Bezafibrate Reduces mRNA Levels of Adipocyte Markers and Increases Fatty Acid Oxidation in Primary Culture of Adipocytes

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The molecular mechanisms by which peroxisome proliferator–activated receptor (PPAR) activation by fibrates reduces fat deposition and improves insulin sensitivity are not completely understood. We report that exposure of a rat primary culture of adipocytes for 24 h to the PPAR activator bezafibrate increased the mRNA levels of crucial genes involved in peroxisomal and mitochondrial β-oxidation. The mRNA levels of the peroxisomal β-oxidation rate-limiting enzyme acyl-CoA oxidase and of the muscle-type carnitine palmitoyl transferase I (M-CPT-I), which determines the flux of mitochondrial β-oxidation, increased by 1.6-fold ($P < 0.02$) and 4.5-fold ($P = 0.001$), respectively. These changes were accompanied by an increase in the transcript levels of the uncoupling protein-2 (UCP-2; 1.5-fold induction; $P < 0.05$) and UCP-3 (3.7-fold induction; $P < 0.001$), mitochondrial proteins that reduce ATP yield and may facilitate the oxidation of fatty acids. Furthermore, bezafibrate increased the mRNA levels of the fatty acid translocase (2-fold induction; $P < 0.01$), suggesting a higher fatty acid uptake into adipocytes. In agreement with these changes, bezafibrate caused a 1.9-fold induction ($P < 0.02$) in 9,10-[^{3}H]palmitate oxidation. Moreover, bezafibrate reduced the mRNA expression of several adipocyte markers, including PPARγ (30% reduction; $P = 0.05$), tumor necrosis factor-α (33% reduction; $P < 0.05$), and the *ob* gene (26% reduction). In contrast, adipocyte fatty acid binding protein mRNA levels increased (1.5-fold induction; $P < 0.01$), pointing to a mobilization of fatty acids to mitochondria and peroxisomes. The reduction of the adipocyte markers caused by bezafibrate was accompanied by an increase in the mRNA levels of the preadipocyte marker Pref-1 (1.6-fold induction; $P < 0.01$). Some of the changes observed in the primary culture of rat adipocytes also were studied in the epididymal white adipose tissue of bezafibrate-treated rats for 7 days. In vivo, M-CPT-I mRNA levels increased (4.5-fold induction; $P = 0.001$) in epididymal white adipose tissue of bezafibrate-treated rats. Similarly, fatty acid translocase (2.6-fold induction; $P = 0.002$) and Pref-1 (5.6-fold induction) mRNA levels increased, although differences in the latter were not significant because of huge individual variations. These results indicate that exposure of adipocytes to bezafibrate, independent of its hepatic effects, increases the degradation of fatty acids, reducing their availability to synthesize triglycerides. As a result, some degree of dedifferentiation of adipocytes to preadipocyte-like cells is achieved. These changes may be involved in the reduction in fat depots and in the improvement of insulin sensitivity observed after bezafibrate treatment. *Diabetes* 50:1883–1890, 2001

Fibrates are hypolipidemic agents whose effects are mediated by activation of specific transcription factors called peroxisome proliferator–activated receptors (PPARs). PPAR activation by ligands leads to dimerization, usually with retinoic X receptor, and cooperative binding to peroxisome proliferator responsive elements located in the 5′-upstream promoter region of the PPAR-target genes. Three different PPAR subtypes have been identified to date (α, δ/β, and γ). PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism, such as liver, brown fat, kidney, heart, and skeletal muscle (1,2); at low levels, it also is present in fat depots and adipocytes (3–7). PPARβ is expressed ubiquitously, whereas PPARγ has a restricted pattern of expression, mainly in white and brown adipose tissues (8). Regarding the metabolic roles of PPAR subtypes, PPARα and PPARγ seem to be involved in antagonist pathways. Indeed, PPARα activation by classical peroxisome proliferators (such as fibrates) stimulates catabolic pathways of fatty acids in liver, whereas activation of PPARγ by antidiabetic thiazolidinediones promotes lipid storage through its effects on adipocyte differentiation and function in white adipose tissue (1). In contrast, the functions of PPARβ are poorly understood. In addition to their well-known hypolipidemic effects, there is growing evidence that fibrates improve glucose homeostasis and influence body weight and energy homeostasis (rev. in 9). For example, bezafibrate administration to rats decreases fat depots (4) and improves glucose tolerance in diabetic patients (10–12) and rats (13). The molecular mechanisms responsible for the changes in body weight and glucose homeostasis after bezafibrate administration are not understood. It has been proposed that these effects of bezafibrate may be mediated by PPARα activation in liver (9). However, it has not been tested whether bezafibrate has direct effects on adipocytes.

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ACO, acyl-CoA oxidase; aP2, adipocyte fatty acid-binding protein-2; APRT, adenosyl phosphoribosyl transferase; BSA, bovine serum albumin; FAT/CD36, fatty acid translocase; HSL, hormone-sensitive lipase; M-CPT-I, muscle-type carnitine palmitoyltransferase I; PPAR, peroxisome proliferator–activated receptor; TNFα, tumor necrosis factor-α; UCP, uncoupling protein.

