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Original Contribution

Mitochondrial ATP-sensitive K^+ channels as redox signals to liver mitochondria in response to hypertriglyceridemia

Luciane C. Alberici [a], Helena C.F. Oliveira [b], Bruno A. Paim [a], Camila C. Mantello [a], Amanda C. Augusto [c], Karina G. Zecchin [a], Sonia A. Gurgueira [c], Alicia J. Kowaltowski [d], Anibal E. Vercesi [a,*]

[a] Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, 13083-887 Campinas, SP, Brazil
[b] Departamento de Fisiologia e Biofísica, Instituto de Biologia, Universidade Estadual de Campinas, 13083-887 Campinas, SP, Brazil
[c] Laboratório de Estresse Oxidativo, Universidade São Francisco, Bragança Paulista, SP, Brazil
[d] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil

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ABSTRACT

We have recently demonstrated that hypertriglyceridemic (HTG) mice present both elevated body metabolic rates and mild mitochondrial uncoupling in the liver owing to stimulated activity of the ATP-sensitive potassium channel (mitoK$_{ATP}$). Because lipid excess normally leads to cell redox imbalance, we examined the hepatic oxidative status in this model. Cell redox imbalance was evidenced by increased total levels of carbonylated proteins, malondialdehydes, and GSSG/GSH ratios in HTG livers compared to wild type. In addition, the activities of the extramitochondrial enzymes NADPH oxidase and xanthine oxidase were elevated in HTG livers. In contrast, Mn-superoxide dismutase activity and content, a mitochondrial matrix marker, were significantly decreased in HTG livers. Isolated HTG liver mitochondria presented lower rates of H_2O_2 production, which were reversed by mitoK$_{ATP}$ antagonists. In vivo antioxidant treatment with *N*-acetylcysteine decreased both mitoK$_{ATP}$ activity and metabolic rates in HTG mice. These data indicate that high levels of triglycerides increase reactive oxygen generation by extramitochondrial enzymes that promote mitoK$_{ATP}$ activation. The mild uncoupling mediated by mitoK$_{ATP}$ increases metabolic rates and protects mitochondria against oxidative damage. Therefore, a biological role for mitoK$_{ATP}$ as a redox sensor is shown here for the first time in an in vivo model of systemic and cellular lipid excess.

Introduction

Hypertriglyceridemia is a common feature of metabolic disorders and may also occur because of primary inherited disorders [1]. In addition, high plasma levels of triglycerides (TGs) are well established as an independent risk factor for atherosclerosis [2]. Oxidative stress plays a major role in the pathogenesis of hyperlipidemia-linked disorders such as cardiovascular disease [3], atherosclerosis [4,5], diabetes [6], and metabolic syndrome [7].

Previous studies have shown that accumulation of TG-rich lipoproteins in plasma is associated with elevation of biomarkers of oxidative stress in the serum of humans [8,9] and in the serum and livers of rats fed high-carbohydrate diets [10]. Furthermore, Cardona et al. [11] have shown that hypertriglyceridemic patients, with or without metabolic syndrome, present increased levels of serum lipoperoxides, carbonylated proteins, and oxidized glutathione and low levels of antioxidant enzymes.

Multiple biochemical mechanisms have been proposed to explain the central role of lipid-induced oxidative stress [12,13] but the knowledge of cell redox control disruption in metabolic diseases is still incomplete. In patients with hypertriglyceridemia, with and without diabetes, peripheral mononuclear cells present increased superoxide radical production and low scavenging activity [14,15]. Interestingly, the rate of superoxide radical production by these mononuclear cells correlated positively with plasma triglyceride levels but did not relate to other parameters, such as plasma cholesterol and glycated hemoglobin A1 levels. Reactive oxygen species (ROS) generation was shown to be promoted by upregulation of NADPH oxidase and downregulation of several antioxidant enzymes in the kidney and aorta of rats fed high-fat and high-sugar diets [16] and in the adipose tissue of hyperlipidemic obese mice [17].

We have recently demonstrated that liver mitochondria isolated from genetically hypertriglyceridemic (HTG) mice presented higher resting respiratory rates, which were unrelated to the activity or expression of the uncoupling proteins [18]. Intracellular fatty acid content is probably implicated in this process, because respiratory

Abbreviations: HTG, hypertriglyceridemic; mitoK$_{ATP}$, mitochondrial adenosine triphosphate-sensitive potassium channel; MDA, malondialdehyde; GSSG, oxidized glutathione; GSH, reduced glutathione; WT, wild type; ROS, reactive oxygen species; TG, triglycerides; MnSOD, Mn-superoxide dismutase; 5-HD, 5-hydroxydecanoate; DZX, diazoxide; NAC, *N*-acetylcysteine; FFA, free fatty acid.

* Corresponding author. Fax: +55 19 35217330.
E-mail address: anibal@unicamp.br (A.E. Vercesi).

