on PPARγ and PPARα mRNA levels. No changes were observed after treatment with these drugs, except for fenofibrate, which significantly increased PPARα mRNA levels by 62% ($P < .05$) (Fig 1B). We subsequently studied the effects of bezafibrate, fenofibrate, and troglitazone on the expression of several well-known PPAR target genes involved in fatty acid metabolism, ACO, CPT-I, and aP2.[3] Treatment did not significantly affect the transcript levels of ACO, which catalyzes the rate-limiting step of peroxisomal β-oxidation of fatty acids (Fig 1C). In contrast, the expression of CPT-I, the gene that catalyzes the entry of long-chain fatty acids into the mitochondrial matrix[16] and is regulated by PPARα and PPARγ,[3] significantly increased (by 75%, $P < .05$) after troglitazone treatment (Fig 1D). Bezafibrate caused a 230% induction in aP2 mRNA levels, although differences did not become significant (Fig 1E). Moreover, we examined the effects of these drugs on the expression of two receptors for oxidized

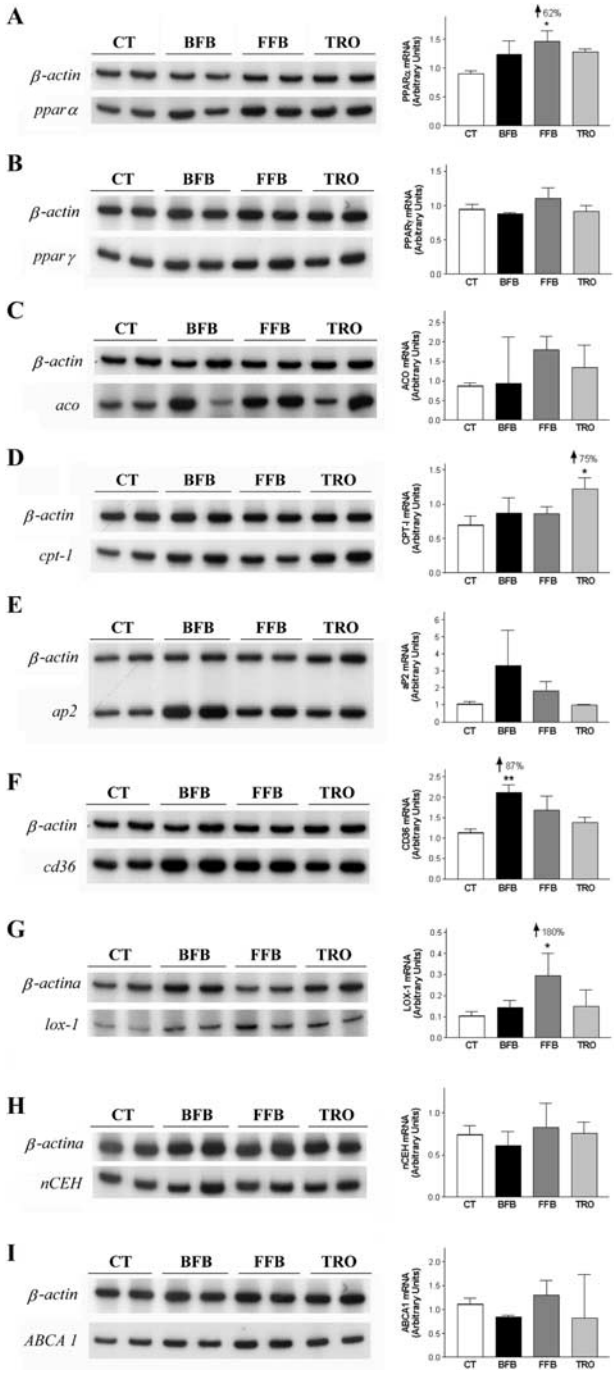

Fig 1. Effects of bezafibrate, fenofibrate and troglitazone on the expression of PPARγ (A), PPARα (B), ACO (C), CPT-I (D), aP2 (E), CD36 (F), LOX-1 (G), nCEH (H), and ABCA1 (I) mRNA levels in primary human monocyte-derived macrophages. Cells were incubated for 24 hours with 100 μmol/L bezafibrate, 100 μmol/L fenofibrate, or 5 μmol/L troglitazone. A 0.5-μg quantity of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification of the β-actin–normalized mRNA levels are shown. Data are expressed as the mean $\pm$ SD of 3experiments. *$P < .05$, **$P < .01$.

