

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

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Abstract. Ciprofibrate is a well-known drug used to normalize lipid parameters and fibrinogen in atherosclerosis patients. In laboratory rodents such as rats or mice, ciprofibrate exhibits peroxisome proliferator activity. However, to date, no clear alterations or side effects caused by ciprofibrate have been noted in humans. In order to further investigate such possible relationships, we studied the effects of sustained ciprofibrate treatment in jerboas (*Jaculus orientalis*). In these rodents, ciprofibrate does not induce hepatomegaly or promote liver cell DNA replication, confirming that this species more closely resembles humans than do rats or mice. The jerboas were treated daily with ciprofibrate at 3 mg/kg body weight for 4 weeks. Subcellular markers, clinical enzymes and enzymatic antioxidant defenses were then assessed. The results showed a strong decrease in peroxisomal catalase activity and an increase in the level of malondialdehyde (a stress biomarker). Moreover, ciprofibrate *in vivo* and *in vitro* inhibited D-3-hydroxybutyrate dehydrogenase, a mitochondrial enzyme of

the ketone body interconversion that is important in redox balance (NAD⁺/NADH+H⁺ ratio). In conclusion, under these conditions, ciprofibrate induced alterations in the liver oxidative metabolism.

Introduction

Fibrate drugs have been used pharmacologically to reduce plasma triglycerides and cholesterol. They are among a diverse group of chemicals called peroxisome proliferators, which produce a huge increase in the size and number of peroxisomes in cells. Peroxisome proliferators include plasticizers, herbicides and hypolipidaemic drugs, and are both species- and tissue-specific (rodent liver). A peroxisome is a cell compartment containing enzymes involved in oxidative metabolism (H₂O₂-producing oxidases, peroxidase/catalase, Mn superoxide dismutase), and is also involved in lipid catabolism via β -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 in hepatic mitochondria and peroxisomes, without clear evidence of cellular proliferation (3). Notably, El Kebbaï *et al* (4) revealed that the jerboa (*Jaculus orientalis*) has unique peroxisome properties in contrast to rats, responding in a moderate manner to peroxisome proliferators such as ciprofibrate and experiencing neither hepatomegaly nor the promotion of liver cell DNA replication. Jerboas are unique nocturnal herbivorous rodents that reside in the sub-desert highlands of Morocco. They are an appropriate organism for the study of peroxisome proliferator-activated receptor (PPAR)-dependent metabolic regulation due to their remarkable tolerance to heat and cold and their limited diet, attributed to hibernation (5-8).

The purpose of this study was to elucidate the impact of ciprofibrate on cellular antioxidants, metabolic pathways, and the effects of these on D-3-hydroxybutyrate dehydrogenase (BDH) activity, protein levels and kinetic parameters in mitochondria. To date, no clear alterations or side effects caused by ciprofibrate have been noted in humans. In order to further investigate such possible relationships, we studied the effect

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Abbreviations: BDH, D-3-hydroxybutyrate dehydrogenase; CPK, creatine phosphokinase; DL-BOH, DL-3-hydroxybutyrate; D-G-3-P, D-glycerol-3-phosphate; EDTA, ethylenediamine tetraacetic acid; GOT, glutamate oxalate transaminase; GPT, glutamate pyruvate transaminase; HDL, high density lipoprotein; KCN, potassium cyanide; LDL, low density lipoprotein; MDA, malondialdehyde; NAD(H), nicotinamide adenine dinucleotide oxidized (reduced) form; NADPH, nicotinamide adenine dinucleotide phosphate reduced form

Key words: ciprofibrate, *Jaculus orientalis*, clinical enzymes, antioxidant enzymes, subcellular marker, D-3-hydroxybutyrate dehydrogenase

of sustained ciprofibrate treatment in jerboas. The jerboas were treated daily with ciprofibrate at 3 mg/kg body weight for 4 weeks. Subcellular markers, clinical enzymes and enzymatic antioxidant defenses were then assessed. The results showed a strong decrease in peroxisomal catalase activity and an increase in the level of malondialdehyde (MDA; a stress biomarker).

Furthermore, we examined the inhibitory effect of ciprofibrate on mitochondrial inner membrane-bound NAD-dependent BDH (EC. 1.1.1.30) from the liver, which plays an important role in redox balance (NAD⁺/NADH+H⁺ ratio) by interconverting aceto-acetate into BDH (9). Any alteration of its activity will affect the energetic metabolism. A catalytic mechanism of the interconversion of BDH and acetoacetate in both the liver and peripheral tissues has been previously proposed by our group (10). Moreover, this enzyme from the Jerboa has been extensively studied at our laboratory (11-16).

