Fenofibrate prevents orotic acid—Induced hepatic steatosis in rats

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Abstract

The experiments performed in this report were designed to investigate the mechanisms involved in the metabolic alterations associated with orotic acid-induced hepatic steatosis and the effect of fenofibrate, a stimulant of peroxisome proliferators-activated receptor α (PPARα), on these alterations. Male Wistar rats were divided into three experimental groups: 1) fed a balanced diet (C); 2) fed a balanced diet supplemented with 1% orotic acid (OA); 3) fed OA diet containing 100 mg.kg⁻¹ bw.day⁻¹ fenofibrate (OA + F), for 9 days.

Administration of OA to rats induced significant increase in the hepatic total lipids content, marked microvesicular steatosis and decrease in plasma lipids concentrations compared to control group. Fenofibrate treatment prevented fatty liver induction, caused an additional reduction on plasma lipids concentrations and caused a 40% decrease in the lipogenic rate in adipose tissue. The results also showed a 40% increase in lipoprotein lipase (LPL) activity in adipose tissue from OA treated group and fenofibrate administration induced a 50% decrease in LPL activity. The liver mRNA expression of PPARα and ACO (acyl CoA oxidase) were 85% and 68% decreased in OA group when compared to control, respectively. Fenofibrate treatment increased the PPARα and ACO expressions whereas the CPT-1 (carnitine palmitoyl transferase-1) expression was not altered. Our results have shown that fenofibrate treatment decreases the hepatic lipid content induced by OA which is mediated by an important increase in fatty acid oxidation consequent to an increase in hepatic mRNA expression of PPARα and ACO.

Keywords: Hepatic steatosis; Fenofibrate; PPARα; Lipid metabolism; Adipose tissue

Introduction

Nonalcoholic fatty liver disease is a clinicopathological term that encompasses a disease spectrum ranging from simple triglyceride accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation (steatohepatitis), fibrosis and cirrhosis (Neuschwander-Tetri and Caldwell, 2003). Excess hepatic triglyceride accumulation is associated with various drugs, nutritional factors, and multiple genetic defects in energy metabolism. Prevention of fatty liver is very important for guarding against degenerative disease. In rats, this condition is induced by administration of large amounts of orotic acid (Standerfer and Handler, 1955; Aoyama and Wada, 2000), an intermediate in the pyrimidine nucleotide synthetic pathway. Accumulation of lipids (mainly triglycerides) within hepatocytes, induced by orotic acid, is due to, at least in part, inhibition of lipoprotein synthesis. Lipoprotein precursors, i.e. the apoprotein and lipids moieties, are actually synthesized, but the apoprotein is deficient in N-acetylglucosamine, galactose
and N-acetyl neuramic acid (Martin et al., 1982) and the conjunction of the two moieties in the liver is impeded (Roheim et al., 1965), resulting in a progressive fatty liver with concomitant loss in serum very low density lipoproteins (VLDL) and triglyceride (TG) (Windmueller and Levy, 1967; Hay et al., 1988).

In addition to the effect of orotic acid supplementation on hepatic VLDL secretion, it has been reported that this component is capable of altering the transcription of genes involved in fatty acid synthesis (Buang et al., 2005; Griffin et al., 2004), growth and inflammation control (Griffin et al., 2004). Other studies have reported that orotic acid administration produces alterations on metabolism of tryptophan to niacin (Fukuwatari et al., 2002) and improves cardiac function (Ferdinandy et al., 1998).

The pathogenesis of fatty liver is intimately related to derangements in the regulation of the fatty acid, triglyceride and carbohydrate metabolism. Recent study (Degrace et al., 2007) has shown that during the development of hepatic steatosis in carnitine-depleted rats there is a strong regulation of lipid flux between liver and adipose tissue. Kolak et al. (2007) also have shown that the adipose tissue is inflamed and have increased ceramide content in subjects with high fat liver content independent of obesity. However, to our knowledge, the relationship between the fatty liver induced by orotic acid and the adipose tissue metabolism remains poorly understood.

