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Sensitivity of liver metabolism from jerboa (*Jaculus orientalis*) to ciprofibrate, a peroxisome proliferator

**Driss Mountassif^{1,2}, Mostafa Kabine², Karima Mounchid³, Khadija Mounaji⁴,
Norbert Latruffe^{1(*)} and M'Hammed Saïd El Kebbaj²**

¹ INSERM U866; Université de Bourgogne, Laboratoire de Biochimie Métabolique et Nutritionnelle, Faculté des Sciences, 6 Bd Gabriel, 21000 Dijon cedex, France.

² Laboratoire de Biochimie et Biologie Moléculaire, ³ Laboratoire d'Histologie et Embryologie and ⁴ Laboratoire de Physiologie et Génétique Moléculaire, Université Hassan II - Aïn Chock, Faculté des Sciences, Casablanca, Morocco.

(*) Corresponding author. latruffe@u-bourgogne.fr; tel +33 3 80 39 62 36 ; Fax +33 3 80 39 62 50

Abstract

Ciprofibrate is one of the well known drug used to normalize lipid parameters and fibrinogen in atherosclerosis-developing patients. On the other hand it exhibits a peroxisome proliferator activity in laboratory rodents animals (mice, rats). So far no clear alteration or lack of side effect of ciprofibrate were claimed in human species. In order to further investigate these possible relationships we studied the consequence of a sustained ciprofibrate treatment of jerboa (*Jaculus orientalis*) knowing that in this species ciprofibrate lacks to induce hepatomegaly and to promote liver cells DNA replication (El Kebbaj et al., 1996, *Eur J Cell Biol* 70: 150-6) making this species closer to human than as it to rat or mice. The Jerboas were daily treated with ciprofibrate at 3 mg/kg body weight for 4 weeks. Then, subcellular markers, clinical enzymes and enzymatic antioxidants defences were assessed. Our results show a strong decrease of peroxisomal catalase activity and an increase in MDA (malondialdehyde) level (stress biomarker). Moreover, ciprofibrate *in vivo* and *in vitro* inhibits D-3-hydroxybutyrate dehydrogenase (BDH) a mitochondrial enzyme of the ketone bodies interconversion and important in the redox

balance (NAD⁺/NADH+H⁺ ratio). In conclusion, in these conditions ciprofibrate induces an alteration of liver oxidative metabolism.

Key words

Ciprofibrate, *Jaculus orientalis*, clinical enzymes, antioxidant enzymes, subcellular marker, D-3-hydroxybutyrate dehydrogenase.

Abbreviations

BDH, D-3-Hydroxybutyrate dehydrogenase; CPK, Creatine phosphokinase; DL-BOH, DL-3-hydroxybutyrate ; DCIP, Dichloroindophenol; EDTA, Ethylenediamine tetraacetic acid; D-G-3-P, D-Glycerol-3-phosphate; GOT, Glutamate Oxalate Transaminase; GPT, Glutamate Pyruvate Transaminase; HDL, High Density Lipoprotein; KCN, Potassium cyanide; LDL, Low Density Lipoprotein; NAD(H), Nicotinamide Adenine Dinucleotide oxidized (reduced) forms; NADPH, Nicotinamide Adenine Dinucleotide Phosphate reduced form.

1. Introduction

Fibrate drugs have been used pharmacologically to reduce plasma triglycerides and cholesterol. They are among a diverse group of chemicals called peroxisome proliferators that produce huge increase in both the size and number of peroxisomes in cells. Peroxisome proliferators which include plasticizers, herbicides and hypolipidaemic drugs, are both species- and tissue-specific (rodent liver). Peroxisome is a cell compartment containing enzymes involved in the oxidative metabolism (H_2O_2 -producing oxidases, peroxidase/catalase, Mn superoxide dismutase) and is also involved in lipid catabolism via beta-oxidation [1].