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rates were totally corrected by treatment of HTG mice with fibrates, which activate fatty acid β-oxidation [18]. The increase in mitochondrial resting respiration was shown to be mediated by higher activity of the mitochondrial ATP-sensitive K^+channels (mitoK_{ATP}) [19]. Potassium uptake into the mitochondrial matrix through mitoK_{ATP} is accompanied by phosphate and water and results in mitochondrial swelling. This activates K^+/H^+antiporters generating a futile cycling of K^+across the inner mitochondrial membrane [20]. The concomitant action of mitoK_{ATP} and K^+/H^+antiporter results in the entrance of a proton for each K^+exchange and a slight decrease in membrane potential, which is accompanied by an increase in mitochondrial respiratory rate (mild uncoupling). Accordingly, these mice presented increased whole-liver oxygen consumption, increased whole-body metabolic rate, and higher food intake, without enhanced weight gain [19].

Mitochondria continuously generate superoxide radical anions and other ROS owing to monoelectronic reduction of oxygen molecules at intermediary stages of the electron transport chain (mainly through complexes I and III) [21]. Superoxide radicals are normally removed by Mn-superoxide dismutase (MnSOD), which promotes the generation of H_2O_2. H_2O_2 is then reduced to water mostly by the glutathione and thioredoxin systems. Faster electron transport rates decrease ROS formation by keeping mitochondria in a low oxygen tension microenvironment and decreasing the lifetime of intermediates capable of donating electrons toward superoxide radical formation [22]. Indeed, in vitro mild uncoupling induced by mitoK_{ATP} agonists decrease ROS release in mitochondria isolated from heart, brain, and liver [23].

In this work, we hypothesized that genetically hypertriglyceridemic mice could be protected from oxidative stress because they present mild mitochondrial uncoupling in the liver, mediated by higher mitoK_{ATP} activity.

Experimental procedures

Animals

The human apolipoprotein CIII transgenic (line 3707) mouse model was described elsewhere [18,19]. Experiments were approved by the ethics committee of the Universidade Estadual de Campinas and are in accordance with the *Guidelines for Handling and Training of Laboratory Animals* published by the university's Federation for Animal Welfare. Mice had access to standard laboratory rodent chow (Nuvital CR1, Parana, Brazil) and water ad libitum and were housed at 22 ± 2°C, on a 12-h light/dark cycle. Male and female heterozygous apolipoprotein CIII transgenic (HTG) and nontransgenic (WT) littermates, age 4 to 6 months, were used in this study. Transgenic mice present fasting plasma triglyceride levels above 300 mg/dl and WT mice below 100 mg/dl. A group of mice received *N*-acetylcysteine (NAC) in the drinking water (daily intake of 1 g/kg body wt) for 2 weeks or 4 months. Lipids from the livers (60 mg) were extracted with chloroform:methanol:water (1:2:0.8) according to Bligh and Dyer [24]. The total lipid extract was dried under a stream of nitrogen and dissolved in 0.4 ml of warm isopropanol (60°C). Liver triglycerides and cholesterol levels were then determined by enzymatic–colorimetric method (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany).

Isolation of mouse liver mitochondria

Mitochondria were isolated by conventional differential centrifugation [19]. The protein content of the mitochondrial suspensions was determined by the biuret assay in the presence of 0.2% deoxycholate, using bovine serum albumin as standard.

Mitochondrial respiratory rates

Oxygen consumption was measured using a temperature-controlled computer-interfaced Clark-type oxygen electrode from Hansatech Instruments equipped with magnetic stirring, at 28°C. Respiratory activities with NAD-linked substrates and lipid substrates were determined by a method modified from Iossa et al. [25]. Briefly, mitochondria were allowed to oxidize their endogenous substrates for 4 min in the presence of 250 nmol ADP mg protein^{-1}. After substrate depletion (stabilization of respiration in a very low rate, around 5 nmol O_2 mg^{-1} min^{-1}), a new addition of ADP (450 nmol ADP mg protein^{-1}) plus malate (2 mM) was done, resulting in no increments in the respiration rates. Substrates (0.01 mM palmitoylcarnitine or 2 mM glutamate) were then added and the phosphorylating respiration rates were determined.

Reactive oxygen species

Mitochondrial H_2O_2 production was followed by measuring the conversion of amplex red (Molecular Probes), in the presence of horseradish peroxidase (HRP), to highly fluorescent resorufin [26]. Mitochondria (0.5 mg/ml) were incubated at 37°C, in medium containing 150 mM KCl, 5 mM Hepes, 2 mM K_2HPO_4, 0.05 mM EGTA, 2 mM succinate, 1 μg/ml oligomycin, 10 μM amplex red, and 1 U/ml horseradish peroxidase, pH 7.4. Fluorescence was monitored over time using a Hitachi 4010 fluorescence spectrophotometer operating at excitation and emission wavelengths of 563 and 587 nm, respectively, slit widths of 3 nm, with continuous stirring.

