Fenofibrate reduces serum retinol-binding protein-4 by suppressing its expression in adipose tissue

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Fenofibrate reduces serum retinol-binding protein-4 (RBP4) by suppressing its expression in adipose tissue. Am J Physiol Endocrinol Metab 296: E628–E634, 2009. First published December 16, 2008; doi:10.1152/ajpendo.90526.2008.—Fenofibrate is a peroxisome proliferator-activated receptor-α (PPARα) activator that has been clinically used to treat dyslipidemia and insulin resistance. To better understand the molecular mechanisms underlying fenofibrate action, we investigated whether fenofibrate affects serum levels of retinol-binding protein-4 (RBP4), an adipocytokine that has recently been shown to link obesity and insulin resistance. Fenofibrate treatment significantly decreased serum RBP4 levels of dyslipidemic patients, which correlated with reduced body weight and increased insulin sensitivity. To elucidate the biochemical mechanisms of fenofibrate action, we investigated the effect of fenofibrate on RBP4 expression in obese rats. Fenofibrate greatly decreased RBP4 mRNA levels in adipose tissue but not in the liver, which correlated with decreased serum RBP4 levels and increased insulin sensitivity in obese rats. Consistent with a direct effect on RBP4 expression, fenofibrate treatment significantly reduced the mRNA expression levels of RBP4 in 3T3-L1 adipocytes. Together, our results demonstrate for the first time that fenofibrate inhibits RBP4 expression in dyslipidemic human subjects and suggest that inhibition of RBP4 expression in adipocytes may provide a mechanism by which fenofibrate improves insulin sensitivity in dyslipidemic patients.

Clinical study. Fifteen nondiabetic male patients with insulin resistance and dyslipidemia were recruited in this study. The diagnosis of dyslipidemia was based on Adult Treatment Panel III: serum triglyceride (TG) levels >2.26 mmol/l, total cholesterol levels >6.22 mmol/l, low-density lipoprotein-cholesterol (LDL-C) levels >4.14 mmol/l, and/or high-density lipoprotein (HDL) levels <1.04 mmol/l (5). The diagnosis of nondiabetic subjects was based on fasting plasma glucose levels <6.1 mmol/l and no family history of type 2 diabetes mellitus (T2DM). The diagnosis of T2DM was based on the World Health Organization criteria (1). Patients with a history of urinary tract infection, urolithiasis, liver cirrhosis, congestive heart failure, macrovascular disease, overt proteinuria, or any other known major diseases were excluded on the basis of interview, physical examination, and urinalysis. The subjects were given 200 mg of fenofibrate (Solvay) daily for 8 wk. Blood samples were collected before and after the treatment on each morning under fasting conditions. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters. Waist and hip circumferences were measured as well. The quantifying insulin sensitivity check index (QUICKI) was calculated to estimate insulin sensitivity from fasting insulin and glucose by the following equation: QUICKI = 1/[log(FINS) + log(FPG)], where FINS is fasting serum insulin concentration (μU/ml) and FPG is fasting plasma glucose (mmol/l) (15). The study was approved by the human research ethics committee of the hospital, and informed consent was obtained from each participant.

Animals. Male Sprague-Dawley rats (n = 30, 8 wk old) were purchased from Fudan University Experimental Animal Center. After 1 wk of quarantine (day 0), the rats were randomly assigned into two groups: standard chow diet (Slac Laboratory Animal; 3.8 kcal/g, 55% of energy in carbohydrate, 21% in protein, and 14% in fat) (n = 10) and high-fat diet (HFD; 5.2 kcal/g, 20% of energy in carbohydrate, 21% in protein, and 59% in fat) (n = 20). These diets contained sufficient amounts of vitamin A (20–25 IU/g). After 6 wk on HFD, 50% of the rats in the HFD group were treated with fenofibrate.
The end of the 2-wk treatment with an intraperitoneal injection of fenofibrate dosage was determined by a previous study (19). All animals had free access to diets and water under a controlled environment (19–22°C, 30–40% humidity, and 12:12-h light-dark cycle). Daily energy intake was measured in all groups during the 2-wk study.

The test was conducted at 15, 30, 60, 90, and 120 min after administration of the exogenous insulin. Plasma glucose half-life (t_{1/2}) and glucose disappearance rate [K = (0.693 × 100)/t_{1/2}] were calculated and used to estimate insulin sensitivity (25).