Ciprofibrate is one of the basic drugs used to lower risk values of lipid parameters and fibrinogen in atherosclerosis patients [2]. The treatment of primates with ciprofibrate leads to an increase of hepatic mitochondria and peroxisomes without proved evidence of cellular proliferation [3]. Interestingly, El Kebbaj et al. [4] reveal that the jerboa shows unique peroxisome properties and responds in a moderate manner to a peroxisome proliferator such as ciprofibrate, neither leading any hepatomegaly nor to promote liver cells DNA replication in contrast to rat. Jerboa (*Jaculus orientalis*) is an unique nocturnal herbivorous rodent living in the subdesert highland of Morocco. It is an appropriate organism to study PPAR (peroxisome proliferator-activated receptor)-dependent metabolic regulation due to its remarkable tolerance to heat, cold and scarce diet by hibernating [5-8].

The purpose of this study was to provide the impact of ciprofibrate on the cellular antioxidants, metabolic pathways and their effects on the D-3-hydroxybutyrate dehydrogenase activity, protein level and kinetic parameters in mitochondria. So far no clear alteration or lack of side effect of ciprofibrate were claimed in human species. In order to further investigate these possible relationships we studied the consequence of a sustained ciprofibrate treatment of jerboa (*Jaculus orientalis*). The Jerboas were daily treated with ciprofibrate at 3 mg/kg body weight for 4 weeks. Then the subcellular

markers, the clinical enzymes and enzymatic antioxidants defences were assessed. Our results show a strong decrease of peroxisomal catalase activity and an increase in MDA (malondialdehyde) level (stress biomarker).

Furthermore, we pay attention to the inhibitory effect of ciprofibrate on the mitochondrial inner membrane bound NAD-dependent D-3-hydroxybutyrate dehydrogenase (BDH, EC. 1.1.1.30) from liver which play an important role in the redox balance ($\text{NAD}^+/\text{NADH}+\text{H}^+$ ratio) by interconverting acetoacetate into D-3-hydroxybutyrate [9]. Any alteration of its activity will affect the energetic metabolism. A catalytic mechanism of the interconversion of D-3-hydroxybutyrate and acetoacetate in both liver and peripheral tissues has been previously proposed by our group [10]. Moreover, this enzyme from Jerboa has been extensively studied in our lab [11-16].

2. Materials and Methods

2.1. Chemicals

Ciprofibrate (2-[4-(2, 2-dichlorocyclopropyl) phenoxyl]-2-methyl propanoic acid) was a gift from Sanofi Sterling Winthrop, Dijon. DL-3-hydroxybutyrate (sodium salt) was purchased from Fluka (Buchs Switzerland); NAD^+ (free acid) and NADH were from Boehringer (Mannheim, Germany); succinate and cytochrome c were from Sigma (St Louis, USA) and all other chemicals were of analytical grade.

2.2. Animal and administration of ciprofibrate

Adult jerboas (120 - 150 g), 4-6 months old, were captured in the area of Engil Aït Lahcen (in subdesert East Moroccan highland). They were adapted to laboratory conditions during 3 weeks at a temperature of 22°C with food (salad and rat diet), and water ad libitum. The light cycle during the entire experiment was set to 14 hours light and 10 hours

dark. The ciprofibrate was dissolved in 1 ml sterile water and administered daily by intraperitoneal injection for four weeks. Four jerboas were given ciprofibrate (3 mg/kg/day) while corresponding groups were given sterile water serving as control. All the experiments were done in compliance with the Guide for The Care and Use of Laboratory Animals.

2.3. Blood analysis

The determination of the total cholesterol, HDL, LDL, triglycerides, CPK and transaminases (glutamate oxalate transaminase (GOT) and glutamate pyruvate transaminase (GPT)) was carried out by Centre National de Transfusion Sanguine, Casablanca, using the conventional methods.

2.4. Liver mitochondria isolation

The jerboas were decapitated and the liver were rapidly removed for mitochondrial extraction according to Fleischer et al. [17]. This method allowed the preparation of high yield and pure mitochondria light fraction. We previously reported by using markers enzymes that purity of mitochondria were estimated to 86% [18].

2.5. Biochemical assays

All assays were conducted at 37°C using Jenway 6405 UV/Visible spectrophotometer and done with liver homogenate.

2.5.1. Thiobarbituric acid reactive substances

Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents according to the method described by Samokyszyn and Marnett [19].

One ml of liver homogenate samples was added to 1 ml solution (0.375 % thiobarbituric acid and 15 % trichloroacetic acid in 0.25 M hydrochloric acid). The tubes were heated at 100°C during 15 min. Then, they were cooled in the ice to stop the reaction. One then carried out a centrifugation with 1000g during 10 min. The reading of supernatant was made to 535 nm.