Mitochondrial swelling

Mitochondrial swelling was estimated from the decrease in absorbance of the mitochondrial suspension measured at 520 nm in an SLM Aminco DW 2000 spectrophotometer (SLM Instruments, Urbana, IL, USA). Mitochondria (0.5 mg/ml) were added to medium containing 100 mM KCl, 5 mM Hepes, 2 mM K_2HPO_4, 0.05 mM EGTA, 2 mM succinate, and 1 μg/ml oligomycin, pH 7.4, at 37°C.

Malondialdehyde (MDA) determination by HPLC

Mitochondria (2 mg) or liver (30 mg) was homogenized in 0.5 ml of cooled pH 7.4 medium containing 0.1 M Tris, 2 mM K_2HPO_4, and 5 mM $MgCl_2$ and mixed with 0.15 ml of 0.2% butylated hydroxytoluene solution in 95% ethanol, 0.1 ml of 10% SDS, and 1 ml of 0.4% thiobarbituric acid (TBA) solution in 0.2 N HCl:H_2O (2:1). The mixture was heated to 90°C for 45 min, cooled on ice, and extracted with 1.5 ml of isobutanol. The isobutanolic phase was injected through a Shimadzu autoinjector Model SIL-10AD/VP (Shimadzu, Kyoto, Japan) in a Shimadzu HPLC system, consisting of two LC-6AD pumps connected to a Lichrosorb 10 RP-18 (Phenomenex, Torrance, CA, USA) reversed-phase column (250×4.6-mm i.d., particle size 10 μm). The flow rate of the isocratic eluent (25 mM K_2HPO_4 buffer, pH 7, with 40% of methanol) was 1 ml/min. An RF-10A/XL fluorescence detector was set at an excitation wavelength of 515 nm and an emission wavelength of 550 nm. The data were processed using the Shimadzu Class-VP 5.03 software. Malonaldehyde–bisdiethylacetal was used for calibration of the fluorescence data, yielding a quantitative adduct of the malonaldehyde–TBA product.

Liver enzyme activities

MnSOD [27], aconitase [28], and fumarase [29] activities were measured in liver as previously described. Xanthine oxidoreductase activity was measured in supernatants prepared according Stirpe and Della Corte [30]. Xanthine oxidase converts xanthine to uric acid using O_2 as an electron acceptor, generating superoxide radicals ($O_2^{\cdot -}$) and

H_2O_2. NADPH oxidase activity was measured in microsomal vesicles prepared according Oliveira et al. [31]. NADPH oxidase catalyzes the production of $O_2^{\cdot -}$ from O_2 and NADPH. Oxygen consumption rates by xanthine and NADPH oxidase activities were measured using a Clark-type electrode as described above.

Reduced (GSH) and oxidized (GSSG) glutathione levels

GSH and GSSG were assayed in liver homogenates and isolated mitochondria separately according to the fluorimetric *ortho*-phthalaldehyde method of Hissin and Hilf [32].

Protein carbonyls

Liver protein carbonyl content was estimated according to Reznick and Packer [33], as modified by Schild et al. [34].

Western blot

Livers (10 mg) were homogenized in 1 ml buffer (10% sucrose, 1% NP-40, 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4, and protease inhibitors 100 mM PMSF, 10 mM STI, 1 mg/ml aprotinin, and 1 mg/ml leupeptin) and centrifuged at 14,000 rpm for 15 min. Supernatants (40 μg protein) were resolved on SDS–polyacrylamide gels (12%), transferred onto nitrocellulose membranes (Protran; Schleicher and Schuell, Keene, NH, USA), and stained with Ponceau S (Sigma) to verify the transfer efficiency and equal sample loading. The membranes were blocked with 5% nonfat milk in Tris–HCl, pH 7.6, containing 150 mM NaCl and 0.1% Tween 20 (TBST) and probed for 2 h at room temperature with antibodies against MnSOD (1:2000; Abcam) or β-actin (1:60,000; Sigma). Membranes were washed in TBST and then incubated with HRP-conjugated secondary antibodies diluted at 1:1000, and washed again and the reactions were developed with an enhanced chemiluminescence detection system (ECL detection kit; Amersham Pharmacia Biotech, Arlington Heights, IL, USA) according to the manufacturer's instructions. Membranes were exposed to high-performance chemiluminescence films (Hyperfilm ECL; Amersham Biosciences, Buckinghamshire, UK).

Metabolic parameters

Liver respiratory rates (slices chopped into 1-mm cubes, 50 mg) were measured using a Clark-type electrode and CO_2 production in vivo was measured in a temperature-controlled respirometer described by Calegario et al. [35] as previously described [19].

