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Hypertriglyceridemia Increases Mitochondrial Resting Respiration and Susceptibility to Permeability Transition

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High plasma level of triglycerides (TGs) is a common feature in atherosclerosis, obesity, diabetes, alcoholism, stress, and infection. Since mitochondria have been implicated in cell death under a variety of metabolic disorders, we examined liver mitochondrial functions in hypertriglyceridemic transgenic mice. Hypertriglyceridemia increased resting respiration and predisposed to mitochondrial permeability transition (MPT). Ciprofibrate therapy reduced plasma TG levels, normalized respiration, and prevented MPT. The higher resting respiration in transgenic mitochondria remained in the presence of the adenine nucleotide carrier inhibitor, carboxyatractyloside, bovine serum albumin, and the uncoupling proteins (UCPs) inhibitor, GDP. UCP2 content was similar in both control and transgenic mitochondria. We propose that faster resting respiration represents a regulated adaptation to oxidize excess free fatty acid in the transgenic mice.

KEY WORDS: Hypertriglyceridemia; transgenic mice; mitochondrial permeability transition; uncoupling protein; respiratory control; proton leak; fibrates.

INTRODUCTION

Hypertriglyceridemia and high free fatty acids (FFA) concentrations are manifestations of primary genetic disorders or features of several pathological states such as obesity, diabetes, alcoholism, stress, and infection (Assmann and Brewer, 1991; Mancini *et al*., 1991; Reaven, 1994). In addition, high plasma levels of triglyceride- (TG) rich lipoproteins and their remnants are independent risk factors for coronary heart disease (Malloy and Kane, 2001). Clinical trials have shown that fibrate therapy diminishes plasma TG levels by 20–50% (Miller, 2000) and reduces coronary heart disease events (Faergeman, 2000). Fibrates and long-chain fatty acids are ligands of the peroxisome proliferator-activated receptors, subtype alpha (PPARα), which increase the transcription of several genes, including the fatty acid binding proteins and mitochondrial and peroxisomal oxidative enzymes (Schoonjans *et al*., 1996). These actions result in increased cellular lipid catabolism, decreased liver TG secretion (Fruchart *et al*., 1999; Staels *et al*., 1998) and reduction of body weight gain in diet-induced obesity (Guerre-Millo *et al*., 2000; Mancini *et al*., 2001).

Mitochondrial uncoupling protein (UCP) genes contain PPAR-responsive elements (Acin *et al*., 1999; Silva and Rabelo, 1997) and may be targets of fibrates. It has previously been shown that, after fenofibrate treatment, rat UCP2 mRNA may be upregulated (Nakatani *et al*., 2002; Tsuboyama-Kasaoka *et al*., 1999) or not altered in liver and downregulated in muscle (Mancini *et al*., 2001). On the other hand, Lanni *et al*. (2002) have shown

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Key to abbreviations: $\Delta\psi$, transmembrane electrical potential; apo CIII, apolipoprotein CIII; BSA, bovine serum albumin; CAT, carboxyatractyloside; Chol, cholesterol; CsA, cyclosporin A; FFA, free fatty acids; MPT, mitochondrial permeability transition; TG, triglycerides; TPP^+, tetraphenylphosphonium; UCP, uncoupling protein.

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that fenofibrate treatment was able to induce UCP3 expression in the rat liver, a tissue where it is normally silenced.

Mice genetically modified to overexpress the human apolipoprotein (apo) CIII develop severe hypertriglyceridemia and increased FFA plasma levels (Ito *et al*., 1990), but normal glucose homeostasis (Amaral *et al*., 2002; Reaven *et al*., 1994). The overexpression of apo CIII impairs the plasma removal of the TG-rich lipoprotein remnants by their specific liver receptors (Aalto-Setala *et al*., 1992). Thus, the extended lipoprotein half lifes in the plasma compartment results in increased generation of FFA.