2.5.2. Catalase

The consumption of 7.5 mM H₂O₂ by peroxisomal catalase made in 50 mM potassium phosphate buffer (pH 7) was monitored at 240 nm according to Aebi [20].

2.5.3. Acyl-CoA oxidase

This peroxisomal enzyme was assayed according to Lazarow and De Duve [21] with assay conditions: 50 mM phosphate buffer (pH 7.4), 0.2 mM NAD⁺, 50 μM FAD⁺, 12 mM, 1 mM KCN, 200 μg of BSA and 100 μg of protein. The mixture was preincubated 10 min at 37°C before adding 50 μM of palmitoyl-CoA (Sigma) as final concentration. The measure of activity was done at 340 nm.

2.5.4. Glutathione reductase

The procedure of Di Ilio et al. [22] was used to measure cytosolic glutathione reductase. Assay mixture contained 0.5 mM oxidized glutathione, 1 mM EDTA, 0.1 mM NADPH and 50 mM potassium phosphate buffer (pH 7.4) and NADPH consumption was monitored at 340 nm.

2.5.5. Superoxide dismutase

The Mn-dependent peroxisomal enzyme was assayed according to Paoletti et al. [23] with assay conditions: 5 mM EDTA, 2.5 mM MnCl₂, 0.27 mM NADH, 3.9 mM 2-mercaptoethanol in 50 mM potassium phosphate buffer (pH 7), monitored at 340 nm. The activity was started by the addition of NADH to 0.27 mM final concentration.

2.5.6. NADPH-cytochrome c reductase

Endoplasmic reticulum-linked NADPH-cytochrome c reductase was measured as described by Williams and Kamin [24]. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.7), 1.25 mg/ml cytochrome c, 0.1 mM EDTA and 100 µg of protein were incubated during 2 min at 37°C, then the NADPH at 100 µM final concentration was added and the increase in absorbance due to the reduction of cytochrome c was measured.

2.5.7. Glycerol 3-phosphate dehydrogenase

This cytosolic enzyme was assayed according to Bentley et al. [25] with assay conditions: 100 mM glycine - NaOH buffer (pH 9), 5 mM NAD⁺ and 100 µg of protein. The mixture was preincubated 2 min at 37°C before adding 5 mM of glycerol-3-phosphate (Sigma) as final concentration. The activity was recorded at 340 nm.

2.5.8. Glyceraldehyde 3-phosphate dehydrogenase

Cytosolic GAPDH activity was determined by monitoring NADH generation at 340 nm as described by Serrano et al. [26]. The reaction mixture of 1ml contained 50 mM Tricine-NaOH buffer pH 8.5, 10 mM sodium arsenate, 1 mM NAD⁺ and 2 mM D-G-3-P.

2.5.9. Succinate dehydrogenase

This mitochondrial membrane-bound enzyme was assayed according to King [27] with assay conditions: 100 mM potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.053 mM DCIP and 100 µg of mitochondrial proteins. The mixture was preincubated 10 min at 37°C before adding 50 µl of KCN-succinate (containing 3.25 mg/ml of KCN in 0.5 M succinate). The measure of activity was done at 625 nm.

2.5.10. D-3-hydroxybutyrate dehydrogenase

Mitochondrial BDH activity was measured as described by Lehninger [28] following NADH production at 340 nm ($\epsilon = 6.22 \times 10^3 \text{ M}^{-1}.\text{cm}^{-1}$) using 100 μg of frozen/thawed liver mitochondrial protein in a medium containing: 6 mM potassium phosphate pH 8, 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol, 0.3 mM dithiothreitol, in the presence of 2 mM NAD^+ (Sigma) and 2.5 μg rotenone (final addition) to prevent NADH reoxidation by the respiratory chain. The activity was started by the addition of DL-3-hydroxybutyrate (Sigma) to 10 mM final concentration.

2.5.10.1. *In vitro* BDH inhibition and BDH kinetic studies

For the determination of ciprofibrate concentration giving 50% inhibition (IC_{50}) of BDH, the activities were measured as described previously in the absence and the presence of different ciprofibrate concentrations after preincubation for 5 minutes. The ciprofibrate was added at zero time of incubation. Aliquots were removed at different times during the incubation for the enzymatic activity measurement. Aliquots of the control assay were removed at the same time in order to calculate the percentage of BDH activity.