Data analysis

Data shown in the figures represent –four to eight independent experiments and are shown as means ± SEM. Statistical analysis was done using the Student *t* test for two-mean comparison or one-way ANOVA for multiple comparisons using the Origin 7.0 software. $P<0.05$ was considered significant.

Table 1
Phosphorylating respiratory rates (State 3) supported by glutamate plus malate or palmitoylcarnitine (PC) plus malate in liver mitochondria isolated from wild-type (WT) and HTG mice

	Glutamate plus malate	PC plus malate	PC relative to glutamate (%)
WT	55.54 ± 0.76	25.30 ± 1.66	45.5 ± 3.01
HTG	61.66 ± 0.93	34.71 ± 4.18*	56.4 ± 6.83*

Means ± SEM. Respiratory rates in nmol oxygen mg protein^{-1} min^{-1}. State 3 respiration was stimulated by 700 nmol ADP mg protein^{-1}.
* $P<0.05$ vs WT.

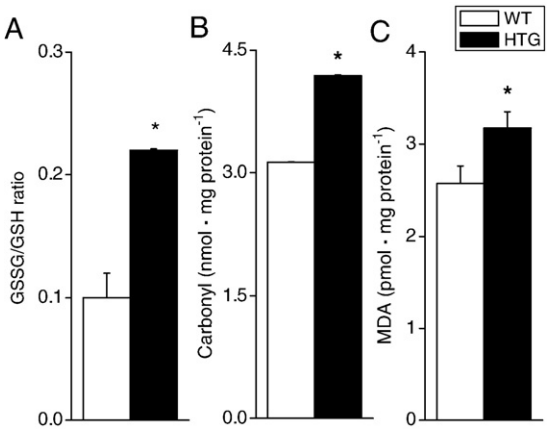

Fig. 1. HTG livers present extramitochondrial oxidative stress. (A) GSSG/GSH ratios, (B) carbonyl proteins, and (C) malondialdehyde (MDA) levels were measured in livers of WT and HTG mice, as described under Experimental procedures. *$P<0.05$ vs WT.

Results

HTG mice presented an approximately eightfold increase in their plasma levels of triglycerides compared to WT mice (559.6 ± 54.7 vs 70.0 ± 7.3 mg/dl, respectively, $P<0.0001$). Next, we examined whether the elevated plasma levels of TG were accompanied by liver lipid accumulation. Once taken up by the liver cell, FFAs can be either esterified into TGs in the cytoplasm compartment or oxidized mainly via mitochondrial β-oxidation. Compared to WT mice, HTG mice had a twofold increase in liver TG content (5.52 ± 1.17 vs 2.76 ± 0.11 mg g^{-1} wet liver, respectively, $P<0.05$), but similar cholesterol levels (1.88 ± 0.08 vs 1.84 ± 0.07 mg g^{-1} wet liver, respectively). Mitochondrial β-oxidation rate was assessed by measuring respiration rates supported by lipid substrates compared to nonlipid substrate in isolated liver mitochondria, which represents an index of fatty acid oxidation [36]. During phosphorylation (State 3 respiration), the rate of respiration supported by palmitoylcarnitine plus malate is 56% compared to that supported by glutamate plus malate in HTG mitochondria, whereas it is 45% in WT mitochondria (Table 1). These results indicate a higher β-oxidation rate in mitochondria from HTG mice, including the rate-limiting step mediated by carnitine palmitoyltransferase. Thus, increases in both FFA esterification and β-oxidation rates indicate FFA overaccumulation in HTG livers.

To assess the redox state of HTG mouse livers, we measured the levels of reduced and oxidized glutathione and the content of oxidized proteins and lipids in this tissue. Fig. 1A shows that the GSSG/GSH

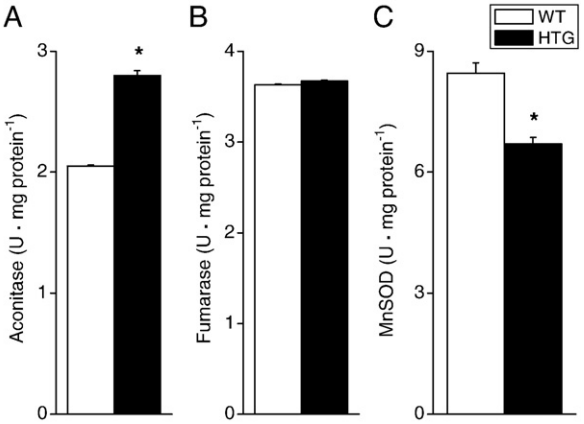

Fig. 2. Mitochondrial enzyme activities in wild-type and HTG livers. Activities of (A) aconitase, (B) fumarase, and (C) MnSOD were measured in livers of WT and HTG mice, as described under Experimental procedures. *$P<0.05$ vs WT.