Intracellularly, FFA may disturb mitochondrial energy-linked functions. They have the ability to directly uncouple oxidative phosphorylation (Pressman and Lardy, 1956) or to act as UCP's substrates (Garlid *et al*., 1996; Skulachev, 1991), and also to induce mitochondrial permeability transition (MPT) in Ca^{2+} loaded mitochondria (Catisti *et al*., 2000; Chavez *et al*., 1999; Wieckowski and Wojtczak, 1998). Mild uncoupling of oxidative phosphorylation may represent an energy dissipating mechanism and/or protection against oxidative stress (Skulachev, 1991). MPT may result from oxidative stress and lead to cell death, by either necrosis or apoptosis (Kowaltowski *et al*., 2001).

In this study, we have analyzed respiration rates, efficiency of oxidative phosphorylation, UCP2 content and activity, and susceptibility to MPT in liver mitochondria isolated from control and hypertriglyceridemic transgenic mice treated with placebo or ciprofibrate.

MATERIAL AND METHODS

Animals, Treatment, and Plasma Biochemical Analyzes

Human apo CIII transgenic (line 3707) (Walsh *et al*., 1993) founders were donated by Dr Alan R. Tall (Columbia University, NY) and bred in the animal facilities of the Department of Physiology and Biophysics, State University of Campinas, SP, Brazil. The experiments were approved by the university's Ethics Committee and were in accordance with the *Guidelines on the Handling and Training of Laboratory Animals* published by the Universities Federation for Animal Welfare (1992). The mice had access to standard laboratory rodent chow diet (Nuvital CR1, PR, Brazil) and water *ad libitum* and were housed at $22 \pm 2^{\circ}$C on a 12-h light–dark cycle. Male and female heterozygous apo CIII transgenic and nontransgenic littermates, aged 4–6 months, were used in this study. Ciprofibrate (Oroxadin, Sanofi Winthorop, RJ, Brazil) or placebo (2% arabic gum) were given daily through gavage, 10 mg/kg BW, during 21 days. Blood samples from overnight fasted mice were taken from the retro-orbital plexus of anesthetized mice (50 mg/kg ketamine, Parke-Davis, SP, Brazil, and 10 mg/kg xylazine, Bayer S.A., SP, Brazil) using heparinized hematocrit tubes. Enzymatic colorimetric methods were used to determine plasma concentrations of glucose (Merck®, France), TGs (Boehringer Mannhein®, Germany), total cholesterol (Merck®, Germany), and FFA (Wako Chemicals®, Japan), according to the manufacturer's instructions.

Isolation of Mice Liver Mitochondria and Standard Incubation Procedure

Mitochondria were isolated by conventional differential centrifugation (Kaplan and Pedersen, 1983) from the liver of adult animal fasted overnight. The homogenate was prepared in 250 mM sucrose, 1 mM EGTA, and 10 mM Hepes buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA and the final pellet was diluted in 250 mM sucrose to a final protein concentration of 80–100 mg/mL. The experiments were carried out in standard medium containing 125 mM sucrose, 65 mM KCl, 5 mM potassium succinate, 2 mM inorganic phosphate, 1 mM magnesium cloryde, 2 μM rotenone, 10 mM Hepes buffer (pH 7.2).

Mitochondrial Swelling

Mitochondrial swelling was estimated from the decrease in the absorbance of the mitochondrial suspension measured at 520 nm in a Hitachi U-3000 spectrophotometer.

Mitochondrial Transmembrane Electrical Potential ($\Delta\psi$)

Mitochondria were incubated in the standard medium containing 3 μM tetraphenylphosphonium (TPP^+). The concentration of TPP^+ in the extramitochondrial medium was continuously monitored with a TPP^+ selective electrode prepared in our laboratory according to Kamo *et al*. (1979). The membrane potential was then calculated assuming that the TPP^+ distribution

between mitochondria and medium follows the Nernst equation (Muratsugu *et al*., 1977). Corrections due to binding of TPP^+ to the mitochondrial membrane were made according to Jensen *et al*. (1986).

Mitochondria Respiration Rates and Phosphorylation Efficiency

Oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments Ltd. using software OXIGRAPH V1.10, England) in a 1-mL glass chamber equipped with magnetic stirring. Liver mitochondria (0.5 mg/mL) were added to the standard medium containing 0.5 mM EGTA and 0.1% bovine serum albumin (BSA) at 28°C. Respiration rates are given in natoms oxygen/mg/min. Phosphorylating (state III) respiration was initiated by addition of 200 nmol ADP/mg protein. Phosphorylation efficiency (ADP/O ratio) was calculated from the added amount of ADP and total amount of oxygen consumed during state III.