LDL, CD36 and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). Bezafibrate significantly increased CD36 (by 87%, $P < .01$), whereas surprisingly fenofibrate upregu-

lated LOX-1 mRNA levels (by 180%, $P < .05$) (Fig 1F and G). Finally, we assessed the effects of PPAR activators on the expression of 2 genes, nCEH, involved in the hydrolysis of stored cholesterol esters, and ABCA1, involved in cholesterol efflux. No changes were observed in mRNA levels of either gene after drug treatment of macrophages.

We next studied whether treatment with PPAR activators affected the transcript levels of the genes studied in foam cells. Human macrophage-derived foam cells were obtained by incubation of macrophages with 150 μg/mL acetylated-LDL for 48 hours. Oil Red O staining and determination of intracellular cholesterol ester levels were performed in order to make sure there was lipid-loading in foam cells (data not shown). As expected, foam cells, unlike macrophages, showed the presence of characteristic cholesteryl ester droplets in their cytoplasm. Like macrophages, foam cells were treated with PPAR activators for 24 hours. PPARα and PPARγ mRNA levels were not modified by the treatment (Fig 2A and B). The 3 drugs studied caused a slight increase in ACO mRNA levels, but differences were not significant (Fig 2C). When we assessed the effects of PPAR activators on CPT-I transcripts we observed that troglitazone significantly increased CPT-I by 66% ($P < .01$) (Fig 2D). In these foam cells, bezafibrate, fenofibrate, and troglitazone increased the mRNA levels of aP2 by 112% ($P < .01$), by 100% ($P < .05$), and by 130% ($P < .05$), respectively (Fig 2E). No changes were observed in CD36 mRNA levels after drug treatment (Fig 2F), whereas ABCA1 mRNA levels increased (by 134%, $P < .05$) after troglitazone treatment (Fig 2G).

Finally, we determined whether treatment with PPAR activators for 24 hours affected intracellular cholesteryl ester accumulation in foam cells in the absence of a cholesterol acceptor in the culture media (Fig 3). Bezafibrate and fenofibrate did not affect the levels of esterified cholesterol. However, a 32% reduction ($P < .05$) in intracellular esterified cholesterol was observed after troglitazone treatment. The levels of free cholesterol fell slightly (by 13.5%, $P < .05$) with fenofibrate treatment.