For kinetic studies, initial velocities of the enzymatic reaction (in the presence of ciprofibrate at 0; 0.15; 0.3 and 0.6 mM) were performed by varying the concentration of the substrates, BOH (from 2.5 to 10 mM) or NAD^+ (from 0.5 to 2 mM). Values of the Michaelis constants (K_M), dissociation constants (K_D) and maximal velocity for the oxidation of BOH and the reduction of NAD^+ by the BDH were obtained by mathematical analysis according to the method of Cleland [29].

2.5.10.2. Western-blotting

After SDS-PAGE (12%) [30] and subsequent transfer in nitrocellulose [31], the mitochondrial proteins (50 μg) were exposed to 1/100 dilution of monospecific rabbit polyclonal anti-BDH anti-rat antibody [32] and detected with the secondary antibody of

anti-rabbit, IgG peroxidase conjugate (diluted to 1/2500) (Promega). Control of equivalent loaded amount of proteins in each track were controlled by coomassie blue staining of the overall proteins before and after electrotransfer as alternative to possible changes of single housekeeping protein.

2.5.11. Protein assay

Protein content was measured according to the Bradford procedure, using bovine serum albumin (BSA) as standard [33].

2.6. Statistical data analysis

In each assay, the experimental data represent the mean of four independent assays \pm standards deviations. Means were compared using the Student t-test. Differences were considered significant at the level $p < 0.05$ and very significant at the level $p < 0.01$. The calculation of the inhibition concentrations (IC) is carried out by the analysis of probit [34].

3. Results and discussion

3.1. *In vivo* effect of ciprofibrate on clinical enzymes, lipidic assessment and on response of metabolic and stress biomarkers

The table 1 indicates that plasma parameters i.e. glucose, triglycerides, cholesterol (total, HDL and LDL fractions) remain unchanged following ciprofibrate treatment confirming that fibrates do not exhibit hypolipidaemic activities in normal lipid homeostasis, but they do it only in hyperlipidaemic conditions. However, the treatment modifies the GOT (glutamate-oxaloacetate transaminase) level (+50%) while no significant changes were observed for glutamate-pyruvate transaminase (GPT) and for creatine-phosphokinase (CPK) activities.

For the stress biomarkers, the treatment of the jerboa shows that ciprofibrate induced a significant increase (+20%) in peroxidized lipid level (malondialdehyde-MDA) and a large decrease of catalase activity (-58%) compared with the control (table 2). The decrease of catalase activity is in agreement with the previous results on the treatment of jerboa for two weeks at the same ciprofibrate dosage [4]. Superoxide dismutase and on glutathione reductase activities were decreased but in a non-statistically significant manner.

For the metabolic markers, no changes were observed in the peroxisomal acyl-CoA oxidase activity confirming the lack, or weak, liver peroxisome proliferation in jerboa [4]. Mitochondrial D-3-hydroxybutyrate dehydrogenase activity was very sensitive to *in vivo* treatment by ciprofibrate and strongly decreased (-57%). This aspect is emphasized below in §3.3. No changes were observed for mitochondrial succinate dehydrogenase.

Microsomal NADPH-cytochrome c reductase activity is dramatically decreased (-69%) while a two-week treatment does not significantly modify this activity [4].

No changes were observed for cytosolic glycerol-3-phosphate dehydrogenase (G3PDH) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

In order to explore the possible correlations between the various biomarkers, Principal Component Analysis (PCA) was carried out. This method is a way of identifying patterns of data, and expresses the data in a way which highlights similarities and differences. The figure 1 show the existence of one principal group which explained 62% of total variance, made up of the BDH, catalase and NADPH-cytochrome c reductase. All the components of this group presented a significant reduction caused by the treatment by the ciprofibrate. In results not shown we observed several morphological liver alterations in ciprofibrate exposed jerboa such as centrilobular necrotic areas filled by detritus and connective tissue and dilated central veins.