Interestingly, Zhou et al. (6) recently showed that hyperleptinemia induction in healthy rats depleted adipocyte tissue through downregulation of lipogenic enzymes and their transcription factor PPARγ in epididymal fat. In contrast, enzymes of fatty acid oxidation and their transcription factor PPARα, usually low in adipocytes, were upregulated, as were uncoupling protein-1 (UCP-1) and UCP-2. Moreover, the transformation of the adipocyte phenotype from fat-storing cells to fatty acid–oxidizing cells was accompanied by loss of adipocyte markers, such as adipocyte fatty acid-binding protein-2 (aP2), tumor necrosis factor α (TNFα), and the *ob* gene, and by the appearance of the preadipocyte marker Pref-1 (6). To determine whether bezafibrate causes similar changes, we examined the effects of this fibrate in fatty acid oxidation and in adipocyte markers in a primary culture of rat adipocytes. Treatment with bezafibrate for 24 h significantly increased mRNA levels of genes involved in peroxisomal (acyl-CoA oxidase [ACO]) and mitochondrial (muscle-type carnitine palmitoyltransferase I [M-CPT-I], UCP-2, and UCP-3) fatty acid oxidation. These changes in the mRNA expression of genes involved in fatty acid catabolism correlated well with the increase in palmitate oxidation caused by bezafibrate. In addition, bezafibrate decreased the mRNA levels of several adipocyte markers (*ob* gene, PPARγ, and TNFα) and increased the mRNA expression of the preadipocyte marker Pref-1. In agreement with the results observed in adipocytes, an increase was detected in the mRNA levels of M-CPT-I, fatty acid translocase (FAT/CD36), and Pref-1 in epididymal white adipose tissue of bezafibrate-treated rats. These results may explain the decrease in body fat deposition and, therefore, the improvement in energy homeostasis observed after bezafibrate treatment.

RESEARCH DESIGN AND METHODS

Materials. Bezafibrate was obtained from Sigma (St. Louis, MO), and 9,10-[^{3}H]palmitic acid (40–60 Ci/mmol) was obtained from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cell isolation and primary culture of rat adipocytes. Fibroblastic preadipocytes were isolated from adipose tissue using a method modified from that of Rodbell (14). The epididymal adipose tissue from male Sprague-Dawley rats that weighed 200–250 g was removed under sterile conditions and washed in Hank's balanced salt solution (Life Technologies, Grand Island, NY). Minced tissue was transferred to a sterile polypropylene Erlenmeyer flask containing collagenase (2 mg/ml, type II; Sigma) and 5% bovine serum albumin (BSA). After incubation at 37°C for 45 min in a shaking water bath, the digest was filtered through a sterile 250-μm nylon mesh. The digested tissue was centrifuged at 250*g* for 10 min, and mature adipocytes were removed by aspiration. The pellet was resuspended in Hank's balanced salt solution, filtered through a 25-μm nylon mesh, and centrifuged again. The pellet obtained consisted mainly of preadipocytes. It was resuspended in MEM Alpha medium (Life Technologies) supplemented with penicillin, streptomycin, and amphotericin (100 U/ml, 100 μg/ml, and 2.5 μg/ml, respectively; Life Technologies) and refiltered. To eliminate erythrocytes, we incubated preadipocytes with an erythrocyte-lysing buffer consisting of 0.154 mol/l NH_4Cl, 10 mmol/l $KHCO_3$, and 0.1 mmol/l EDTA for 5 min at room temperature. In these conditions, >95% of erythrocytes were lysed without damaging the nucleus-containing cells as assessed by Trypan blue exclusion. The suspension was centrifuged (250*g* for 10 min), and the cells were counted using a hemocytometer. Cells were plated in 60-mm culture dishes at a density of 1.5×10^5 cells/ml in MEM Alpha medium with 10% fetal calf serum. Medium was changed every 2 days. Cells were maintained in this medium until confluence (usually 3 days). Differentiation was induced by the addition of medium supplemented with isobutylmethylxanthine (0.5 mmol/l), dexamethasone (0.25 μmol/l), and insulin (10 μg/ml). After 48 h, the induction medium was removed and replaced by MEM Alpha medium containing 10% fetal calf serum and insulin (10 μg/ml). This medium was changed every 2 days. Ten days later, cells were regarded as differentiated by morphologic criteria when, after acquiring a round shape, their cytoplasm was completely filled with multiple lipid droplets (assessed by oil red O staining). Differentiated adipocytes in MEM Alpha medium were treated with either vehicle (0.1% DMSO) or 500 μmol/l bezafibrate for 24 h. After incubation, RNA was extracted from the adipocytes as described below.

Animals and treatment. Eight male Sprague-Dawley rats from Letica (Barcelona, Spain), weighing 120–130 g, were maintained in conditions of constant humidity and temperature (22 ± 2°C) and a 12-h light-dark cycle (light from 8:00 A.M. to 8:00 P.M.). They were fed a standard diet (Panlab, Barcelona, Spain) for 5 days before the studies began. The animals were distributed randomly into two groups of four. Each group was fed, respectively, either a control diet or a diet containing bezafibrate (0.45%, wt/wt). The diets were prepared as previously described (15) by soaking in an acetone solution of the drug. To avoid any possible effect of the solvent, we also soaked the control diet in acetone and dried it. Throughout the study, the weight and daily food intake were measured. These parameters were not affected by the treatment. Rats were killed by decapitation after 7 days of treatment. Epididymal white adipose tissue was rapidly dissected and frozen in liquid nitrogen for RNA analysis. Animal handling and disposal were performed in accordance with the law (5/1995, July 21, from the Generalitat de Catalunya).