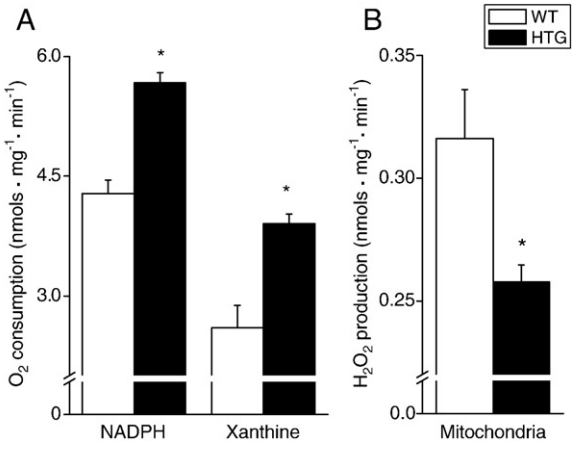

Fig. 3. HTG livers present increased xanthine-and NADPH-oxidase activities and lower mitochondrial H_2O_2 production rates. (A) Oxygen consumption by NADPH oxidase activity in liver microsomal vesicles and oxygen consumption by xanthine oxidase activity in liver cytosol. (B) H_2O_2 production rates in isolated mitochondria. *$P<0.05$ vs WT.

ratio was almost twofold higher in HTG compared to WT livers. Protein and lipid oxidation levels were measured by quantifying protein carbonyl groups (Fig. 1B) and MDA (Fig. 1C). Both protein carbonylation and MDA levels were enhanced in HTG livers, evidencing tissue oxidative stress. However, in isolated mitochondria, there were no differences between the WT and the HTG GSSG/GSH ratios (0.15 ± 0.01 and 0.14 ± 0.02, respectively) or MDA content (31.2 ± 3.0 and 32.9 ± 2.6 pmol mg^{-1}, respectively).

We also measured the activity of aconitase, a mitochondrial matrix enzyme that acts as a superoxide radical anion sensor [37] (Fig. 2A). Surprisingly, aconitase activity was enhanced in HTG livers, suggesting that these mitochondria were actually in a more reduced state. To exclude that enhanced aconitase activity was related to increased mitochondrial biogenesis or globally enhanced citric acid cycle activity, we determined the activity of fumarase, a mitochondrial enzyme that is not a ROS target. Fumarase activity was similar in WT and HTG livers (Fig. 2B). In addition, the activity (Fig. 2C) of the mitochondrial-specific antioxidant enzyme MnSOD was decreased in HTG livers. Thus, these results suggest that HTG liver oxidative stress is caused by extramitochondrial sources.

Next, we measured the activity of the three main sources of hepatic ROS: NADPH oxidase, xanthine oxidase, and mitochondrial respiration (Fig. 3). We found that the ROS-generating enzymes NADPH-oxidase and xanthine-oxidase had significantly increased activities in HTG livers (Fig. 3A), whereas the mitochondrial H_2O_2 release rate was actually decreased in HTG mice (Fig. 3B). These

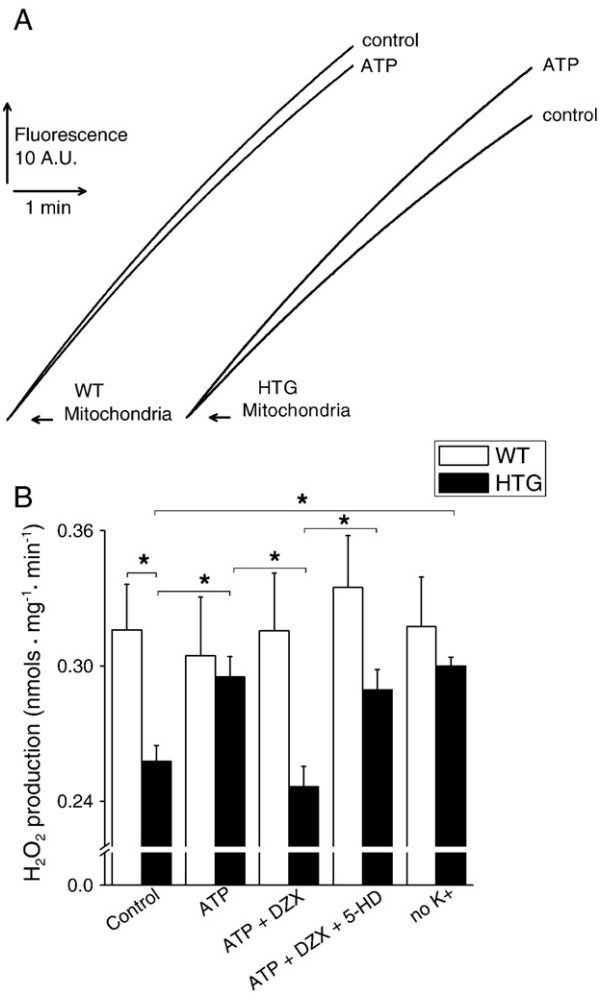

Fig. 4. H_2O_2 release in mitochondria from HTG livers is decreased by stimulated mitoK_{ATP} activity. (A) Typical traces and (B) means ± SEM of H_2O_2 production rates are presented. Mitochondria were added to the reaction medium in the presence of 0.1 mM ATP, 30 μM diazoxide (DZX), and 0.1 mM 5-hydroxydecanoate (5-HD), as indicated. "No K^+" experiments were conducted in medium in which all K^+ salts were substituted by Li^+ salts. *$P<0.05$.