Nutrient metabolism and energy homeostasis are tightly regulated by endocrine, paracrine, and autocrine signals that control the expression and activity of key metabolic enzymes by transcriptional and post transcriptional mechanisms. Lipid mediators play a critical role in metabolic control, and peroxisome proliferator-activated receptors (PPARs), a class of ligand-dependent transcription factors, have emerged as master transcriptional and post transcriptional mechanisms. Lipid mediators play a critical role in metabolic control, and peroxisome proliferator-activated receptors (PPARs), a class of ligand-activated transcription factors, have emerged as master transcriptional regulators of lipid and carbohydrate metabolism. Recent study (Degrace et al., 2007) has shown that during the development of hepatic steatosis in carnitine-depleted rats there is a strong regulation of lipid flux between liver and adipose tissue. Kolak et al. (2007) also have shown that the adipose tissue is inflamed and have increased ceramide content in subjects with high fat liver content independent of obesity. However, to our knowledge, the relationship between the fatty liver induced by orotic acid and the adipose tissue metabolism remains poorly understood.

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Liver samples were fixed at 4 °C in 4% paraformaldehyde dissolved in phosphate-buffer saline. The tissue was dehydrated, embedding in glycol-metacrilate, and cut into sections (3 µm). Liver sections were then stained with toluidine blue for histological and morphometric analyses.

Adipocyte isolation

Adipocytes were isolated from epididymal fat pads by the method of Rodbell, 1964. Digestion was carried out at 37 °C with constant shaking for 45 min. Cells were filtered through nylon mesh and washed three times with buffer containing (mM): 137 NaCl, 5 KCl, 4.2 NaHCO3, 1.3 CaCl2, 0.5 MgCl2, 0.5 MgSO4, 0.5 KH2PO4, 20 HEPES (pH 7.4), plus 1% BSA.

Lipolysis measurements

Lipolysis was measured by following the rate of glycerol release, as previously described (Gasic and Green, 1995). After washing, adipocytes were incubated at 37 °C in a water bath for 60 min, in the presence or absence of the isoproterenol (ISO 0.1 μM) and the effects of insulin on isoproterenol-stimulated lipolysis were determined by constructing dose–response curves using 0–12.5 ng/ml. At the end of the incubation period, an aliquot of the infranatant was removed for enzymatic determination of glycerol released into the incubation medium (LABTEST, Lagoa Santa, MG).

Lipoprotein lipase (LPL) activity

Samples of epididimal adipose tissue (50 mg) were homogenized in buffer containing heparin and detergents (Iverius and Ostlund-Lindqvist, 1986) and total LPL activity was measured.
using a [9,10-\textsuperscript{3}H]triolein-containing substrate emulsified with lecithin (Nilsson-Ehle and Schotz, 1976) and contained 24 h fasted rat plasma as a source of apo CII. The reaction was stopped with extraction mixture (Belfrage and Vaughan, 1969) and liberated [\textsuperscript{3}H]-free fatty acids were quantified by liquid scintillation. The enzyme activity was expressed as nmol of [\textsuperscript{3}H]-fatty acid released/min.

In vitro lipogenesis

Portions of epididymal adipose tissue (200 mg) were incubated at 37 °C for 2 h in the buffer described above, to which had been added 5 mM glucose and 150 μCi/ml of \textsuperscript{3}H\textsubscript{2}O as a radioisotopic tracer. At the end of the incubation period, [\textsuperscript{3}H]-total lipids were extracted with chloroform-methanol 2:1 (Folch et al., 1957) and lipid counting was performed by adding a toluene-diphenyloxazole scintillation fluid (5 g/L). Rates of lipid synthesis were calculated as previously described (Windmueller and Spaeth, 1966).

Real-time quantitative RT-PCR

Total RNA was prepared from frozen liver by the method of Chomczynski and Sacchi (1987). First strand cDNA was generated from 2 μg RNA in a 50 μl final volume of reaction using the oligo (dT) primer, and the reverse primers of PPAR\textsubscript{α}, ACO, CPT-1 and β-actin. After reverse transcription (RT), cDNA was used for real-time PCR using a SYBR\textsuperscript{®}Green PCR buffer. The β-actin was co-amplified as an endogenous normalizing gene. Absolute quantification by real-time amplification was carried out in a ABI PRISM 7000 SDS apparatus as follows: (STEP 1) 52 °C/2 min, (STEP 2) one cycle at 95 °C/10 min, (STEP 3) 40 cycles of 95 °C/30 s, and (STEP 4) 50 °C/1 min. The following primer pairs were used: PPAR\textsubscript{α} forward: 5′-taccactatggagtccacgcatgt-3′, PPAR\textsubscript{α} reverse: 5′-ttgcagcttgagatcacacttgtcg; ACO forward: 5′-atctctgtggttgctgtggagtca 3′, ACO reverse: 5′-tctggatgcttccttctccaaggt-3′; CPT-1 forward: 5′-acgtgagtgactggtgggaagaat-3′, CPT1 reverse: 5′-tctccatggcgtagtagttgctgt-3′; β-actin forward: 5′-catgaagatcaagatcattgctcct-3′, β-actin reverse: 5′-ctgcttgctgatccacatctg-3′. All absolute quantitative values were related to a calibration curve generated for each gene target preparation and were normalized by β-actin mRNA expression calibration curve.