Uncoupling Protein Analyzes by Western Blotting

Isolated mitochondria were homogenized in buffer A (10 mM trisma base, 10 mM EDTA, 10% SDS, 100 mM NaF, 10 mM sodium orthovanadate, and 10 mM sodium pyrophosphate) and centrifuged for 20 min at 12,000 rpm in a 70Ti Beckman rotor at 4°C. Supernatants (125-μg protein) were subjected to SDS-polyacrylamide gel electrophoresis (10% acrylamide). After electrotransfer of proteins, the nitrocellulose membranes were preincubated in blocking buffer (5% BSA, 10 mM Tris, 150 mM NaCl, 0.02% Tween 20) for 2 h at room temperature and incubated with goat polyclonal antibodies against human UCP2 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit antihuman UCP3 (Chemicon International, Temecula, CA) overnight at 4°C. After washing, the UCP2 blots were additionally incubated with rabbit antigoat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and washed again. UCP2 and UCP3 blots were then incubated with [^{125}I]-protein A (Amersham, Buckinghamshire, UK) for 2 h at room temperature and washed several times for 15 min. UCPs bands were detected by autoradiography using preflashed Kodak XAR film with Cronex Lightning Plus intensifying screens at −70 °C for 6 days. Band intensities were quantified by digital densitometry (Scion Image software, ScionCorp).

RESULTS AND DISCUSSION

The apo CIII transgenic mouse model is very useful to study the effect of elevated plasma concentrations of TG and FFA *per se*, without other metabolic confounding factors. Under standard housing and feeding conditions, these animals do not differ from control mice with respect to life cycle, weight gain, growth rate, reproductive function, and general behavior. However, under high-fat and high-cholesterol diet, they develop more severe atherosclerosis than control mice (Hayek *et al*., 1995). Also, when plasma FFA are further elevated by heparin treatment, they show impaired glucose tolerance due to reduced insulin secretion (Amaral *et al*., 2002).

Plasma levels of lipids and glucose in control, transgenic and ciprofibrate-treated transgenic mice are shown in Table I. Transgenic mice exibited concentrations of TGs, FFA, and of cholesterol (Chol) that were, 4.6-, 1.8-, and 1.7-fold, respectively, higher than control mice. Glucose levels were not different between transgenic and control animals. Ciprofibrate treatment of the transgenic mice reduced the concentrations of TG by 33%, FFA by 24%, and total cholesterol by 18%. Glucose levels were slightly but significantly elevated. The rise in the glycemia after the fibrate treatment has been described elsewhere (Tikkanen *et al*., 1998).

Table II shows the respiration and oxidative phosphorylation pattern of liver mitochondria isolated from control, transgenic, and ciprofibrate-treated transgenic mice.

Table I. Fasting Plasma Levels of Cholesterol (Chol), Triglycerides (TG), Free Fatty Acid (FFA), and Glucose (Gluc) in Control Nontransgenic and Hypertriglyceridemic Transgenic Mice Treated With Placebo or Ciprofibrate

	Chol (mg/dL)	TG (mg/dL)	FFA (nmol/L)	Gluc (mg/dL)
Control	74 ± 32^a	93 ± 39^b	1.6 ± 0.6^c	80 ± 23^d
Transgenic (placebo)	124 ± 32^a	429 ± 134^b	2.9 ± 0.4^c	73 ± 19^e
Transgenic (ciprofibrate)	102 ± 39	288 ± 140^b	2.2 ± 0.8	$118 \pm 21^{d,e}$

Note. Mean ± SD (n = 8–12). Same superscripts are statistically different ($p < 0.05$ or better) by Kruskal–Wallis multiple comparison test (Dunn's post-test).