RNA preparation and analysis. Total RNA was isolated using the Ultraspec reagent (Biotecx, Houston, TX). Relative levels of specific mRNAs were assessed by 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 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies), 20 units of 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 min. A 5-μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 25-μl PCR 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 unit of *Taq* polymerase (Ecogen, Barcelona, Spain), 0.5 μg of each primer, and 20 mmol/l Tris-HCl (pH 8.5). To avoid unspecific annealling, we separated cDNA and *Taq* polymerase 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 as follows: ACO, 5′-ACTATATTTGGCCAATTTTGTG-3′ and 5′-TGTGGCAGTGGTTTCCAAGCC-3′; M-CPT-I, 5′-TTCACTGTGACCCCAGACGGG-3′ and 5′-AATGGACCAGCCCCATGGAGA; UCP-2, 5′-AACAGTTCTACACCAAGGGC-3′ and 5′-AGCATGGTAAGGGCACAGTG-3′; UCP-3, 5′-GGAGCCATGGCAGTGACCTGT-3′ and 5′-TGTGATGTTGGGCCAAGTCCC-3′; PPARα, 5′-GGCTCGGAGGGCTCTGTCATC-3′ and 5′-ACATGCACTGGCAGCAGTGGA-3′; PPARγ, 5′-TGGGGATGTCTCACAATGCCA-3′ and 5′-TTCCTGTCAAGATCGCCCTCG-3′; TNFα, 5′-TACTGAACTTCGGGGTGATTGGTCC-3′ and 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′; *ob*, 5′-GTGCTGGAGACCCCTGTGTCG-3′ and 5′-AGAATGGGGTGAAGCCCAGGA-3′; aP2, 5′-GACCTGGAAACTCGTCTCCA-3′ and 5′-CATGACACATTCCACCACCA-3′; Pref-1, 5′-AGCCCTCCTGCGCGTCCTCTT-3′ and 5′-AGTCCCATTGTTGGCGCAGGG-3′; FAT/CD36, 5′-AATGGCACAGATGCAGCCTCC-3′ and 5′-TGCCAATGTCCAGCACACCAT-3′; hormone-sensitive lipase (HSL), 5′-TCAAGCCAAGGTGTCCTCCACATG-3′ and 5′-GGGTGCAAGAGGTCTTTTAGTGCC-3′; β-actin, 5′-TTGTAACCAACTGGGACGATATGG-3′ and 5′-GATCTTGATCTTCATGGTGCTAGG-3′; and adenosyl phosphoribosyl transferase (APRT), 5′-AGCTTCCCGGACTTCCCCATC-3′ and 5′-GACCACTTTCTGCCCCGGTTC-3′. PCR was performed in an MJ Research Thermocycler (Watertown, MA) equipped with a Peltier system and temperature probe. After an initial denaturation for 1 min at 94°C, PCR was performed for 20 (FAT/CD36 in white adipose tissue), 21 (aP2 and FAT/CD36 in isolated adipocytes), 23 (ACO, UCP-2, PPARα, and PPARγ in isolated adipocytes; APRT and M-CPT-I in white adipose tissue), 25 (M-CPT-I, TNFα, and HSL in isolated adipocytes), 28 (Pref-1 in isolated adipocytes), or 30 cycles (UCP-3 and *ob* in isolated adipocytes and Pref-1 in white adipose tissue). Each cycle consisted of denaturation at 92°C for 1 min, primer annealing at 60°C (except for ACO [58°C]), and primer extension at 72°C for 1 min, 50 s. A final 5-min 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 to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (ACO, 195 bp; M-CPT-I, 222 bp; UCP-2, 471 bp; UCP-3, 179 bp; PPARα, 645 bp; PPARγ, 200 bp; TNFα, 284 bp; *ob*, 206 bp; aP2, 349 bp; Pref-1, 281 bp; FAT/CD36, 256 bp; HSL, 353 bp; β-actin, 764 bp; APRT, 329 bp). Preliminary experiments were carried out with various amounts of cDNA to determine nonsaturating conditions of PCR