3.2. *In vivo* and *in vitro* impact of ciprofibrate on BDH expression and activity

In order to know if the decrease of BDH activity following *in vivo*-ciprofibrate treatment (see table 2), was due to variation of BDH protein level, a western-blotting was carried out which revealed no decrease of the BDH immunoreactive band (figure 2). This result suggests that BDH inhibition is not caused by BDH gene expression inhibition either at the transcriptional or the translational level but due to a BDH catalytic inhibition by ciprofibrate. Figure 3 indicates that mitochondrial BDH was *in vitro* sensitive to the presence of ciprofibrate and this sensitivity dose dependent where the inhibition concentration of 50% of BDH activity (IC_{50}) was estimated at $0.75 \text{ mM} \pm 0.08$ following probit analysis [34].

The time course of *in vitro* BDH inhibition by ciprofibrate indicates that BDH is rapidly and strongly inhibited by ciprofibrate in a first order mechanism (results not shown).

The *in vitro* effects of ciprofibrate on BDH kinetic parameters were also studied. BDH kinetic parameters: $K_M\text{BOH}$, $K_M\text{NAD}^+$, K_i (ciprofibrate vs NAD^+) and K_i (ciprofibrate vs BOH) were reported in table 3. Interestingly, they showed that the kinetic constants with respect to the NAD^+ and BOH are modified when BDH is exposed to ciprofibrate: $K_M\text{NAD}^+$ and $K_M\text{BOH}$ were largely increased ($\times 12.3$ and $\times 2.2$ respectively), indicating a binding of ciprofibrate to the active site. On the other hand, K_i (ciprofibrate vs NAD^+) and K_i (ciprofibrate vs BOH) correspond to 0.128 mM and 0.820 mM respectively.

4. Conclusion

Fibrate drugs have been used pharmacologically to prevent cardiovascular diseases by decreasing serum lipids level. For instance the daily dosage of ciprofibrate is usually 100 mg (approximatly 1.5 mg/kg/day) which is a comparable range with the amount used in the present treatment of jerboa (3 mg/kg/day). The hypolipidaemic effect is dependent of PPAR (Peroxisome Proliferator-Activated Receptor); for recent review, see [35]. On the other hand studies reported that prolonged administration of fibrates causes in liver both peroxisome proliferation and cancer in rodents; see for instance ref. [36] or at the opposite to be pro-apoptotic in hepatoblastoma cell lines [37]. Recently Gonzalez and Shah reported a new role of PPAR alpha in hepatocarcinogenesis in rodent [38]. They show that hepatocellular proliferation involves downregulation of the microRNA let-7c gene by PPAR alpha. Let-7c controls levels of proliferative c-myc by destabilizing its mRNA. Thus, upon suppression of let-7c, c-myc mRNA and protein are elevated resulting in enhanced hepatocellular proliferation. In contrast, PPARalpha-humanized mice are resistant to peroxisome proliferator Wy-14,643-induced cell proliferation and cancer and do not exhibit downregulation of let-7c gene expression.

Peroxisome proliferation is considered to be a possible link between the overproduction of intracellular H_2O_2 , a reactive oxygen species and carcinogenic mechanisms at the initiation step [39]. However, experimental evidence for this adverse effect in humans exposed to these drugs is inconclusive [40]. Moreover, effect of ciprofibrate on oxidative stress induction and prevention were not well studied yet except the paper of Mayer et al. reporting that that volunteers exposed to six months-periods of fenofibrate show elevated levels of oxidized lipoproteins and malondialdehyde [41]. To put forward proofs of possible early alterations we used jerboa (*Jaculus orientalis*) knowing that this species responds to peroxisome proliferators closer to human than rat or mice [4]. The present study showed that a middle term treatment with ciprofibrate leads to several negative effects such as liver tissue alteration (centrilobular necrotic areas), circulating transaminase increase (GOT) and oxidative stress modifications including a strong decrease of peroxisomal catalase activity and an increase in MDA (malondialdehyde) level. In addition, as shown before in other species [42], ciprofibrate inhibits jerboa mitochondrial D-3-hydroxybutyrate dehydrogenase at its active site. Since this NAD^+ -dependent enzyme is contribute to the body redox balance through the ($NAD^+/NADH+H^+$ ratio) [42] the direct enzyme inhibitory effect of ciprofibrate will modify the redox balance and will alterate cascade of cellular functions.

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