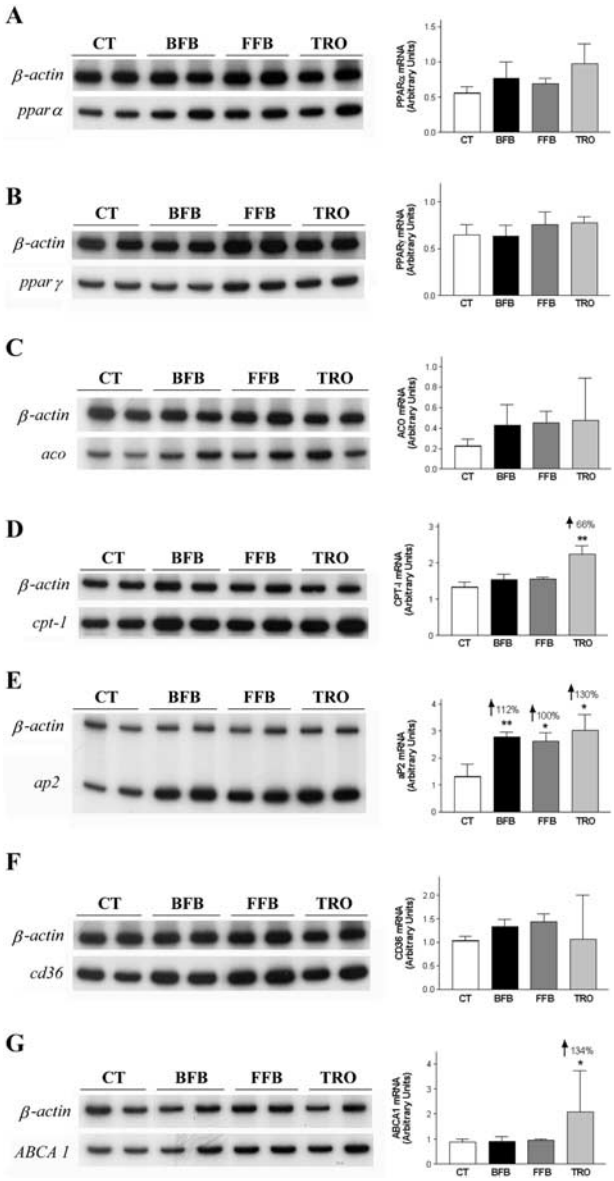

Fig 2. Effects of bezafibrate, fenofibrate, and troglitazone on the expression of PPARγ (A), PPARα (B), ACO (C), CPT-I (D), aP2 (E), CD36 (F), and ABCA1 (G) mRNA levels in primary human macrophage-derived foam cells. Cells were incubated for 24 hours with 100 μmol/L bezafibrate, 100 μmol/L fenofibrate, or 5 μmol/L troglitazone. A 0.5-μg quantity of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification of the β-actin–normalized mRNA levels are shown. Data are expressed as the mean ± SD of 3 experiments. *$P < .05$, **$P < .01$.

DISCUSSION

PPARs are lipid-activated transcription factors that regulate genes involved in lipid and glucose metabolism, cellular differentiation, and inflammation control.[3] The expression of these transcription factors in the arterial wall,[3] in addition to the beneficial effects of PPAR activators (fibrates and thiazolidinediones) in atherosclerosis,[6,7] has prompted researchers to study the role of PPARs in this disease. It has been recently reported that PPARα and PPARγ activators increase apoAI-induced cholesterol efflux from human macrophages.[5] The cholesterol removal from human macrophages after treatment with PPAR activators was mediated by the induction of the expression of the gene encoding ABCA1, a transporter that controls apoAI-mediated cholesterol efflux from macrophages. In this study by Chinetti et al,[5] macrophages were treated with selective PPARα (Wy-14,463) and PPARγ (rosiglitazone) activators for 3 days. In the present research, we treated macrophages for 24 hours with 3 different PPAR activators—bezafibrate, fenofibrate, and troglitazone. In contrast to the study by Chinetti et al, treatment of macrophages for 24 hours with troglitazone did not modify ABCA1 mRNA levels. This lack of effect after 24 hours of treatment suggests that longer expositions are required to observe a PPAR-mediated expression of this gene. The need for a longer exposition of macrophages to PPARγ activators to observe changes in ABCA1 expression confirms that ABCA1 upregulation by PPARγ is mediated indirectly through the oxysterol receptor LXRα.[5] Unlike ABCA1, CPT-I mRNA levels increased in macrophages after troglitazone treatment. CPT-I, which is located on the mito-

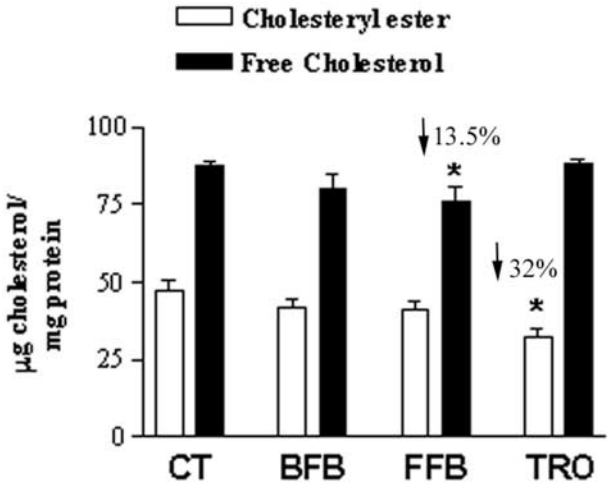

Fig 3. Effects of bezafibrate, fenofibrate, and troglitazone on the intracellular levels of cholesterol in human macrophage-derived foam cells. Monocyte-derived macrophages were converted in foam cells by incubation for 48 hours with 150 μg/mL acetylated-LDL and subsequently treated for 24 hours with 100 μmol/L bezafibrate, 100 μmol/L fenofibrate, or 5 μmol/L troglitazone. Data are expressed as the mean ± SD of 3 experiments. *$P < .05$, **$P < .01$.