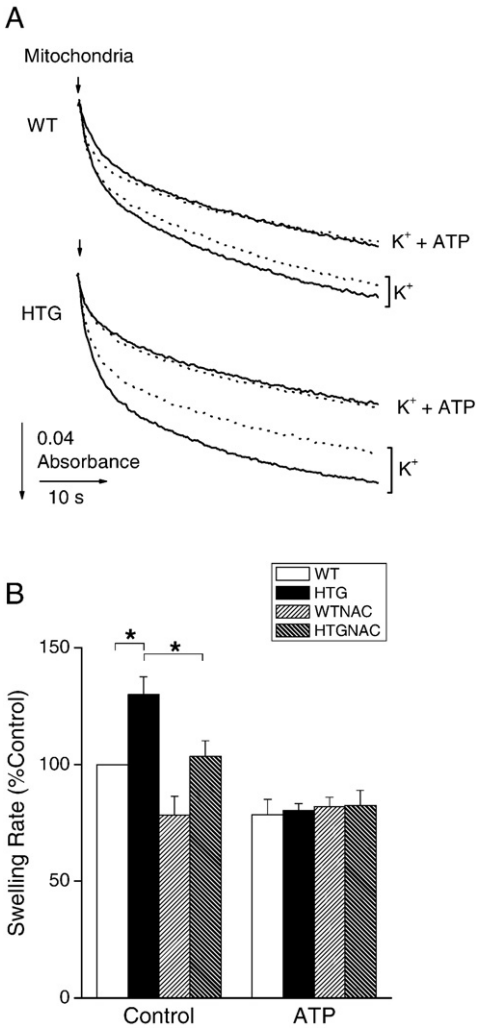

Fig. 5. In vivo treatment with *N*-acetylcysteine (NAC) reverses enhanced ATP-sensitive K^+ uptake in HTG mitochondria. (A) Typical traces and (B) means ± SEM of swelling rates of liver mitochondria isolated from WT and HTG mice (solid lines) and from NAC-treated WT and HTG mice (dotted lines) are shown. Swelling rates were determined during the first 40 s. 0.1 mM ATP was added where indicated. *$P<0.05$.

results strongly suggest that hypertriglyceridemia leads to extra-mitochondrial generation of ROS, whereas the mitochondrial compartment is protected from oxidative stress, as evidenced by the higher aconitase and lower MnSOD activities.

Mitochondrial ROS release is controlled by respiratory rates. In general, faster respiratory rates, such as those found when uncoupling pathways are activated, are accompanied by lower ROS release [22]. We have previously shown that HTG mice present enhanced activity of the mitoK$_{ATP}$, a channel that leads to mild uncoupling [19]. Thus, we investigated whether mitoK$_{ATP}$ channels were responsible for the decreased ROS release observed in HTG liver mitochondria. In mitochondria isolated from WT livers, we found that ROS release was not changed by the presence of the mitoK$_{ATP}$ inhibitors ATP and 5-hydroxydecanoate (5-HD) or the mitoK$_{ATP}$ activator diazoxide (DZX) (Fig. 4B). This result is in line with the finding that the activity of this channel in WT mouse livers is very low [19]. On the other hand, lower ROS release by HTG mitochondria was reversed when mitoK$_{ATP}$ was inhibited by ATP, whereas DZX restored diminished ROS release in HTG mitochondria, in a manner sensitive to the antagonist 5-HD (Fig. 4B). No differences in ROS generation between HTG and WT mitochondria were observed in medium devoid of K^+ salts. Together, these results demonstrate that lower ROS release rates in HTG mitochondria are attributable to enhanced mitoK$_{ATP}$ activity.