Table II. Resting (State IV) and Phosphorylating (State III) Respiration Rates and Phosphorylation Efficiency (ADP/O) in Liver Mitochondria From Control Nontransgenic and Hypertriglyceridemic Transgenic Mice Treated With Placebo or Ciprofibrate

	State III	State IV	State III/State IV	ADP/O
Control	160.8 ± 12.2	36.3 ± 4.9^{a}	4.45 ± 0.46^{c}	1.45 ± 0.15
Transgenic (placebo)	152.2 ± 19.95	$44.8 \pm 5.5^{a,b}$	$3.22 \pm 0.37^{c,d}$	1.37 ± 0.14
Transgenic (ciprofibrate)	150.2 ± 19.6	38 ± 4.4^{b}	3.92 ± 0.44^{d}	1.38 ± 0.14

Note. Mean $\pm$ SD ($n = 11$). Respiration rates given in natoms oxygen/mg protein/min. Same superscripts are statistically different ($p < 0.05$ or better) by Kruskal–Wallis multiple comparison test (Dunn's post-test).

To avoid interference of contaminating FFA, these experiments were carried out in the presence of BSA, a chelator of FFA. It can be observed that although the respiratory control (state III/state IV respiration rates ratio) is significantly lower (28%) in mitochondria isolated from the transgenic mice (placebo), there is no significant difference in the phosphorylation efficiency (ADP/O ratio) as compared to control mice. The lower value of the respiratory control in transgenic (placebo) mice is the result of a higher resting (state IV) respiration in their mitochondria. The ciprofibrate treatment significantly decreased the state IV respiration, and hence, increased the transgenic mitochondria respiratory control almost to the level of the control mice.

Table III shows that, under *in vitro* conditions, only at high concentration (100 μM), ciprofibrate had a slight effect on both respiratory control and ADP/O ratio, in both isolated liver mitochondria preparations. Riley and Pfeiffer (1986) found large amplitude swelling when they incubated rat liver mitochondria in sucrose medium containing 80 μM $CaCl_2$ and the fibrate WY-14643. However, when they used clofibrate, whose chemical structure is closer to ciprofibrate, a 20-fold higher concentration was necessary to observe similar effects. It should be mentioned that these deleterious *in vitro* effects of WY-14643 are opposite to what we observed with the *in vivo* ciprofibrate treatment of transgenic mice which normalized mitochondria function.

Table III. In Vitro Effect of Ciprofibrate on Respiratory Control (RC) and ADP/O Ratio in Liver Mitochondria From Control Nontransgenic and Hypertriglyceridemic Transgenic Mice

	Control		Transgenic	
Dose	RC	ADP/O	RC	ADP/O
0	5.7 ± 0.0	1.3 ± 0.0	4.8 ± 0.0	1.3 ± 0.0
0.1% DMSO	5.7 ± 0.0	1.3 ± 0.0	4.8 ± 0.0	1.3 ± 0.0
1 μM	5.7 ± 0.1	1.3 ± 0.0	4.8 ± 0.0	1.3 ± 0.0
10 μM	5.6 ± 0.2	1.3 ± 0.0	4.8 ± 0.2	1.3 ± 0.0
100 μM	4.9 ± 0.2	1.2 ± 0.0	4.1 ± 0.1	1.2 ± 0.0

Note. Mean $\pm$ SD ($n = 3$) $p > 0.5$ by Kruskal–Wallis multiple comparison test (Dunn's posttest).

The results showing that the transgenic mice present oxidative phosphorylation efficiency similar to the control mice are in accordance with their healthy behavior under standard conditions. Therefore, the observed higher state IV respiration rate could be an adaptation to dissipate extra metabolic energy in animals with a higher content of endogenous FFA. Such an adaptation could be mediated by UCPs. Thus, we performed experiments to ascertain whether UCP2 or 3 could be responsible for this effect. In these experiments mitochondria were isolated in an extraction medium containing 0.2% BSA. The results depicted in Fig. 1(A) show that the differences between control and transgenic mice remained the same, i.e., higher resting respiration in the transgenic mitochondria. Carboxyatractyloside (CAT, 2 μM) was included in the medium before GDP and BSA to rule out the possibility that the adenine nucleotides (ADN) carrier could contribute to the increased resting respiration of transgenic mitochondria by catalyzing a CAT-sensitive futile cycling of FFA (Skulachev, 1991). It can be noticed that none of the compounds CAT, GDP (UCPs inhibitor), and BSA had significant effects on the state IV respiration in both mitochondria as compared to the control experiments (no additions, dotted lines). This supports our interpretation that FFA is not involved in the mechanism of faster resting respiration of transgenic mitochondria via either ADN carrier or UCPs. Accordingly, Western blot analyzes showed no significant differences in the content of UCP2 in mitochondria (Fig. 2) from control, transgenic, and ciprofibrate-treated transgenic mitochondria. As expected (Ricquier and Bouillaud, 2000), liver mitochondria UCP3 bands could not be detected in any of the three mitochondria.