chondrial outer membrane, catalyzes the entry of long-chain fatty acids into the mitochondrial matrix and is one of the main factors that determine the flux of mitochondrial β-oxidation.[16] The induction of CPT-I by troglitazone is consistent with the transcriptional regulation of this gene by PPARγ .[3] In addition to being oxidized in the mitochondria, long-chain fatty acyl-CoA can be joined to cholesterol to form cholesterol esters in a reaction catalyzed by acyl-CoA cholesterol acyltransferase (ACAT).[17] Therefore, it is likely that increased mitochondrial β-oxidation after troglitazone treatment, as suggested by the induction in CPT-I mRNA levels, may result in a reduction in the availability of long-chain fatty acids. As a result, the formation of cholesterol esters would be reduced, leading to higher levels of free cholesterol. In fact, in macrophage-derived foam cells treated with troglitazone we found an induction in CPT-I mRNA levels. In these cells the induction of CPT-I was accompanied by a reduction in the levels of intracellular cholesterol esters, suggesting that CPT-I may have a function in the regulation of cholesterol ester levels in macrophages. Moreover, the potential involvement of CPT-I in the control of cholesterol ester levels in macrophages is supported by previous studies showing that CPT-I inhibition by etomoxir resulted in a 3.5-fold induction in the incorporation of labeled cholesterol into cholesteryl esters in macrophages.[18] Interestingly, the reduction in intracellular cholesteryl esters after troglitazone treatment in macrophage-derived foam cells was not accompanied by an increase in the levels of free cholesterol, indicating that troglitazone treatment may lead to increased cholesterol efflux. This may be mediated by apoE, since apoE secreted by human macrophages may act as an acceptor for free cholesterol.[19] Macrophage-specific expression of apoE, an apolipoprotein involved in reverse cholesterol transport, prevents the development of atherosclerotic lesions in mice in spite of very low plasma apoE concentrations.[20,21] Moreover, apoE expression in macrophages facilitates cholesterol efflux from cholesterol-loaded macrophages.[2] Although we have not determined apoE levels, the potential involvement of apoE in the changes observed in troglitazone-treated macrophages is consistent with the presence of a functional peroxisome proliferator response element (PPRE) in the promoter of the apoE gene that mediates apoE induction in human macrophages after thiazolidinedione treatment.[19] Therefore, it is likely that the increase in free cholesterol levels caused by reduced fatty acid availability is compensated by an increase in apoE-mediated cholesterol efflux.

The reduction in intracellular cholesteryl esters after troglitazone treatment may also occur as a result of changes in the expression of other genes involved in cholesterol metabolism, such as nCEH. This enzyme catalyzes the hydrolysis of stored cholesterol esters, and the free cholesterol thus released moves to the plasma membrane and is subsequently transferred to a cholesterol acceptor, resulting in net cellular cholesterol efflux.[22] Thus, nCEH may be an important factor controlling the mobilization of cholesterol esters from foam cells. Moreover, it has been recently shown that human nCEH contains a functional PPREs.[23] However, in our study, troglitazone, as well as the other PPAR activators assayed, did not modify the mRNA levels of this gene, which made it unlikely that this enzyme contributed to the changes observed.

Treatment of foam cells with the three PPAR activators assayed resulted in the induction of aP2, which is consistent with the reported upregulation of this gene by PPARγ[24] and PPARβ[8] activators. It should be mentioned that aP2 is a cytosolic protein that facilitates the transport of fatty acids to peroxisomes and mitochondria. It is likely that an increase in CPT-I expression, causing a mobilization of fatty acids to mitochondria, leads to an increase in the expression of the substrate provider. Surprisingly, the PPARα activator fenofibrate increased the expression of LOX-1. Further studies are needed to ascertain whether LOX-1, a membrane protein that can act as a cell surface endocytosis receptor for atherogenic oxidized LDL,[25] is regulated by PPARs

In contrast with the beneficial effects of PPARα and PPARγ activation in macrophages, the role of PPARβ in foam cell formation is controversial, since different studies show that agonists of this receptor promote[8] or reduce[9] lipid accumulation in macrophages. In this study we used bezafibrate, a drug that activates PPARβ, as well as PPARα and PPARγ. Treatment of macrophages with bezafibrate resulted in the induction of CD36, a well-known PPARγ target gene.[4] However, troglitazone treatment did not modify the expression of this gene, suggesting that activation of PPARβ is a more potent stimulus for the induction of this gene than PPARγ activation alone. This corroborates the findings of Vosper et al,[8] who showed that treatment with a selective PPARβ activator for 48 hours caused a 6-fold induction in CD36 mRNA levels, compared with the 2-fold induction achieved with a saturating concentration of the thiazolidinedione rosiglitazone.

In summary, we show that troglitazone treatment induces the expression of CPT-I, which in turn may decrease the availabil-

ity of fatty acids for cholesterol esterification, leading to reduced foam cell formation. Further studies will be needed to examine the precise function of CPT-I in intracellular cholesterol ester accumulation in macrophages, since it could be a potential target for reducing foam cell formation.