Plasma analysis and total lipid content of liver

Plasma triglyceride, total cholesterol, HDL-cholesterol and glycerol were assayed by conventional enzymatic methods using kits produced by KATAL (Belo Horizonte, MG). Free fatty acids (FFA) were estimated using commercial kit from Wako (Pure Chemical Industries, Japan). Plasma leptin was determined by radioimmunoassay (Linco Research, St Charles, MO). Total lipids were extracted from hepatic tissue using the method described by Folch et al.

Statistical analysis

All values shown are expressed as mean±S.E. A one-way analysis of variance (ANOVA) was used to assess the statistical significance across all groups. When tested as significant a post hoc (Newman Keuls) test was used to establish differences between the groups. All data and statistical analyses were performed using GraphPad Prism version 4.0 for Windows, GraphPad Software, San Diego California, USA. Differences were considered statistically significant at the \( p < 0.05 \) level.

Results

Administration of orotic acid to rats for 9 days induced the expected modification on hepatic total lipid content (Fig. 1) and plasma lipid concentration (Table 1) with significant increase in total fat liver content (160%) and decreases in plasma
triglyceride concentration (30%), total cholesterol (28%) and HDL-cholesterol (18%), compared to control animals. The orotic acid treated group showed 100% increase in FFA plasma levels, whereas no changes were observed in plasma glycerol concentration (Table 1).

The treatment with fenofibrate (100 mg.Kg\(^{-1}\) bw.day\(^{-1}\)) prevented OA-induced lipid accumulation (Fig. 1) along with additional reduction on plasma triglyceride (50%), cholesterol (46%), HDL-cholesterol (60%) and no changes on plasma FFA or glycerol levels, compared to OA treated group (Table 1). Consistently, histological examination of liver from OA treated rats demonstrated a marked microvesicular steatosis (Fig. 2) which was attenuated by fenofibrate administration (Fig. 2). These results were confirmed by morphometric analysis of liver samples. As shown in Table 2, hepatocyte lipid droplets volume increased in the animals treated with OA when compared to control group, and fenofibrate treatment prevented lipid accumulation induced by orotic acid. Besides its effects on hepatocyte fat droplets volume, the fenofibrate also increased the cellular, nuclear and cytoplasmatic volumes of cells (Table 2).

The orotic acid diet did not change body weight gain, epididymal and retroperitoneal fat pads weight compared to control diet. However, animals fed orotic acid plus fenofibrate showed reductions in body weight gain (58%), in epididymal (33%) and retroperitoneal (53%) adipose tissue mass when compared to OA group. Nevertheless, food intake was similar among the groups (Table 1).

Plasma leptin levels were also measured in these rats. As shown in Table 1, OA administration produced approximately 40% decrease in plasma leptin levels, compared to the control group. Fenofibrate supplementation to OA group for 9 days caused a marked reduction in plasma leptin levels. However, as shown in Fig. 3, positive correlation between leptin levels and adipose tissue weight was not influenced by orotic acid diet and fenofibrate treatment (\(r=0.85\)).

The lipogenic response, shown in Fig. 4, was altered only by the association of orotic acid and fenofibrate treatment as measured by incorporation of \(^3\)H\(_2\)O in total lipids from epididymal fat pads. The result shows a 40% decrease in \(de\\,\,nov\)o lipogenic activity when the two agents were administered simultaneously.

To determine the effect of orotic acid and fenofibrate on lipolysis, adipocytes were incubated in a basal or isoproterenol (ISO)-stimulated conditions additionally to insulin sensitivity of lipolysis measurement. Since basal lipolysis is low, the antilipolytic action of insulin was tested against ISO-stimulated lipolysis. The presence of ISO produced a similar and significant increase in lipolytic rate in all groups (Table 3). These results demonstrate that insulin sensitivity of lipolysis did not change with OA diet nor with fenofibrate plus OA treatments (Fig. 5).