To further examine the possibility of FFA involvement via some other unknown mechanism we did experiments (Fig. 1(B)) adding 2.0 μM linoleic acid to the medium. It can be observed that the presence of FFA equally decreased both respiratory control and ADP/O ratio in control and in transgenic mitochondria at the

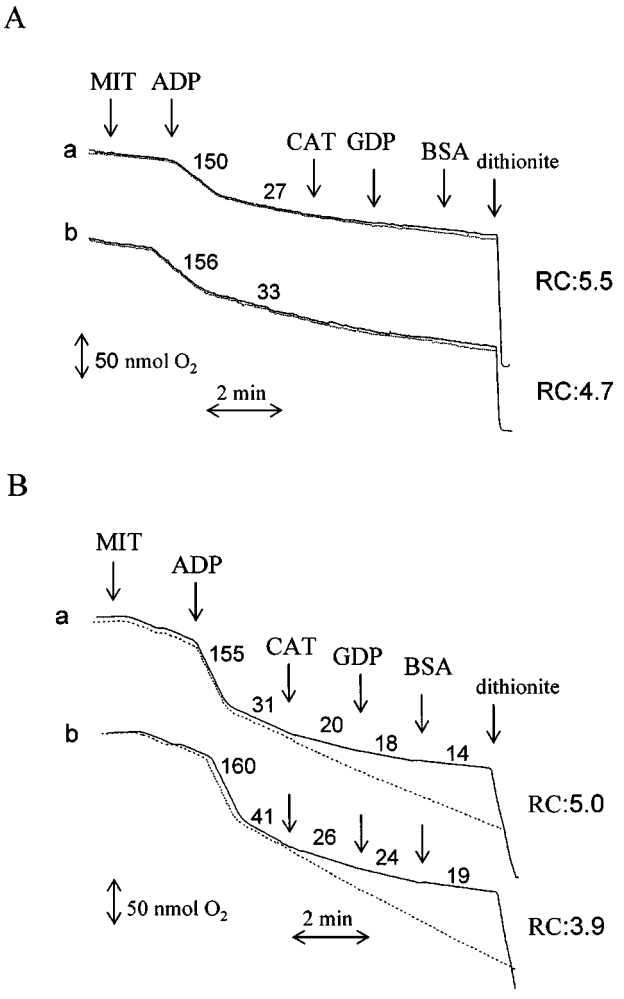

Fig. 1. Lack of effect of CAT, GDP, and BSA on the resting respiration (State IV) of control (a) and transgenic mice (b) mitochondria. Mouse liver mitochondria (0.5 mg/mL) were added to standard reaction medium as described in Material and Methods section. ADP (200 nmol/mg protein), CAT (2.0 μM), GDP (500 μM), and BSA (0.1%) were added where indicated. At the end of the experiments, excess dithionite was added to exhaust medium O_2. Panel A: absence of linoleic acid. Panel B: 2.0 μM linoleic acid was present in the incubation medium. The numbers indicate the respiration rates. Dotted line represents respiration rates without CAT, GDP, and BSA additions. RC: respiratory control (State III/State IV ratio). Mitochondria were isolated in an extraction medium containing 0.2% BSA.

expenses of increased resting respiration. Under these conditions, CAT caused a significant decrease of resting respiration in both types of mitochondria. Thus, the increase in liver mitochondria resting respiration caused by FFA is (i) paralleled by a decrease in ADP/O ratio and (ii) additively inhibited by CAT, GDP, and BSA, in contrast to the increased resting respiration observed in the transgenic mitochondria that is neither associated with lower ADP/O ratio nor modified by CAT, ADP, or BSA.