**Oral glucose tolerance test.** Three days after ITT, all the animals were fasted overnight (12–16 h). The rats were then given 2 g/kg of glucose through oral gavage. Blood was collected from the retroorbital sinus at 0, 30, 60, and 120 min after the glucose challenge. Glucose and insulin levels were measured in the blood collected at each time point. The areas under the curves for blood glucose (AUCBG) and insulin (AUCINS) levels during the oral glucose tolerance test (OGTT) were calculated.

**Cell culture and differentiation.** 3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% (vol/vol) calf serum (31). To induce differentiation, 2-day postconfluent/G1 phase preadipocytes (designated day 0) were fed DMEM containing 10% (vol/vol) fetal bovine serum (FBS), 1 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (MDI) until day 2. Cells were then fed DMEM supplemented with 10% FBS and 1 µg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. We tested adipocyte gene expression on day 0, day 1, day 3, and day 7. RBP4 gene expression was nearly undetectable before adipocyte differentiation, and it was induced in the late phase of adipocyte differentiation. RBP4 mRNA expression was detectable on day 3, and it increased a little bit on day 7, but still at a low level. Fenofibrate was dissolved in DMSO and added into fresh medium on day 3, and day 7, when >90% of the cells had acquired the adipose phenotype, with the doses of 10, 50, and 100 µM (11). Control cells received an equal volume of DMSO. We assayed adipocyte gene expression at 4 h, 12 h, and 24 h after addition of fenofibrate.

**Triglyceride analyses in epididymal adipose tissue and liver.** After OGTT, the animals were anesthetized and killed by inhalation of isoflurane (Abbott Laboratories). Epididymal fat pads and livers were surgically collected and stored at −80°C for further study. For TG measurements, total lipids were extracted from 100 mg of tissue. The extraction was conducted with 30 µl of tert-butyl alcohol and 20 µl of a Triton X-104-methyl alcohol mixture (1:1 vol/vol). TG content was measured with Sigma Triglyceride (GPO-Trinder) kits.

**Quantitative real-time polymerase chain reaction.** Total RNA was isolated from fat pads, livers, and 3T3-L1 adipocytes with TRIzol (Invitrogen), and 2 µg of mRNA was reverse transcribed with random hexamers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). The product (2 µl) was amplified in a final PCR volume of 50 µl with the EvaGreen TaqMan universal PCR master mix (BioRad). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI PRISM 7300 sequence detector (Applied Biosystems) for an initial denaturation at 95°C for 10 min, followed by 40 PCR cycles, each cycle consisting of 95°C for 30s, 60°C for 1 min, and 72°C for 1 min. The following primers were used: RBPA (accession no. XM_215285.4): 5′-ggacaaggtctttcg-3′ (sense) and 5′-agggagccgctacccaa-3′ (antisense), adiponectin (accession no. NM_144744): 5′-aatcgcgccagactaaag-3′ (sense) and 5′-tgttccggagatgccatctc-3′ (antisense); and β-actin (accession no. NM_007393.1): 5′-cagacaggggccccgactc-3′ (sense) and 5′-ttaagctatgtggcactc-3′ (antisense).

**Statistical analysis.** All statistical analyses were performed with SPSS version 11.5 (Statistical Package for the Social Sciences, SPSS, Chicago, IL). Each variable was examined for normal distribution, and significantly skewed variables were log transformed. Results are expressed as means ± SD or means ± SE. Characteristics of subjects among or between groups were compared by one-way ANOVA or covariance. Comparisons between groups before and after treatment were calculated by Wilcoxon signed-rank test. Spearman correlation was used to investigate main factors influencing serum RBP4. All reported P values were two-tailed, and P values < 0.05 were considered statistically significant.

**RESULTS**

**Regulation of serum RBP4 and adiponectin by fenofibrate in humans.** The patients with insulin resistance and dyslipidemia were treated with fenofibrate for 8 wk. Administration of fenofibrate significantly reduced RBP4 (34.8% vs. 40.0% vs. 24.4 ± 2.1 µg/ml; P < 0.01) and increased adiponectin (11.3 ± 6.7 vs. 16.5 ± 7.2 µg/ml; P < 0.01). This pattern change was associated with a decrease in serum insulin levels (Table 1). Before the treatment, all of the patients were hyperinsulinemic. Fenofibrate treatment decreased body weight, waist circumference, BMI, and LDL-C but increased QUICKI and HDL-C (Table 1). These data imply that fenofibrate may improve...
insulin sensitivity by reducing serum RBP4 levels in patients with insulin resistance and dyslipidemia.