To determine whether the enhanced mitoK$_{ATP}$ activity observed in HTG mitochondria was related to liver oxidative stress, we compared the activity of this channel in WT and HTG mitochondria isolated from mice chronically treated with the antioxidant NAC. The effect of in vivo NAC treatment on the mitoK$_{ATP}$ activity is shown in Fig. 5. As observed previously [19], HTG mitochondria present significantly increased swelling in K^+-containing medium compared with WT, in which swelling rates are very low. However, mitoK$_{ATP}$-mediated swelling in NAC-treated HTG mouse mitochondria was significantly lower than in untreated HTG and was similar to that of WT mitochondria, demonstrating that the activation of this channel in these HTG mice involves redox signaling. When the channel inhibitor ATP was present in the medium, no differences in mitochondrial swelling were detected among all groups, regardless of NAC treatment (Fig. 5B). In addition, in vivo treatment with NAC normalized all liver oxidative stress markers, namely GSSG/GSH ratio and carbonyl and MDA contents (Figs. 6A–6C). As expected for an antioxidant agent, NAC treatment increased aconitase activity significantly in the WT livers (Fig. 6D compared to Fig. 2A). Because HTG livers already had higher aconitase activity, no significant changes were observed after this treatment (Fig. 6D). Fumarase activity was unaltered by NAC (Fig. 6D). Regarding MnSOD, the Western blot analysis showed that its liver content was indeed significantly reduced in HTG mice. This difference between WT and HTG liver content of MnSOD disappeared after NAC treatment (Fig. 6E).

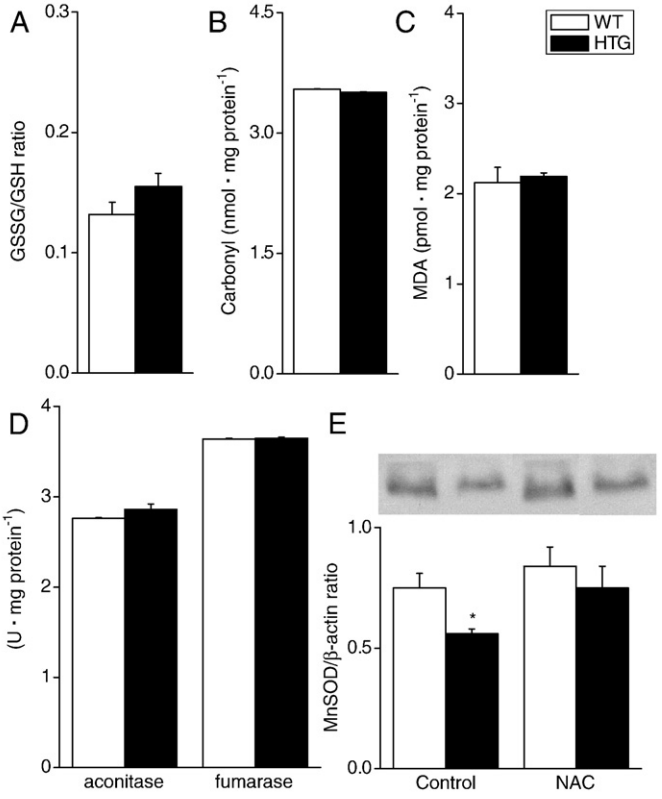

Fig. 6. Effects of in vivo treatment with NAC on the liver redox markers and mitochondrial enzymes. (A) GSSG/GSH ratio, (B) carbonyl protein content, (C) MDA content, (D) aconitase and fumarase activities, (E) MnSOD content normalized to β-actin measured by Western blot. *P<0.05 vs WT under control conditions.

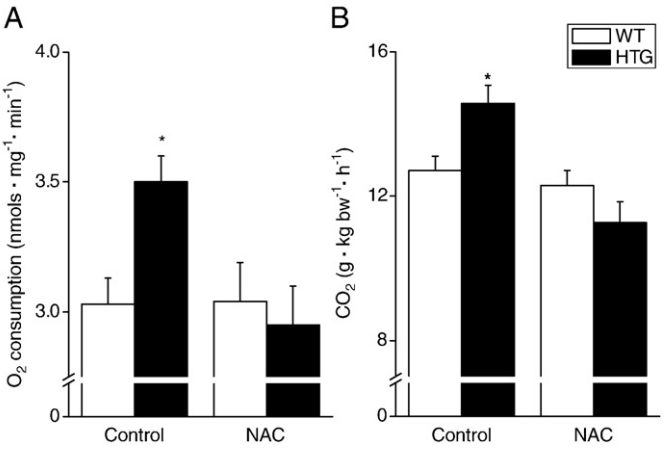

Fig. 7. In vivo treatment with NAC reverses enhanced rates of O_2 consumption in HTG livers and CO_2 production in HTG mice. (A) Respiratory rates of liver fragments incubated at 37°C in Krebs-Henseleit solution. (B) CO_2 production rates in WT and HTG mice. *P<0.05 vs WT under control conditions.