Materials and methods

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

Animals and administration of ciprofibrate. Adult 4-6 month old jerboas (120-150 g) were captured in the Engil Ait Lahcen region (subdesert East Moroccan highlands) then acclimatized to laboratory conditions for 3 weeks at a temperature of 22°C with *ad libitum* access to food (salad and rat diet) and water. The light cycle during the entire experiment was set to 14 h light and 10 h dark. Ciprofibrate was dissolved in 1 ml sterile water and administered daily for 4 weeks by intraperitoneal injection. Four jerboas were administered ciprofibrate (3 mg/kg/day), while corresponding groups serving as the control were given sterile water. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

Blood analysis. Determination of total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), triglycerides, creatine phosphokinase (CPK) and transaminases (GOT, glutamate oxalate transaminase; GPT, glutamate pyruvate transaminase) was carried out at the Centre National de Transfusion Sanguine de Casablanca using conventional methods.

Liver mitochondria isolation. The jerboas were decapitated and their livers rapidly removed for mitochondrial extraction according to the method described by Fleischer *et al* (17), which allows for the preparation of high-yield and pure mitochondrial light fractions. We previously estimated, using marker enzymes, the purity of mitochondria to be 86% (18).

Biochemical assays. All assays were conducted at 37°C with a Jenway 6405 UV/Visible spectrophotometer, and were carried out using a liver homogenate.

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

Liver homogenate sample (1 ml) was added to a 1 ml solution composed of 0.375% thiobarbituric acid and 15% trichloroacetic acid in 0.25 M hydrochloric acid. The tubes were heated at 100°C for 15 min, then cooled on ice to halt the reaction. Centrifugation was conducted at 1000 x g for 10 min, and the reading of the supernatant was carried out at 535 nm.

Catalase. The consumption of 7.5 mM H₂O₂ by peroxisomal catalase in 50 mM potassium phosphate buffer (pH 7.0) was monitored at 240 nm according to Aebi (20).

Acyl-CoA oxidase. The acyl-CoA oxidase peroxisomal enzyme was assayed according to Lazarow and De Duve (21) under the following conditions: 50 mM phosphate buffer (pH 7.4), 0.2 mM NAD⁺, 50 μM FAD⁺, 12 mM DTT, 1 mM potassium cyanide (KCN), 200 μg bovine serum albumin (BSA) and 100 μg protein. The mixture was preincubated for 10 min at 37°C before the addition of 50 μM of palmitoyl-CoA (Sigma) as a final concentration. Measurement of activity was carried out at 340 nm.

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

Superoxide dismutase. The Mn-dependent peroxisomal enzyme was assayed according to Paoletti *et al* (23) under the following conditions: 5 mM EDTA, 2.5 mM MnCl₂, 0.27 mM NADH and 3.9 mM 2-mercaptoethanol in 50 mM potassium phosphate buffer (pH 7.0), and was monitored at 340 nm. Activity was initiated by the addition of NADH, for a final concentration of 0.27 mM.

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

Glycerol 3-phosphate dehydrogenase. The G3PDH cytosolic enzyme was assayed according to Bentley *et al* (25) under the following conditions: 100 mM glycine - NaOH buffer (pH 9.0), 5 mM NAD⁺ and 100 μg protein. The mixture was preincubated for 2 min at 37°C before the addition of 5 mM of glycerol-3-phosphate (Sigma) as a final concentration. Activity was recorded at 340 nm.

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

Succinate dehydrogenase. This mitochondrial membrane-bound enzyme was assayed according to King (27) under the following conditions: 100 mM potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.053 mM dichloroindophenol and

100 μg of mitochondrial proteins. The mixture was preincubated for 10 min at 37°C before the addition of 50 μl KCN-succinate (containing 3.25 mg/ml KCN in 0.5 M succinate). Measurement of activity was carried out at 625 nm.

D-3-hydroxybutyrate dehydrogenase. Mitochondrial BDH activity was measured as described by Lehninger *et al* (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), 0.5 mM EDTA, 1.27% (v/v) redistilled ethanol and 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 initiated by the addition of DL-BOH (Sigma) to a 10-mM final concentration.