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REFERENCES

1. Guyton JR: The arterial wall and the atherosclerotic lesion. Curr Opin Lipidol 5:376-381, 1994

2. Mazzone T, Reardon C: Expression of heterologous human apolipoprotein E by J774 macrophages enhances cholesterol efflux to HDL3. J Lipid Res 35:1345-1353, 1994

3. Desvergne B, Wahli W: Peroxisome proliferator-activated receptors: Nuclear control of metabolism. Endocr Rev 20:649-688, 1999

4. Tontonoz P, Nagy L, Alvarez J, et al: PPARγ promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell 93:241-252, 1998

5. Chinetti G, Lestavel S, Bocher V, et al: PPARα and PPARγ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway. Nat Med 7:53-58, 2001

6. Ericsson CG, Hamsten A, Nilsson J, et al: Angiographic assessment of effects of bezafibrate on progression of coronary artery disease in young male postinfarction patients. Lancet 347:849-853, 1996

7. Rosen ED, Spiegelman BM: Peroxisome proliferator-activated receptor-γ ligands and atherosclerosis: Ending the heartache. J Clin Invest 106:629-631, 2000

8. Vosper H, Patel L, Graham TL, et al: The peroxisome proliferator-activated receptor delta promotes lipid accumulation in human macrophages. J Biol Chem 276:44258-44265, 2001

9. Oliver WR, Shenk JL, Snaith MR, et al: A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. Proc Natl Acad Sci USA 98:5306-5311, 2001

10. Vázquez M, Zambón D, Hernández Y, et al: Lipoprotein composition and oxidative modification during therapy with gemfibrozil and lovastatin in patients with combined hyperlipidaemia. Br J Clin Pharmacol 45:265-269, 1998

11. Basu SK, Goldstein JL, Anderson RG, et al: Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc Natl Acad Sci USA 73:3178-3182, 1976

12. Freeman WM, Walker SJ, Kent EV. Quantitative RT-PCR: Pitfalls and potential. Biotechniques 26:112-125, 1999

13. Klansek JJ, Yancey P, St Clair RW, et al: Cholesterol quantitation by GLC: Artifactual formation of short-chain steryl esters. J Lipid Res 36:2261-2266, 1995

14. Guardiola F, Codony R, Rafecas M, et al: Selective gas chromatographic determination of cholesterol in eggs. J Am Oil Chem Soc 71:867-871, 1994

15. Brown PJ, Winegar DA, Plunket KD, et al: A ureido-thioisobutyric acid (GW9578) is a subtype-selective PPARalpha agonist with potent lipid-lowering activity. J Med Chem 42:3785-3788, 1999

16. McGarry JD, Brown NF: The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 244:1-14, 1997

17. Buhman KF, Accad M, Farese RV: Mammalian acyl-CoA: cholesterol acyltransferases. Biochim Biophys Acta 1529:142-154, 2000

18. Moinat M, Kossovsky M, Chevey JM, et al: Balance between fatty acid degradation and lipid accumulation in cultured smooth muscle cells and IC-21 macrophages exposed to oleic acid. Comp Biochem Physiol 98:147-150, 1991

19. Galetto R, Albajar M, Polanco JI, et al: Identification of a peroxisome-proliferator-activated-receptor response element in the apolipoprotein E gene control region. Biochem J 357:521-527, 2001

20. Boisvert WA, Spangenberg J, Curtiss LK: Treatment of severe hypercholesterolemia in apolipoprotein E-deficient mice by bone marrow transplantation. J Clin Invest 96:1118-1124, 1995

21. Linton MF, Atkinson JB, Fazio S: Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. Science 267:1034-1037, 1995

22. Ghosh S: Cholesteryl ester hydrolase in human monocyte/macrophage: Cloning, sequencing, and expression of full-length cDNA. Physiol. Genomics 2:1-8, 2000

23. Ghosh S, Natarajan R: Cloning of the human cholesteryl ester hydrolase promoter: Identification of functional peroxisomal proliferator-activated receptor responsive elements. Biochem Biophys Res Commun 284:1065-1070, 2001

24. Pelton PD, Zhou L, Demarest KT, et al: PPARγ activation induces the expression of the adipocyte fatty acid binding protein gene in human monocytes. Biochem Biophys Res Commun 261:456-458, 1999

25. Kume N, Kita T: Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in atherogenesis. Trends Cardiovasc Med 11:22-25, 2001