**Regulation of RBP4 and adiponectin by fenofibrate in rats.**

To understand the mechanism underlying RBP4 regulation by fenofibrate, we used HFD-induced obese rats. qRT-PCR experiments revealed RBP4 mRNA expression in both the liver and adipose tissue of rats fed with normal chow diet (Fig. 1). HFD feeding greatly increased the body weight of the rats, which was significantly reduced by fenofibrate treatment (Table 2). The body weight reduction was associated with a decrease in plasma TG and LDL-C (Table 2). The total food consumption over 2 wk of fenofibrate treatment was reduced as determined by weight or caloric intake in the HFD-fenofibrate treatment (HFD-FT) group (Table 2). HFD treatment greatly increased serum RBP4 levels in non-fenofibrate-treated (HFD-UT) rats, and the HFD-induced increase in RBP4 levels was significantly reduced by fenofibrate treatment (HFD-FT) (Fig. 2A). HFD treatment reduced the serum adiponectin levels in rats, which was partially prevented (20%) by fenofibrate (Fig. 2B). To determine the relative contribution of adipose tissue and liver to serum RBP4 in response to fenofibrate treatment, we assessed the mRNA levels of RBP4 in adipose tissue and liver by real-time PCR. HFD feeding increased RBP4 mRNA levels in adipose tissue, but the increase was significantly attenuated by fenofibrate treatment (50%) (Fig. 3A). On the other hand, RBP4 mRNA levels were not significantly increased in the liver in the HFD-induced obese rats compared with control rats (Fig. 3A). In adipose tissue adiponectin mRNA was reduced almost 40% by HFD, and this reduction was attenuated by fenofibrate (Fig. 3B).

**Reduction of triglyceride accumulation in adipose tissue and liver by fenofibrate.** To determine whether fenofibrate has an effect on TG mass, we examined TG contents in adipose tissue and liver of treated and untreated rats. TG levels were higher in HFD-fed rats than in chow diet-fed rats. TG was reduced in both epididymal adipose tissue (144.0 ± 17.3 vs. 72.9 ± 17.8 mg/g; P < 0.01) and liver (25.0 ± 6.2 vs. 9.3 ± 4.0 mg/g; P < 0.01) of the fenofibrate-treated rats (Fig. 4).

**Effects of fenofibrate treatment on insulin sensitivity in rats.** Insulin sensitivity was reduced by HFD in rats. Fasting insulin, AUCBG, and AUCINS, and K values were higher in the HFD-UT group than in the lean control group (1.7 ± 0.4 vs. 0.5 ± 0.1 ng/ml, 1,136.3 ± 96.9 vs. 925.1 ± 96.6 mmol·min⁻¹·l⁻¹, 373.7 ± 18.2 vs. 187.6 ± 37.9 mg·min⁻¹·ml⁻¹, 8.4 ± 0.9 vs. 11.3 ± 0.8 min⁻¹, respectively; all P < 0.01). In the dietary obese rats, fenofibrate treatment enhanced insulin sensitivity. In the HFD-FT group, fasting insulin levels (0.7 ± 0.2 ng/ml), AUCBG (1,026.8 ± 171.2 mmol·min⁻¹·l⁻¹), and AUCINS (219.2 ± 24.8 mg·min⁻¹·ml⁻¹) were all decreased, while K values (10.8 ± 0.6 min⁻¹) were increased (P < 0.01 in all comparisons) (Fig. 5).

**Relationship of serum RBP4 and insulin resistance, triglycerides, fat distribution, and adiponectin.** Serum RBP4 levels were positively correlated to AUCBG (R = 0.44, P < 0.01) and AUCINS (R = 0.8, P < 0.01) but negatively correlated to K values (R = −0.56, P < 0.01). Serum RBP4 levels were also significantly correlated to TG contents in serum, adipose tissue, liver, and epididymal adipose tissue.