In vitro D-3-hydroxybutyrate dehydrogenase inhibition and kinetic studies. To determine the ciprofibrate concentration of BDH exhibiting 50% inhibition (IC_{50}), the activities were measured as described previously in the absence and presence of different ciprofibrate concentrations after preincubation for 5 min. The ciprofibrate was added at zero time of incubation. Aliquots were removed at different times during incubation for the measurement of enzymatic activity. Aliquots of the control assay were removed at the same time to calculate the percentage of BDH activity.

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

Western blotting. After SDS-PAGE (12%) (30) and subsequent transfer to nitrocellulose (31), the mitochondrial proteins (50 μg) were exposed to a 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). The equivalent loaded amount of proteins in each track was controlled by Coomassie blue staining of the overall proteins before and after electrotransfer, as an alternative to possible changes of single housekeeping protein.

Protein assay. Protein content was measured according to the Bradford procedure using BSA as a standard (33).

Statistical data analysis. In each assay, the experimental data represent the mean of four independent assays \pm standard deviation. Means were compared using the Student's t-test. Differences were considered significant at $p < 0.05$ and very significant at $p < 0.01$. Calculation of the inhibition concentrations (IC) was carried out by probit analysis (34).

Results and discussion

In vivo effect of ciprofibrate on clinical enzymes and lipidic assessment, and on the response of metabolic and stress

Table I. Plasmatic parameters from control and ciprofibrate-treated jerboa.

	Control	Ciprofibrate
Glycemia (g/l)	1.25 \pm 0.12	1.19 \pm 0.08
Triglycerides (mg/l)	1.44 \pm 0.46	0.8 \pm 0.297
Total cholesterol (mg/l)	1.11 \pm 0.22	1.175 \pm 0.091
HDL cholesterol (mg/l)	0.745 \pm 0.063	0.685 \pm 0.077
LDL cholesterol (mg/l)	0.185 \pm 0.077	0.22 \pm 0.099
GOT (U/l)	159 \pm 5.65	239 \pm 15.5 ^a (+50%)
GPT (U/l)	6 \pm 2.24	4 \pm 2.01
CPK (U/l)	1143 \pm 280	1067.5 \pm 280.1

Values represent the means \pm standard deviation from four different jerboas. ^a $p < 0.05$ (Student's t-test). GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; CPK, creatine phosphokinase.

biomarkers. Table I indicates that plasma parameters, i.e., glucose, triglycerides and cholesterol (total, HDL and LDL fractions), remained unchanged following ciprofibrate treatment. This confirms that fibrates do not exhibit hypolipidaemic activities under normal lipid homeostasis, doing so only under hyperlipidaemic conditions. However, treatment modified GOT levels (+50%), while no significant changes were observed in terms of GPT or CPK activities.

Concerning stress biomarkers, treatment of the jerboa indicated that ciprofibrate induced a significant increase (+20%) in the peroxidized lipid level (MDA) and a large decrease in catalase activity (-58%) compared with the controls (Table II). The decrease in catalase activity was in agreement with previous results, involving the treatment of jerboa for two weeks at the same ciprofibrate dosage (4). Superoxide dismutase and glutathione reductase activities were decreased, but not in a statistically significant manner.

Regarding the metabolic markers, no changes were observed in peroxisomal acyl-CoA oxidase activity, confirming the lack of, or weak, liver peroxisome proliferation in jerboa (4). Mitochondrial BDH activity was very sensitive to *in vivo* treatment with ciprofibrate and was strongly decreased (-57%). This finding is discussed in detail below. No changes were observed in mitochondrial succinate dehydrogenase.

Microsomal NADPH-cytochrome c reductase activity was dramatically decreased (-69%). A two-week treatment cycle did not significantly modify this activity (4).

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

In order to explore possible correlations between the various biomarkers, principal component analysis was carried out. This method allows for the identification of patterns of data, and expresses the data in a way which highlights their similarities and differences. Fig. 1 shows the existence of one principal group composed of BDH, catalase and NADPH-cytochrome c reductase, which explains 62% of the total variance. All the components of this group exhibited a significant reduction caused by ciprofibrate treatment. Several

Table II. *In vivo* effect of ciprofibrate on response of oxidative stress and metabolic biomarkers.