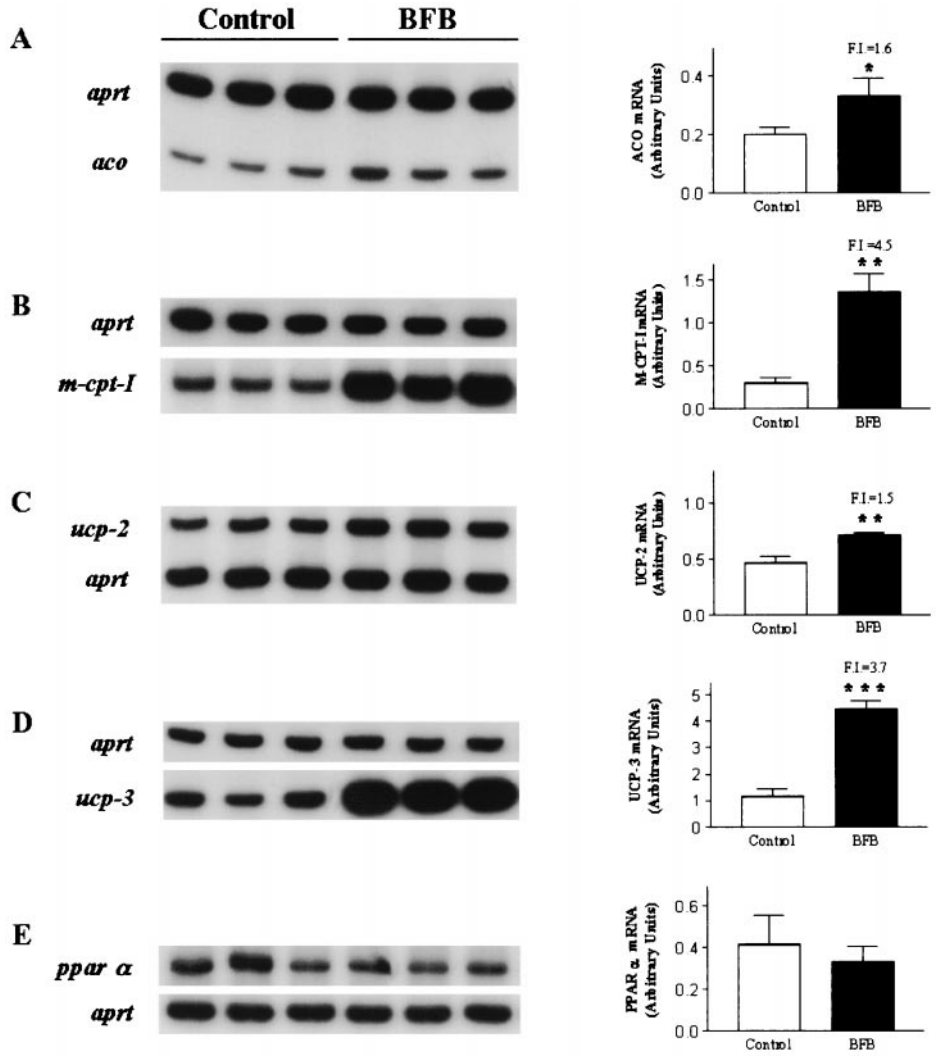

FIG. 1. Effects of bezafibrate on the expression of ACO (*A*), M-CPT-I (*B*), UCP-2 (*C*), UCP-3 (*D*), and PPARα (*E*) mRNA in rat primary culture of adipocytes. Cells were incubated for 24 h with vehicle or 500 μmol/l bezafibrate. Total RNA (0.5 μg) was analyzed by RT-PCR. A representative autoradiogram and the quantification of the *aprt*-normalized mRNA levels are shown. Data are expressed as means ± SD of four experiments. *$P < 0.05$; **$P < 0.01$, and ***$P < 0.001$ compared with control experiments. F.I. = fold induction.

amplification for all of the genes studied. Therefore, under these conditions, the RT-PCR method used in this study permits relative quantification of mRNA (16). Radioactive bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*β-actin* or *aprt*).

9,10-[^{3}H]palmitate oxidation. Palmitate oxidation was measured by incubating differentiated adipocytes in MEM Alpha medium containing 0.3 mmol/l palmitate (9,10-[^{3}H]palmitate, 25 μCi/60-mm dish), 2% BSA, 0.25 mmol/l L-carnitine, and 3 mmol/l glucose. After 24 h of treatment with either vehicle (0.1% DMSO) or 500 μmol/l bezafibrate, palmitate oxidation was assessed by measuring ^3H_2O produced in the incubation medium. Excess [^{3}H]palmitate in the medium was removed by precipitating twice with an equal volume of 10% trichloroacetic acid with 2% BSA. The supernatants (0.5 ml) were extracted by addition of 2.5 ml of methanol:chloroform (2:1) and 1 ml of 2 mol/l KCl:HCl, followed by centrifugation at 3,000*g* for 5 min. The aqueous phase (1 ml) was treated once more, and an aliquot (0.5 ml) was taken for counting.

Statistical analysis. Results are expressed as means ± SD of *n* experiments. Significant differences were established by Student's *t* test, using the computer program GraphPad Instat (GraphPAD Software).

RESULTS

Effects of bezafibrate on mRNA levels of genes involved in fatty acid oxidation and on UCPs in rat adipocytes. Fatty acid β-oxidation occurs in both peroxisomes and mitochondria. We first tested the effect of bezafibrate on the mRNA levels of ACO, the rate-limiting enzyme of the peroxisomal β-oxidation pathway, the transcription of which is controlled by PPARα (1). Treatment of rat primary culture of adipocytes with bezafibrate caused a 1.6-fold induction ($P < 0.02$) in ACO mRNA levels (Fig. 1*A*). One of the main factors that determine the flux of mitochondrial β-oxidation is M-CPT-I, which is located on the mitochondrial outer membrane and catalyzes the entry of long-chain fatty acids into the mitochondrial matrix (17). Bezafibrate treatment of rat adipocytes strongly increased M-CPT-I mRNA levels (4.5-fold induction; $P = 0.001$; Fig. 1*B*). Enhanced mitochondrial β-oxidation provides higher amounts of reducing equivalents (NADH), which are oxidized by the respiratory chain, resulting in the generation of an electrochemical gradient of protons (18). This is dissipated by inner mitochondrial membrane transporters, which act as a pore for H^+ ions, called UCPs. As a result, the efficiency of ATP synthesis is reduced (19). To study a possible link between fatty acid oxidation and UCP activity, we tested the effect of bezafibrate on these UCPs. Bezafibrate treatment significantly increased UCP-2 (1.5-fold induction; $P < 0.05$) and UCP-3 (3.7-fold induction; $P < 0.001$) mRNA levels (Fig. 1*C* and *D*). According to the proposed role for UCPs, which could facilitate the oxidation of fatty acids (20), a high correlation was observed between M-CPT-I and UCP-3 mRNA levels in adipocytes ($r = 0.97$, $r^2 = 0.95$, $P < 0.0001$ for $n = 8$). To study whether the changes mentioned above were

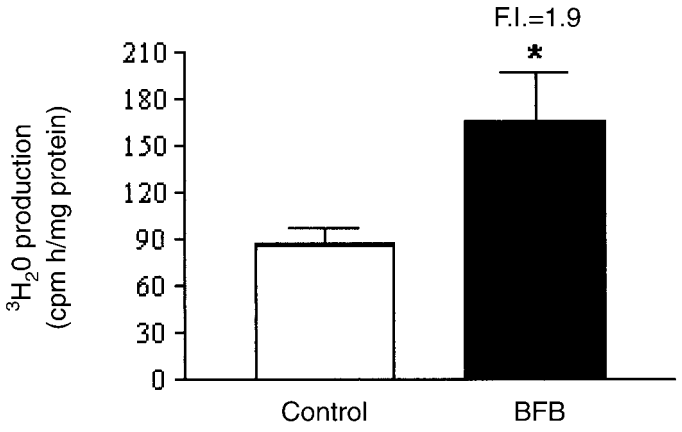

FIG. 2. Effect of bezafibrate on fatty acid oxidation in rat primary culture of adipocytes. Cells were incubated for 24 h with either vehicle or 500 μmol/l bezafibrate. 9,10-[^{3}H]palmitate oxidation was measured as counts per minute of ^3H_2O produced per hour per milligram of protein. Data are presented as means ± SD of three experiments. *P < 0.05 compared with control group. F.I. = fold induction.

related to changes in the expression of PPARα, we determined the mRNA levels of this transcription factor. Bezafibrate did not significantly modify PPARα mRNA levels, although a slight reduction was observed (Fig. 1*E*).