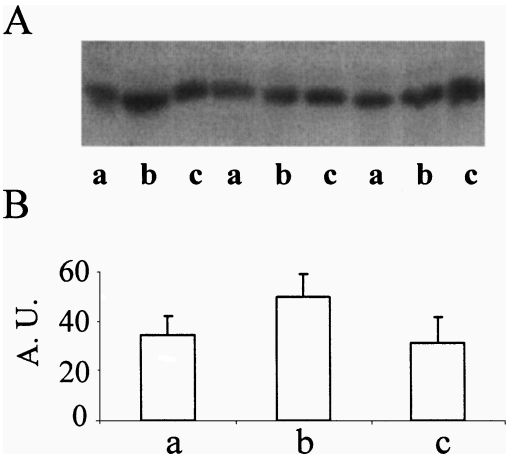

Fig. 2. Western blotting detection of UCP2 content in isolated liver mitochondria from control (a), transgenic (b), and ciprofibrate-treated transgenic (c) mitochondria (see details in Material and Methods section). Panel A: UCP2 immunoblot autoradiography. Panel B: UCP2 bands densitometry (A.U., arbitrary units). Mean $\pm$ SD ($n = 4$).

Since the higher proton leak observed in the transgenic mitochondria state IV respiration disappears under phosphorylating conditions allowing for similar ADP/O ratios in control and transgenic mitochondria, we propose that the changes in respiratory control represents a regulated adaptation to oxidize excess FFA in the transgenic mice. According to the results mentioned above, this adaptation cannot be attributed to FFA-mediated higher activity of either ADN carrier or UCPs which would decrease the ADP/O ratio. Thus, other structural or functional differences in the transgenic inner mitochondria membranes may be present.

To ascertain whether these putative membrane alterations could affect other transport functions, we studied the ability of mitochondria to accumulate Ca^{2+} and retain the transmembrane electrical potential ($\Delta\psi$). Figure 3 (Panel A) shows that when mice liver mitochondria were added to the standard medium containing 5 mM succinate and 3 μM TPP^+, there was a fast deflexion of the trace compatible with the formation of a membrane potential and TPP^+ accumulation by mitochondria. In mitochondria isolated from control mice (*line a*), the addition of Ca^{2+} was acompanied by a decrease in $\Delta\psi$ followed by an almost complete return to the previous level remaining unchanged during the following period of analyzes. In contrast, in mitochondria isolated from the transgenic mice (*line b*) the drop in $\Delta\psi$ was only partially recovered after the Ca^{2+} pulse. This was followed by a continuous drop of $\Delta\psi$. It can be observed that cyclosporin A (CsA), an inhibitor of the permeability transition pore opening, totally prevented the elimination of $\Delta\psi$ in mitochondria