Circulating triglyceride-fatty acid uptake was estimated by measuring the LPL activity, the enzyme that hydrolyzes the core of triglyceride-rich lipoproteins into free fatty acids and monoglyceride in epididymal fat pads. The results showed a 40% increase in LPL activity in epididymal adipose tissue from OA treated rats when compared to control groups. Fenofibrate treatment reduced in 50% LPL activity compared to OA treated animals (Fig. 6).

We examined gene expression of important liver peroxisomal and mitochondrial \(\beta\)-oxidation enzymes, ACO and CPT-I respectively, and PPAR\(\alpha\) in each group (Fig. 7). Real-time PCR absolute quantification targets were performed to measure mRNA using specific primers for each ACO, CPT-I and PPAR\(\alpha\), and the housekeeping gene \(\beta\)-actin was used as internal control after reverse transcription analyses. Expression of PPAR\(\alpha\) mRNA decreased (85%) in OA treated group when compared to control animals. Interestingly, fenofibrate treatment increased 14 folds the liver PPAR\(\alpha\) mRNA expression compared to OA group (Fig. 7A). Since transcription rate of ACO is regulated by PPAR\(\alpha\), its mRNA expression was also measured in this work. Orotic acid treatment decreased ACO mRNA in 68% when compared to control animals. This orotic acid effect

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**Table 3**

Basal and isoproterenol-stimulated (ISO) glycerol release by epididymal adipocytes isolated from control, orotic acid and fenofibrate treated rats

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<thead>
<tr>
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<th>Glycerol release ((\mu)mol/h)</th>
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<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>Control (C)</td>
<td>0.73±0.08</td>
</tr>
<tr>
<td>Orotic acid (OA)</td>
<td>0.71±0.06</td>
</tr>
<tr>
<td>Fenofibrate (OA+F)</td>
<td>0.70±0.1</td>
</tr>
</tbody>
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Data are means±SEM for \(n=6\)–7 rats; *\(p<0.05\) vs Basal.

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**Fig. 5.** Antilipolytic action of insulin on 0.1 \(\mu\)M ISO-stimulated lipolysis in the absence and presence of increasing amounts of insulin. Data are reported as means±SEM for \(n=6\)–7 animals.

**Fig. 6.** Effect of orotic acid diet and fenofibrate treatment on lipoprotein lipase activity of epididymal fat pads. Data are reported as means±SEM for \(n=6\) animals. *\(p<0.05\) vs C, #\(p<0.05\) vs OA.
was prevented by fenofibrate treatment (100 mg·Kg\(^{-1}\) bw. day\(^{-1}\)) yielding 300% increase in ACO mRNA compared to the OA treated group. Liver mitochondrial β-oxidation enzyme CPT-1 was also evaluated, however no difference was found among groups (Fig. 7C).

Discussion

In the present study, we demonstrated that short-term (9 days) administration of orotic acid to rats caused marked lipid accumulation in liver, confirming data from previous work (Aoyama and Wada, 2000; Windmueller and Levy, 1967). Microvesicular steatosis was evident in hepatic sections from rats receiving 1% of OA diet. This form of fatty liver injury is closely associated with hepatotoxicity produced by drugs such as valproic acid, tetracycline, and some nonsteroidal anti-inflammatory agents (Breen et al., 1975; Fromenty et al., 1990).

In addition to the effect of OA on hepatic lipid accumulation, we also found an increase in adipose tissue LPL activity when compared to the control animals. This increase in the LPL activity might be a parallel contributory factor to the observed reduction in plasma TG concentration so markedly induced by orotic acid ingestion. Furthermore, the increased LPL activity on OA-treated rats could contribute to the maintenance of TG stores in adipose tissue.

Another study has demonstrated that fibrate-activated PPAR\(\alpha\) controls LPL activity by inducing its expression in the liver and by inhibiting the hepatic expression of apo C-III, an LPL activity inhibitor (Staels et al., 1995). These effects promote hydrolysis of triglyceride-rich lipoproteins, thus decreasing the plasma levels of triglycerides.