Effects of bezafibrate on palmitate oxidation in rat adipocytes. To correlate the increase in ACO, M-CPT-I, and UCP mRNA expression caused by bezafibrate with changes in lipid metabolism, we determined the rate of fatty acid oxidation in primary culture of rat adipocytes for 24 h (Fig. 2). The oxidation of exogenously administered 9,10-[^{3}H]palmitate, measured by ^3H_2O production, increased from 87 ± 11 cpm · h^{-1} · mg^{-1} protein in control adipocytes to 166 ± 31 cpm · h^{-1} · mg^{-1} protein in bezafibrate-treated adipocytes (1.9-fold induction; $P < 0.02$). This finding indicates that bezafibrate induction of the mRNA expression of genes involved in fatty acid catabolism is accompanied by an increase in the catabolic state of the adipocytes. Furthermore, because it has been reported that the rate of fatty acid oxidation falls during adipose differentiation (21), we measured the rate of fatty acid oxidation in preadipocytes and adipocytes. The rate of fatty acid oxidation was higher in preadipocytes than in adipocytes (168 ± 18 vs. 77 ± 5 cpm · h^{-1} · mg^{-1} protein; $P < 0.0002$ [$n = 3$]). This result suggests that bezafibrate increases fatty acid oxidation to a level similar to that observed in preadipocytes.

Effects of bezafibrate on mRNA levels of HSL and FAT/CD36 in rat adipocytes. We studied the effects of bezafibrate treatment in rat adipocytes on the mRNA levels of two genes involved in fatty acid metabolism: FAT/CD36 and HSL. The former is a membrane-associated protein that facilitates the uptake of long-chain fatty acids into adipocytes, and its level of expression determines the fatty acid utilization by a tissue. FAT/CD36 mRNA levels were significantly increased after bezafibrate treatment (2-fold induction; $P < 0.01$; Fig. 3*A*). HSL, which catalyzes the hydrolysis of triglycerides stored in the adipose tissue, showed a 2.6-fold induction in mRNA levels after bezafibrate treatment, although differences were not significant (Fig. 3*B*).

Effects of bezafibrate on mRNA levels of adipocyte and preadipocyte markers in rat adipocytes. Adipogenesis develops through the induction of several adipocyte-enriched genes, such as PPARγ, TNFα, *ob* gene, and aP2. During the late phase, adipogenesis also involves the disappearance of preadipocyte markers, such as Pref-1 (22). Here we determined whether bezafibrate treatment for 24 h modified the mRNA levels of these adipocyte and preadipocyte markers. Bezafibrate reduced PPARγ mRNA levels by 30% ($P = 0.05$; Fig. 4*A*), TNFα mRNA by 33% ($P < 0.05$; Fig. 4*B*), and *ob* mRNA by 26% (Fig. 4*C*). In contrast to the general trend observed in adipocyte markers, aP2 mRNA levels were increased by bezafibrate (1.5-fold induction; $P < 0.01$; Fig. 4*D*). It is interesting that exposure of adipocytes to bezafibrate for 24 h caused a 1.6-fold induction ($P < 0.01$) in the mRNA levels of Pref-1 (Fig. 4*E*).

Effects of bezafibrate on M-CPT-I, FAT/CD36, and aP2 occur at the transcriptional level. To gain insight into the mechanism by which bezafibrate upregulates the expression of some of the genes studied in primary culture of adipocytes, we determined the effects of this fibrate in the presence or the absence of the transcriptional inhibitor actinomycin D (Fig. 5). After 16 h of treatment, bezafibrate increased the mRNA levels of M-CPT-I, FAT/CD36, and aP2, whereas no changes in the transcripts of these genes were observed in the presence of actinomycin D. These results indicate that the action of bezafibrate occurs at the transcriptional level.

Changes on the mRNA levels of M-CPT-I, FAT/CD36, and Pref-1 in epididymal white adipose tissue of bezafibrate-treated rats. Finally, we studied whether some of the changes caused by bezafibrate in the primary culture of adipocytes also were observed in the epididymal white adipose tissue of bezafibrate-treated rats. We reported previously (4) that bezafibrate increased the mRNA levels of UCP-3 and UCP-1 in epididymal white adipose

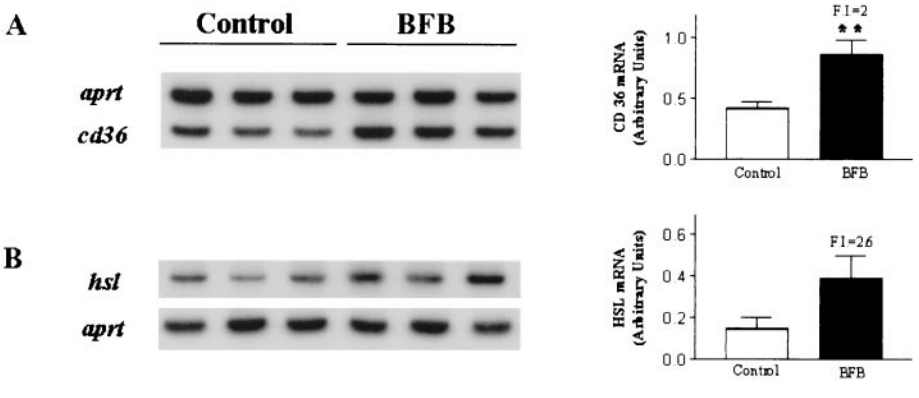

FIG. 3. Effects of bezafibrate on the expression of FAT/CD36 (*A*) and HSL (*B*) mRNA in rat primary culture of adipocytes. Cells were incubated for 24 h with vehicle, 500 μmol/l bezafibrate. Total RNA (0.5 μg) was analyzed by RT-PCR. A representative autoradiogram and the quantification of the *aprt*- or *β-actin*–normalized mRNA levels are shown. Data are expressed as means ± SD of four experiments. **P < 0.01 compared with control experiments. F.I. = fold induction.