Liver markers	Control	Ciprofibrate
Thiobarbituric acid reactive substances (nmol/mg of protein)	0.29±0.02	0.35±0.03 ^a (+20%)
Superoxide dismutase (μ mol/min/mg of protein)	5.54±1.04	4.99±1.17
Glutathione reductase (nmol/min/mg of protein)	6.95±0.47	4.77±0.86
Catalase (μ mol/min/mg of protein)	0.162±0.04	0.068±0.04 ^a (-58%)
Acyl-CoA oxidase (nmol/min/mg of protein)	8.4±2.4	10.5±3.2
D-3-hydroxybutyrate dehydrogenase (nmol/min/mg of protein)	0.21±0.012	0.089±0.006 ^b (-57%)
Succinate dehydrogenase (absorbance/min/mg of protein)	3.08±0.43	2.01±0.38
NADPH cytochrome c reductase (nmol/min/mg of protein)	104±20	32±7 ^a (-69%)
Glyceraldehyde-3-phosphate dehydrogenase (μ mol/min/mg of protein)	0.53±0.12	0.58±0.08
Glycerol-3-phosphate dehydrogenase (nmol/min/mg of protein)	1.13±0.09	1.40±0.3

Values represent the means \pm standard deviation from four different jerboas. For experimental conditions, see Materials and methods. ^ap<0.05; ^bp<0.01 (Student's t-test).

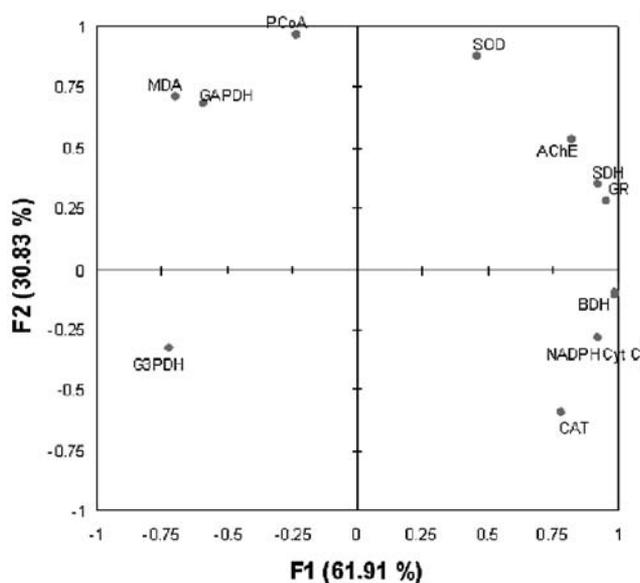


Figure 1. Principal component analysis including all studied parameters.

morphological liver alterations in ciprofibrate-exposed jerboa, such as centrilobular necrotic areas filled by detritis and connective tissue and dilated central veins, were observed (data not shown).

In vivo and in vitro impact of ciprofibrate on D-3-hydroxybutyrate dehydrogenase expression and activity. In order to ascertain whether the decrease in BDH activity following *in vivo* ciprofibrate treatment (Table II) was due to a variation in BDH protein levels, Western blot analysis was carried out. No decrease in the BDH immunoreactive band was detected (Fig. 2). This suggests that BDH inhibition was not caused by the inhibition of BDH gene expression at the transcriptional or translational level, but rather was due to the inhibition of BDH catalysis by ciprofibrate. Fig. 3 shows that mitochondrial BDH was sensitive *in vitro* to the presence of ciprofibrate, and that this sensitivity was dose dependent when



Figure 2. Western blot of the BDH liver mitochondria from control and ciprofibrate-treated jerboas. SDS-PAGE was assayed with 50 μ g of protein. Lanes C and T represent mitochondria extracts from the control and ciprofibrate-treated individual jerboas, respectively. For experimental conditions, see Materials and methods.

the IC_{50} of BDH activity was estimated at 0.75 ± 0.08 mM following probit analysis (34).

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

The *in vitro* effects of ciprofibrate on BDH kinetic parameters were also examined. BDH kinetic parameters K_M BOH, K_M NAD⁺, K_I (ciprofibrate vs. NAD⁺) and K_I (ciprofibrate vs. BOH) are listed in Table III. Notably, it was revealed that the kinetic constants with respect to NAD⁺ and BOH were modified when BDH was exposed to ciprofibrate: K_M NAD⁺ and K_M BOH were largely increased (12.3- and 2.2-fold, 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) corresponded to 0.128 and 0.820 mM respectively.