**Table 1. Effects of fenofibrate on anthropometric parameters, metabolic values, and adipocytokines in humans**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline</th>
<th>Treatment</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, kg</td>
<td>75.7 ± 9.7</td>
<td>72.0 ± 9.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>24.9 ± 3.4</td>
<td>22.4 ± 5.2</td>
<td>&lt;0.01</td>
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<tr>
<td>Waist circumference, cm</td>
<td>87.8 ± 9.4</td>
<td>84.0 ± 7.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fasting plasma glucose, mmol/l</td>
<td>6.0 ± 0.5</td>
<td>5.7 ± 0.7</td>
<td>0.08</td>
</tr>
<tr>
<td>Fasting insulin, mU/l</td>
<td>37.5 ± 3.6</td>
<td>15.8 ± 4.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.43 ± 0.01</td>
<td>0.52 ± 0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.7 ± 0.5</td>
<td>6.0 ± 0.7</td>
<td>0.08</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>6.4 ± 2.1</td>
<td>2.1 ± 0.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL-C, mmol/l</td>
<td>1.7 ± 0.2</td>
<td>2.5 ± 0.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDL-C, mmol/l</td>
<td>3.7 ± 0.5</td>
<td>2.0 ± 0.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>35.6 ± 10.5</td>
<td>45.1 ± 20.4</td>
<td>0.05</td>
</tr>
<tr>
<td>Adiponectin, µg/ml</td>
<td>11.3 ± 6.7</td>
<td>16.5 ± 7.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>RBP4, µg/ml</td>
<td>34.8 ± 4.0</td>
<td>24.4 ± 2.1</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 15 subjects. After fenofibrate treatment for 8 wk, the parameters were compared with those before treatment. The difference was analyzed by Wilcoxon signed-rank test. BMI, body mass index; TG, triglyceride; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; QUICKI, quantifying insulin sensitivity check index; ALT, alanine aminotransferase; RBP4, retinol-binding protein-4.
and liver ($R = 0.62$, $R = 0.64$, and $R = 0.62$, respectively; all $P < 0.01$). However, there was no significant association between serum RBP4 levels and serum ALT levels ($R = 0.18$, $P = 0.39$).

Serum RBP4 levels were significantly and positively correlated with serum adiponectin levels ($R = -0.44$, $P < 0.05$) and adiponectin mRNA levels in adipose tissues ($R = -0.41$, $P < 0.05$).

Regulation of RBP4 and adiponectin by fenofibrate in 3T3-L1 adipocytes. To determine whether fenofibrate has a direct effect on RBP4 mRNA expression, we treated 3T3-L1 adipocytes with fenofibrate. Treating the cells with 10, 50, and 100 μM fenofibrate for 24 h suppressed RBP4 mRNA levels by 5%, 15%, and 22%, respectively (Fig. 7A). Under the same conditions, adiponectin mRNA levels were increased by 20%, 33%, and 46% (Fig. 7B), respectively.

**DISCUSSION**

In the present study, we demonstrate for the first time that fenofibrate can markedly suppress serum RBP4 levels in both obese humans and rats. RBP4, a recently discovered adipocyte protein, may increase blood glucose by inhibiting insulin sensitivity in skeletal muscle and the liver. Reduction of serum RBP4 through enhanced urinary excretion of RBP4 improves insulin sensitivity and glucose intolerance (34). Rosiglitazone and pioglitazone, well-established insulin sensitizers, reduce
serum RBP4 and its mRNA expression in adipose tissue (10, 14). Metformin, another insulin sensitizer, also decreases serum RBP4 in patients with insulin resistance from polycystic ovary syndrome (12). These findings suggest that RBP4 may be a target gene for the therapy of insulin resistance.

PPARα activators have been shown to improve insulin sensitivity and influence adipogenic gene expression. Increase in plasma adiponectin and its mRNA expression in adipose tissue may contribute to insulin sensitization by a PPARα activator (11). Here we provide evidence showing that reduction of the serum levels of RBP4 may be a mechanism underlying the insulinsensitizing effect of the PPARα activator fenofibrate. This conclusion is supported by the findings that fenofibrate reduces serum RBP4 levels in dyslipidemic patients and diet-induced obese rats. In addition, fenofibrate treatment directly reduces RBP4 mRNA levels in 3T3-L1 adipocytes.