Although the fatty liver produced by orotic acid administration seems to result from inhibition of VLDL synthesis and secretion (Windmueller and Levy, 1967; Hay, 1988), the possibility that alterations in fatty acid β-oxidation cannot be excluded since mRNA expression of an important transcriptional factor and an enzyme involved on peroxisomal β-oxidation, PPAR\(\alpha\) and ACO, respectively, was down regulated, as showed by this work. Previous studies have shown that alterations of peroxisomal proliferation and fatty acid metabolism contribute to nonalcoholic fatty liver disease (Everett et al., 2000; Aoyama et al., 1998; Fan et al., 1996; Leone et al., 1999; Costet et al., 1998). Additionally, it can be postulated that the increased plasma free fatty acid levels found in OA-treated rats may be a consequence of the decreased hepatic peroxisomal β-oxidation, since the ACO mRNA levels were low, and no differences were found in the expression of CPT1 neither in the lipolytic activity of epididymal adipocytes.

The treatment with fenofibrate was able to prevent hepatic steatosis induced by orotic acid by activating the ACO expression, an enzyme involved in peroxisomal fatty acid turnover. Similar antisteatotic actions of PPAR\(\alpha\) agonists were observed in other models of nonalcoholic fatty liver disease, such as Shionogi mice (Harano, 2006), choline-deficient diet-fed mice (Ip et al., 2003) and high-fat diet-fed mice (Mancini et al., 2001).

As an alternative or concomitant mechanism, PPAR\(\alpha\) activation might inhibits pro-inflammatory genes as showed by other authors (Shiri-Sverdlov et al., 2006; Stienstra et al., 2007). In support for this hypothesis is the demonstration that PPAR\(\alpha\) activators inhibit NF\(\kappa\)B signaling, resulting in lowered production of cytokines by smooth muscle cells and decreased plasma concentrations of cytokines (Staels et al., 1998). Whether the hepatic steatosis induced by orotic acid is related to the reduction on anti-inflammatory effects of PPAR\(\alpha\) and whether the anti-steatotic actions of fenofibrate involves the activation of anti-inflammatory genes remains to be determined.

In line with the anti-steatotic role, it can be proposed that PPAR\(\alpha\) plays a central role in a pathway that serves to minimize fat storage in adipose tissue as showed by us (Ferreira et al., 2006) and other (Unger and Orci, 2000). The reductions in body weight and adiposity induced by fenofibrate treatment can be, at least partially, consequent to decreased triglyceride uptake, resulting from reductions in LPL activity in adipose tissue, and reduced de novo lipogenesis, one of the pathways providing fatty acyl-CoA for tissue triglyceride synthesis and storage in adipose tissue (Ferreira et al., 2006).

Furthermore, we provide evidence that fenofibrate acts as a “weight-stabilizer” through enhancement of lipid catabolism in rat liver. This effect is mediated by PPAR\(\alpha\), mainly through the

Fig. 7. Effect of orotic acid diet and fenofibrate treatment on liver mRNA expression of PPAR\(\alpha\) (A), ACO (B) and CPT-1 (C). Data are reported as means ±SEM for \(n=3\) animals. *\(p<0.05\) vs C, †\(p<0.05\) vs OA.
induction of target enzymes involved in hepatic lipid metabolism. Although both peroxisomal and mitochondrial compartment contribute significantly to increase oxidation of FFA, our data do not support an important mitochondrial role in wasting energy, which is, instead, an intrinsic property of peroxisomal β-oxidation, as also shown by other author (Mancini et al., 2001). Fan et al. (1996) have shown that deficiency of the enzymes of peroxisomal β-oxidation has been recognized as an important cause of microvesicular steatosis and steatohepatitis. In addition, another study has demonstrated that OA administration did not affect hepatic enzyme activity and mRNA expression of CPT1, the rate-limiting step for mitochondrial fatty acid oxidation (Buang et al., 2005). Our data, along with that of Buang et al. (2005), argues that hepatic steatosis produced by orotic acid administration is not associated with an impairment of mitochondrial oxidation of fatty acids, since the activity and expression of CPT1 were not altered by the treatments used in these studies.

The increased PPARα expression and consequent upregulation of ACO mRNA expression in the liver induced by fenofibrate, indicate an important role of this hypolipidemic agent in the reduction of fat liver content induced by orotic acid administration. It is possible that both the increase in liver PPARαs and ACO expression induced by fenofibrate can also be an important factor in reducing fat storage in adipose tissue observed in this work and previously reported by us (Ferreira et al., 2006). Whether other similar compounds exhibit the same effects remains to be determined. This study unveils a potential therapeutic function of fenofibrate in hepatic steatosis.

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