Fibrate drugs have been used pharmacologically to prevent cardiovascular diseases by decreasing serum lipid levels. The daily dosage of ciprofibrate is usually 100 mg (~1.5 mg/kg/day), a range comparable with the 3 mg/kg/day amount administered to jerboas in the present study. The hypolipidaemic effect is dependent on PPAR (reviewed in ref. 35). On the other hand, studies have found that prolonged administration of fibrates causes both peroxisome proliferation and cancer in the liver of rodents (36) or, conversely, has proapoptotic effects on hepatoblastoma cell lines (37). Recently,

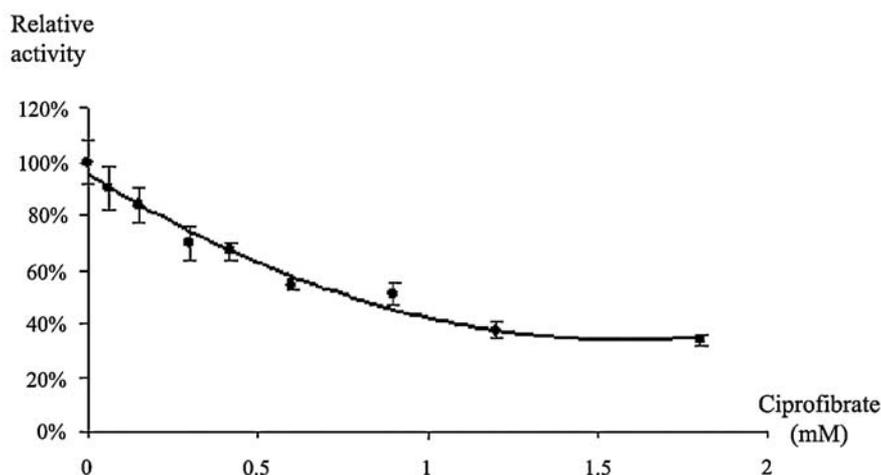


Figure 3. *In vitro* effect of ciprofibrate concentration on BDH activity. Values represent the means from four individual jerboas. For experimental conditions, see Materials and methods.

Table III. Determination of the kinetic parameters of liver D-3-hydroxybutyrate dehydrogenase in the presence and absence of ciprofibrate.

	Control	Ciprofibrate
K_M NAD ⁺ (mM)	0.2±0.01	2.6±0.28 ^a (x 12.3)
K_i ciprofibrate vs. NAD ⁺ (mM)	None	0.128±0.01
K_M BOH (mM)	1.6±0.22	3.55±0.5 ^a (x 2.2)
K_i ciprofibrate vs. BOH (mM)	None	0.82±0.04

Experiments used varying concentrations of NAD⁺ (0.5, 1, 1.5 and 2 mM) or BOH (2.5, 5, 7.5 and 10 mM) and ciprofibrate (0, 0.15, 0.3 and 0.6 mM). Values represent the means ± standard deviation from four different jerboas. ^ap<0.01 (Student's t-test).

Gonzalez and Shah reported a new role of PPAR α in hepatocarcinogenesis in rodents (38), demonstrating that hepatocellular proliferation involves the downregulation of the microRNA let-7c gene by PPAR α . 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, PPAR α -humanized mice are resistant to peroxisome proliferator Wy-14,643-induced cell proliferation and cancer, and do not exhibit any downregulation of let-7c gene expression.

Peroxisome proliferation is considered to be a possible link between the overproduction of intracellular H₂O₂, a reactive oxygen species, and the initial step involved in carcinogenic mechanisms (39). However, experimental evidence for this adverse effect in humans exposed to these drugs is inconclusive (40). Moreover, the effect of ciprofibrates on oxidative stress induction and prevention has yet to be thoroughly examined, with the exception of a study by Mayer *et al*, which reported that volunteers exposed to 6-month periods of fenofibrate exhibited elevated levels of oxidized lipoproteins and malondialdehyde (41). In order to attempt to verify possible early alterations, we used jerboas, as the response of this

species to peroxisome proliferators more closely resembles that of humans than that of rats or mice (4). The present study demonstrated that middle-term treatment with ciprofibrate had several negative side effects, such as alterations in liver tissue (centrilobular necrotic areas), an increase in circulating transaminases (GOT) and oxidative stress modifications, including a strong decrease in peroxisomal catalase activity and an increase in MDA levels. In addition, as previously demonstrated in other species (42), ciprofibrate inhibited jerboa mitochondrial BDH at its active site. Since this NAD⁺-dependent enzyme contributes to the body redox balance through the NAD⁺/NADH⁺ ratio (42), the direct inhibitory effect of ciprofibrate on this enzyme modifies the redox balance and alters the cascade of cellular functions.

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