Several studies have demonstrated that RBP4 is a marker of insulin resistance and that its expression can be reduced by insulin sensitizers. Our findings have confirmed the crucial effects of the PPARα activator fenofibrate on alleviation of insulin resistance. Excessive TG results in fat accumulation in visceral fat and the liver, which leads to the development of insulin resistance (16). Studies from this (14) and other groups (17, 21) consistently suggest that visceral adiposity might be a main source of serum RBP4 and a linkage between RBP4 and insulin resistance. Meanwhile, weight loss by lifestyle intervention and surgery has been shown to decrease serum RBP4 (2, 7, 9). It is proposed that PPARα activators may reduce body fat content through several mechanisms, including increase in mitochondrial β-oxidation of fatty acid, inhibition of fatty acid synthesis, and mediation of negative energy homeostasis by a marked reduction of food intake (8, 18, 23). In the present

Fig. 4. Reduction of triglyceride accumulation in adipose tissue and liver by fenofibrate. Total lipids were extracted from 100 mg of epididymal fat pad or from liver of rats, and triglyceride content was quantified. Fenofibrate treatment was for 2 wk. Each bar represents mean ± SE (n = 10).

Fig. 5. Fenofibrate improved insulin sensitivity in obese rats. Values of blood glucose (A) and serum insulin (B) during oral glucose tolerance test (OGTT) and blood glucose levels during insulin tolerance test (ITT) (C) are shown. Each point represents mean ± SD (n = 10). *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. HFD-UT group.
study, we found that fenofibrate reduced food intake, TG contents locally and systemically, and body weight in both obese humans and rats. Therefore, reduction of body adiposity may be the potential mechanism underlying the inhibitory effect of fenofibrate on RBP4 expression.

Our study suggests that fenofibrate may reduce serum RPB4 through inhibition of RBP4 mRNA in the adipose tissue. However, RBP4 is predominantly expressed in the liver under normal conditions (3), and this feature was confirmed in the present study. Our data showed that RBP4 mRNA in the liver is six times higher than in the adipose tissue in rats (Fig. 1). Recent studies implied that serum RBP4 is associated with liver diseases, such as nonalcoholic fat liver disease and liver cirrhosis (29, 32, 33), suggesting that RBP4 gene expression in liver may be influencing serum RBP4. However, we found that hepatic RBP4 was not significantly affected by fenofibrate. In contrast, adipose RBP4 was significantly reduced by fenofibrate in visceral fat. Our observation is consistent with findings that hepatic RBP4 expression is not involved in the development of insulin resistance (29, 32). Moreover, correlation analyses showed that serum RBP4 is correlated to RBP4 mRNA in adipose tissue but not in liver (Fig. 6). Consistent with these results, fenofibrate directly and moderately reduced

Fig. 6. Relationship of serum RBP4 with its mRNA expression in adipose tissue and liver. A: serum RBP4 is significantly correlated to its mRNA levels in the adipose tissue of rats ($R = 0.56$, $P < 0.01$). B: serum RBP4 is not significantly correlated to its mRNA levels in the liver ($R = 0.39$, $P = 0.07$). Spearman correlation analysis was conducted on data pooled from all rats.

Fig. 7. Effects of fenofibrate on mRNA expressions of RBP4 and adiponectin in 3T3-L1 adipocytes. A: fenofibrate downregulated RBP4 mRNA expression in 3T3-L1 adipocytes. B: fenofibrate upregulated adiponectin mRNA expression in 3T3-L1 adipocytes. Fenofibrate was dissolved in DMSO and added into fresh medium on day 7, when >90% of the cells had acquired the adipose phenotype, with the doses of 10, 50, and 100 µM. Control cells received an equal volume of DMSO. Each point represents mean ± SE ($n = 3$). *$P < 0.05$, **$P < 0.01$ vs. control cells.
RBP4 mRNA level in 3T3-L1 adipocytes (Fig. 7). These data suggest that suppression of RBP4 expression levels in adipose tissue may play a major role by which fenofibrate reduces serum RBP4.

In conclusion, our study suggests that reduction of RBP4 levels by fenofibrate may contribute to insulin sensitization in both humans and rats. The mechanism is related to reduction of body adiposity and direct suppression of RBP4 expression in the adipose tissue. This study provides new evidence for fenofibrate action in the improvement of insulin sensitivity. Our observation suggests a new molecular mechanism for the therapeutic activities of the PPARα activator.

REFERENCES