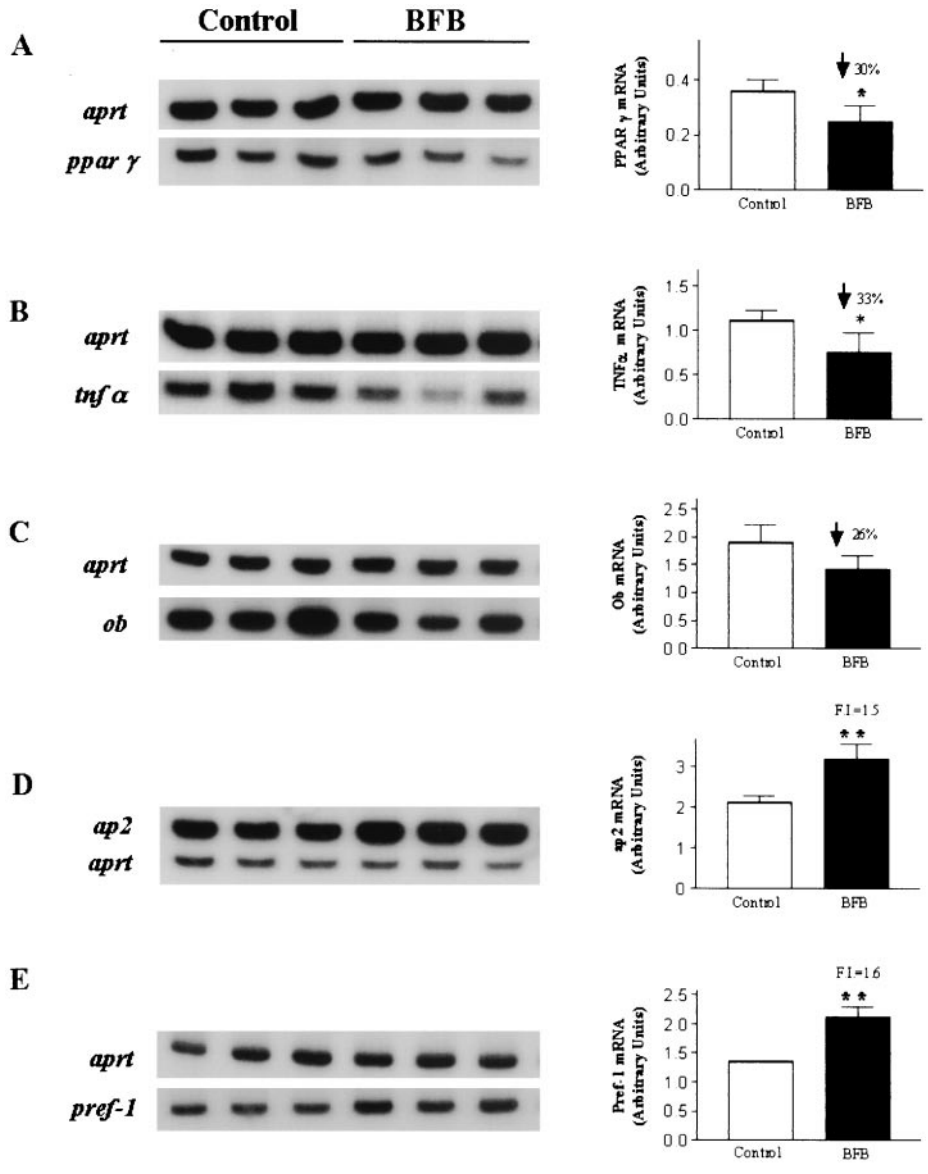

FIG. 4. Effects of bezafibrate on the expression of PPARγ (*A*), TNFα (*B*), *ob* (*C*), aP2 (*D*), and Pref-1 (*E*) mRNA levels in rat primary culture of adipocytes. Cells were incubated for 24 h with vehicle or 500 μmol/l bezafibrate. Total RNA (0.5 μg) was analyzed by RT-PCR. A representative autoradiogram and the quantification of the *aprt*-normalized mRNA levels are shown. Data are expressed as mean ± SD of four experiments. **P* < 0.05 and ***P* < 0.01 compared with control experiments. F.I. = fold induction.

tissue. In the present study, we found that in vivo, bezafibrate caused a 4.5-fold induction ($P < 0.001$) in the mRNA levels of M-CPT-I in epididymal white adipose tissue (Fig. 6*A*). The mRNA levels of FAT/CD36 (2.6-fold induction; $P = 0.002$) and Pref-1 (5.6-fold induction) also increased, although the latter was not significant because of individual variations between animals (Fig. 6*B* and *C*).