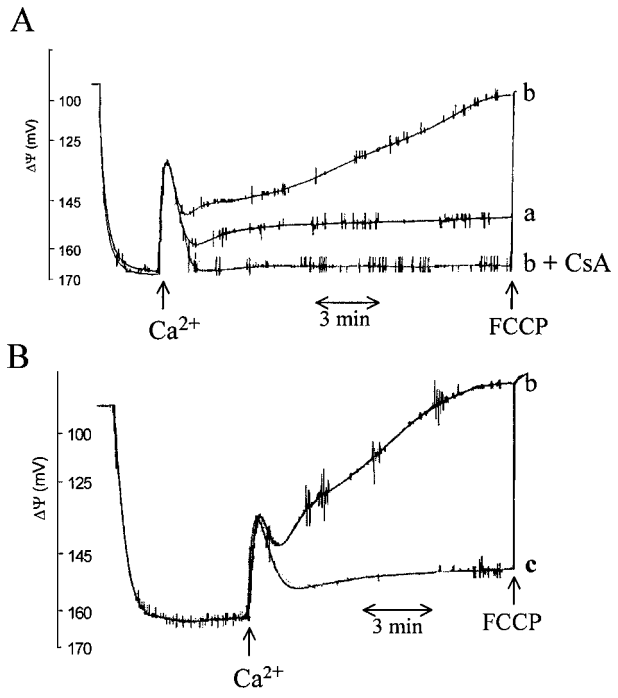

Fig. 3. Calcium-induced changes in isolated mitochondria transmembrane electrical potential ($\Delta\psi$). Mouse liver mitochondria (0.5 mg/mL) were added to standard reaction medium containing 3 μM TPP^+ as described in Material and Methods section. Hundred micromolass of $CaCl_2$ was added where indicated. Panel A: liver mitochondria isolated from control mice (*line a*), transgenic mice (line b), and transgenic mice in the presence of cyclosporin A. Panel B: mitochondria from placebo- (*line b*) and ciprofibrate- (*line c*) treated transgenic mice. Representative of seven experiments.

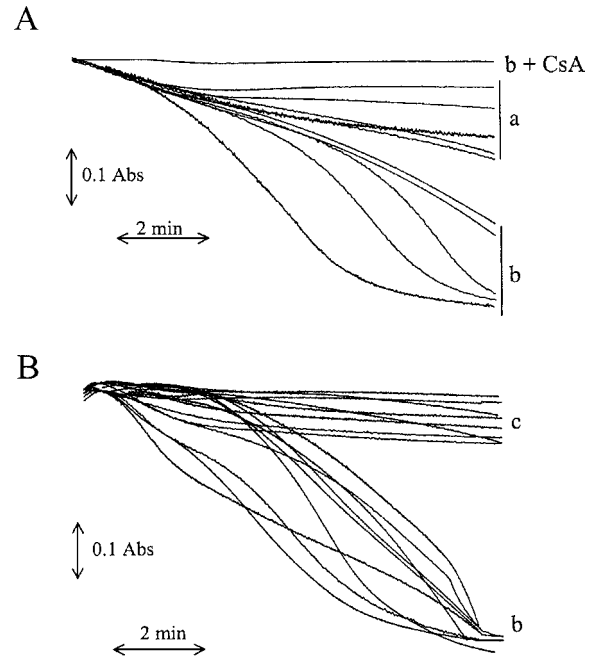

Fig. 4. Mitochondrial swelling induced by 100 μM $CaCl_2$. Mouse liver mitochondria (0.5 mg/mL) isolated from control (a) and transgenic mice (b) (panel A) and from placebo (b) and ciprofibrate (c) treated transgenic mice (panel B). CsA: cyclosporin A.

isolated from the transgenic mice. Figure 3 (Panel B) illustrates that ciprofibrate treatment (*line c*) significantly enhanced the ability of the transgenic mice mitochondria to retain $\Delta\psi$ after Ca^{2+} accumulation as compared to the placebo-treated transgenic mitochondria (*line b*). The CsA effect observed in Fig. 3(A) indicates that the mitochondria isolated from transgenic mice are more susceptible to Ca^{2+}-induced MPT than that isolated from control mice. Indeed, the experiments depicted in Fig. 4 (Panel A) show that, in contrast to control, mitochondria isolated from the transgenic mice underwent extensive swelling when challenged by calcium. Similarly to the $\Delta\psi$ experiments, the presence of CsA (Fig. 4(A)) and ciprofibrate treatment (Fig. 4(B)) prevented the Ca^{2+}-induced mitochondrial swelling. In contrast, the placebo did not change the effects of Ca^{2+} either on membrane potential (Fig. 3(B), *line a*) or on swelling (Fig. 4(B)). It should be mentioned that *in vitro* additions of ciprofibrate (5–140 μM) to transgenic mitochondria preparations had no effect on calcium-induced changes in membrane potential (data not shown).

To conclude, the results presented here indicate that while hypertriglyceridemia decreases respiratory control and predispose to mitochondrial permeability transition, ciprofibrate therapy reduces plasma TG levels, normalizes mitochondrial respiration, and prevents mitochondrial dysfunction under stressed conditions such as calcium overload.

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