DISCUSSION

Leptin administration to rats (6) or to isolated adipocytes (7) induces changes in adipocytes, including downregulation of adipocyte markers, such as the transcription factor PPARγ. In contrast, leptin treatment provoked upregulation of PPARα and its target genes involved in fatty acid oxidation (M-CPT-I, ACO) as well as the induction of thermogenic proteins (UCPs) and the preadipocyte marker Pref-1. These results may provide a therapeutic advantage for leptin treatment of obesity and obesity-related metabolic disorders, such as type 2 diabetes. However, because obese humans have high plasma levels of leptin, suggesting the presence of leptin resistance (23), it remained to be studied whether direct PPAR activation by ligands of this receptor may be an alternative way of inducing similar changes. Here we reported that bezafibrate caused equivalent changes to those observed after leptin treatment. Thus, bezafibrate significantly induces the mRNA levels of genes involved in peroxisomal (ACO) and mitochondrial (M-CPT-I) β-oxidation. The changes observed in ACO mRNA levels were similar, ~2-fold induction, after addition of leptin (20 ng/ml) (7) or bezafibrate to adipocytes. It is interesting that M-CPT-I expression was strongly increased by bezafibrate (4.5-fold induction) compared with the 2-fold induction reached by leptin (7). These changes are consistent with the increase in 9,10-[^{3}H]palmitate oxidation after bezafibrate treatment and would result in a reduced availability of fatty acids, which are the building blocks used by the adipocytes to synthesize triglycerides and may contribute to the decrease in the growth of fat depots observed after bezafibrate treatment (4,24). Furthermore, we showed that induced fatty acid oxidation is accompanied by an increased expression of genes (UCPs) involved in ATP depletion, which may result in a less efficient fatty acid synthesis (an ATP-dependent process) and reduced adiposity. In addition to the effects of beza-

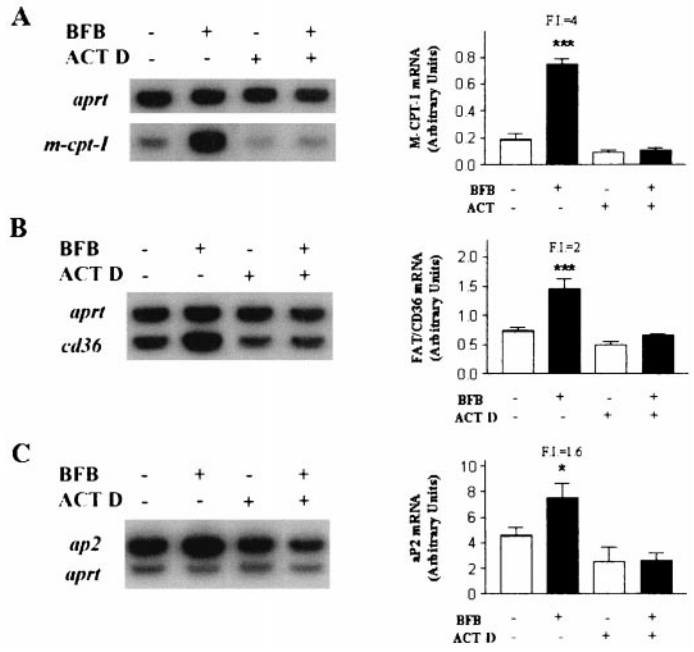

FIG. 5. Bezafibrate effects on M-CPT-I (*A*), FAT/CD36 (*B*), and aP2 (*C*) mRNA levels in rat primary culture of adipocytes. Cells were incubated for 16 h with vehicle or 500 μmol/l bezafibrate in the presence or in the absence of 2.5 μg/ml actinomycin D. Total RNA (0.5 μg) was analyzed by RT-PCR. A representative autoradiogram and the quantification of the *aprt*-normalized mRNA levels are shown. Data are expressed as mean ± SD of four experiments. *$P < 0.05$ and ***$P < 0.001$ compared with control experiments. F.I. = fold induction.

fibrate on UCP-2 and UCP-3 shown in the present study, we reported previously that the reduction of white fat depots in bezafibrate-treated rats was accompanied by induction of UCP-3 and ectopic UCP-1 expression in white adipose tissue (4). The existence of a possible link between mitochondrial UCPs and regulation of body weight was confirmed recently by Rossmeisl et al. (25). These authors generated transgenic *aP2-Ucp1* mice, which induced ectopic expression of *UCP1* gene in white adipose tissue. They showed that induction of mitochondrial uncoupling reduced white fat depots as a result of increased energy expenditure and depressed fatty acid synthesis. Therefore, these results support the idea that treatments that lead to increased fatty acid oxidation (through induction of fatty acid β-oxidation) and ATP depletion (through induced UCP expression) may result in a reduction in the growth of adipose tissue. This is the case for bezafibrate, which after administration to rats reduced white fat depots (4,24) and increased fatty acid β-oxidation and UCP expression. A similar explanation (coordinate induction of ACO and UCP-3 mRNA levels) has been proposed for the decrease in the growth of adipose tissue after administration of dietary fish oil to rats (26). Additional effects of bezafibrate, such as the induction of HSL expression, which would reduce the size of the triglyceride droplets by releasing fatty acids, may contribute to the reduction of fat deposits.

Furthermore, bezafibrate treatment of adipocytes caused a similar profile of changes in adipocyte markers, although of lower intensity, to those observed after leptin treatment in a previous study (6). After fibrate treatment, the mRNA levels of several adipocyte markers, such as PPARγ, *ob*, and TNFα, were reduced, although differences were significant only for PPARγ and TNFα. As in the case of leptin, the reduction of adipocyte markers may be the signal of several changes in the metabolism of the adipocyte, which would result in a change from triglyceride-storing cells to preadipocyte-like cells with a higher capacity to oxidize fatty acids (the present study and ref. 21). It is likely that these changes involve some degree of dedifferentiation of adipocytes to preadipocytes, as shown by the increase in the mRNA levels of Pref-1. Moreover, the reduction in the expression of TNFα is in agreement with previous studies (27), and it has been proposed to be one of the mechanisms involved in the improvement of insulin resistance produced by PPARα activators (9). In fact, TNFα has well-documented effects on lipid and glucose metabolism by regulating lipolysis, lipid biosynthesis, and insulin sensitivity (28) and can regulate leptin production from the adipose tissue and contribute to obesity-associated hyper-

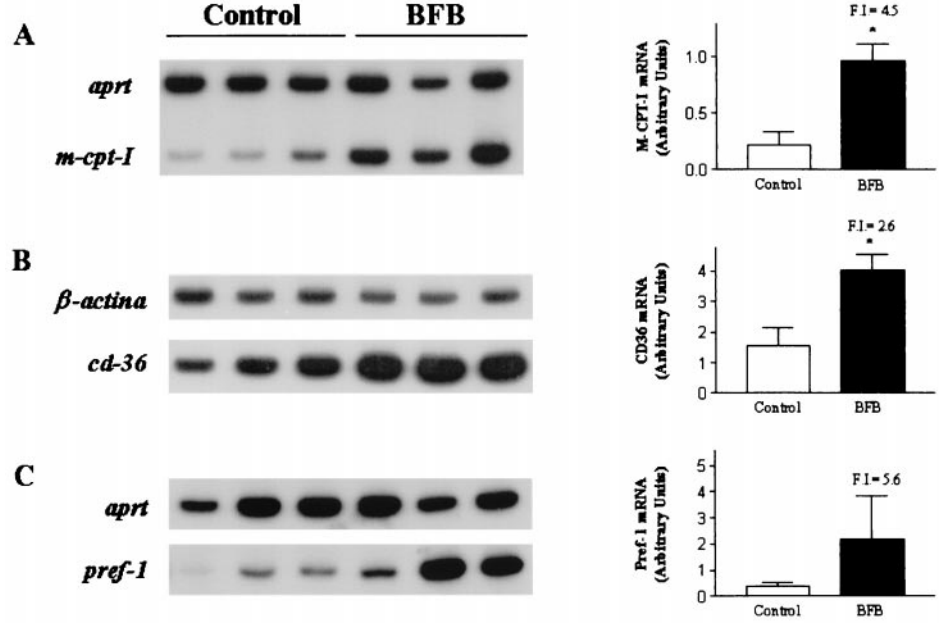

FIG. 6. Bezafibrate effects on M-CPT-I (*A*), FAT/CD36 (*B*), and Pref-1 (*C*) mRNA levels in epididymal white adipose tissue. Rats were treated with either a control diet or a diet supplemented with bezafibrate (0.45%, wt/wt) for 7 days. Total RNA (0.5 μg) isolated from epididymal white adipose tissue was subjected to RT-PCR analysis. A representative autoradiogram and the quantification of the normalized mRNA levels are shown. Data are expressed as means ± SD of four experiments. *$P < 0.01$ compared with control experiments. F.I. = fold induction.

leptinemia (29). Likewise, it was proposed recently that reduced expression of PPARγ improves insulin sensitivity (30). In contrast to the other adipocyte markers, aP2 mRNA levels were increased by bezafibrate. It should be noted that aP2 is a cytosolic protein that facilitates the transport of fatty acids to peroxisomes and mitochondria. Thus, it is reasonable that in a generalized context of fatty acid uptake (through FAT/CD36) and fatty acid mobilization from triglyceride droplets (through HSL activation) to mitochondria and peroxisomes, the levels of the substrate provider should be increased. In addition, we cannot rule out a possible interaction between aP2 and HSL that may result in fatty acid efflux as reported previously (31).

As stated at the beginning of this article, it has been proposed that the effects of fibrates on body weight and glucose homeostasis may be mediated by PPARα activation in the liver (9). To avoid the influence of the liver actions of fibrates, we studied the effects of bezafibrate in a primary culture of adipocytes. In our study, bezafibrate treatment increased fatty acid oxidation (as shown by the increase in palmitate oxidation) and decreased the expression of adipocyte markers. These results are not in agreement with those reported by Guerre-Millo et al. (32), who recently studied the effect of selective PPARα activators (fenofibrate, ciprofibrate, and GW9578) on glucose homeostasis and body weight control in animal models of insulin resistance. They showed that selective PPARα activators reduced insulin resistance and white fat depots through their effects on liver, whereas no effects were observed in adipose tissue. These discrepancies should be attributed to the different selectivity of the fibrates studied in both studies. Bezafibrate, similar to fatty acids (33) and in contrast to other fibrates, such as ciprofibrate and fenofibrate, activates PPARα, PPARβ, and PPARγ with comparable EC_{50} values (34). Therefore, although it is not the subject of the present study, it is likely that bezafibrate may influence body weight and glucose homeostasis through the activation of PPARβ and/or PPARγ subtypes in adipocytes. This idea is in agreement with the recent results presented by Deluca et al. (35), who reported that compounds with PPARγ and/or PPARβ activity were capable of inducing markers of the peroxisome proliferation (including ACO) in the liver of PPARα-null mice, whereas Wy-14643, a selective PPARα activator, had no effect. Thus, it is conceivable that in tissues that express low levels of PPARα, activation of PPARβ/γ subtypes may result in the activation of fatty acid β-oxidation. However, it is noteworthy that, whereas selective activation of PPARγ by thiazolidinediones leads to increased fat deposition (36), bezafibrate reduces fat depots (4,23). Therefore, it is likely that the changes observed after treatment of adipocytes with 500 μmol/l bezafibrate, which is within the plasma levels of fibrates reported in humans (37), may involve mainly the PPARβ subtype. In addition, other effects caused by bezafibrate not necessarily mediated by PPARs could be involved.

In summary, exposure of adipocytes to bezafibrate increases the mobilization of fatty acids to mitochondria and peroxisomes, where they are oxidized. Moreover, the expression of several adipocyte markers is downregulated. These changes may be involved in the reduction of fat depots attained by bezafibrate and in the improvement of insulin sensitivity. The profile of changes after exposure of adipocytes to bezafibrate is similar to those observed after leptin treatment. Because fibrates are low-affinity PPAR ligands, high-affinity agonists may have clinical application in obesity-related metabolic disorders, avoiding the resistance to leptin present in obese humans.

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