The finding that in vivo NAC treatment reverses enhanced mitoK$_{ATP}$ activity in HTG liver mitochondria reveals an interesting experimental tool for verifying the effects of this channel on energy metabolism at the level of the organ and of the whole animal. We have previously shown that HTG mice have increased liver oxygen consumption and whole-body CO_2 production and proposed that these effects were related to enhanced uncoupling promoted by mitoK$_{ATP}$ [19]. Here, we measured these parameters in NAC-treated HTG mice in which mitoK$_{ATP}$ activity was inhibited by NAC. Plasma levels of TG in mice were not significantly modified by in vivo NAC treatment (74.8 ± 12.6 and 81.3 ± 19.4 mg/dl for basal and post-treatment WT mice, respectively; and 513.6 ± 84.0 and 427.8 ± 26.5 mg/dl for basal and post-treatment HTG mice, respectively). Thus, NAC effects cannot be attributed to major changes in circulating TG levels in these mice. In contrast, NAC treatment completely reversed enhanced liver oxygen consumption and whole-body CO_2 production observed in untreated HTG mice (Fig. 7), strongly supporting the proposal that this increased oxidative metabolism is due to mild mitochondrial uncoupling promoted by mitoK$_{ATP}$. Indeed, respiration of liver mitochondria isolated from NAC-treated HTG mice (Table 2) indicates that NAC significantly decreased higher resting (State 4) respiration and, hence, increased respiratory control ratios (State 3/State 4) in HTG mitochondria.

Discussion

High plasma FFA and triglyceride levels lead to increased uptake of FFA into nonadipose tissues and contribute to intracellular lipid accumulation. Chronic accumulation of fat in liver is often seen in

Table 2
Phosphorylating (State 3) and resting (State 4) respiratory rates in liver mitochondria isolated from wild-type (WT) and HTG mice, treated or not with NAC

	State 3	State 4	State 3/State 4
WT	64.47 ± 6.16	14.69 ± 0.74	4.75 ± 0.16
HTG	70.87 ± 1.41	19.65 ± 0.72*	3.61 ± 0.09*
WT NAC	73.52 ± 6.42	15.29 ± 2.01	4.87 ± 0.25
HTG NAC	52.85 ± 0.88	10.08 ± 1.41**	5.36 ± 0.84**

Means ± SEM. Respiratory rates in nmol oxygen mg protein^{-1} min^{-1}.
* $P<0.05$ vs WT.
** $P<0.05$ vs HTG.

rodent models of genetic obesity and in some obese humans [38] and is shown here to occur in genetic hypertriglyceridemia. Although triglycerides stored in lipid droplets may be relatively inert in liver cells, FFAs may also overaccumulate [39]. FFA accumulation may evoke hepatocyte damage by several mechanisms, including activation of ROS-generating microsomal enzymes such as cytochromes P450 2E1 and 4A [40]. Oxidized fatty acids themselves can catalyze lipid peroxidation reactions that are directly cytotoxic. For instance, 4-hydroxynonenal, a by-product of these reactions, may cause the conversion of xanthine dehydrogenase into a xanthine oxidase form in liver [41]. Finally, because certain fatty acids, particularly polyunsaturated and their derivatives and oxidized fatty acids, function as endogenous ligands for transcription factors, such as PPAR and HNF4, their intracellular accumulation might affect global changes in liver gene expression [42]. Regarding the mitochondrial compartment, FFAs may exert dual effects on ROS production [12]. Owing to direct interactions within respiratory complex structures, FFAs may increase the generation of ROS. On the other hand, owing to their protonophoric action on the inner mitochondrial membrane, FFAs may decrease ROS production. In addition, Bakker et al. [43] have hypothesized that the cytotoxic effects of FFAs are related to their effect on blocking adenine nucleotide translocator activity. This would lead to an intramitochondrial ADP deficit with consequent increase in membrane potential and mitochondrial ROS production.

Our previous work showed that mitoK$_{ATP}$ activity is augmented in the livers of HTG mice [19]. This enhanced mitoK$_{ATP}$ activity increases mitochondrial respiratory rates under nonphosphorylating conditions, in a manner dependent on the intracellular pool of FFAs [18]. This work provides evidence that mitoK$_{ATP}$ activation decreases mitochondrial but not whole-cell oxidative stress in HTG livers. Whereas HTG livers had elevated levels of oxidized proteins, lipids, and glutathione, mitochondria presented higher aconitase activity, preserved GSSG/GSH ratios and MDA levels, and decreased H_2O_2 production rates and MnSOD content and activity. Because antioxidant enzymes are substrate-inducible [44], the decreased activity and content of MnSOD is probably the result of downregulation caused by low exposure to an oxidant microenvironment inside the mitochondria [45]. It is important to emphasize that diminished ROS generation by HTG mitochondria not only protects the organelles but also avoids amplification of oxidative stress initiated by extramitochondrial oxidases.

Liver oxidative state found in HTG mice can be explained by increased cytosolic and microsomal ROS production promoted by higher activities of xanthine and NADPH oxidases, respectively. In agreement with our results, a significant increase in xanthine oxidase activity was found in patients with hypertriglyceridemia compared with normolipidemic controls [46]. Furthermore, these authors observed a positive correlation between xanthine oxidase activity and plasma triglyceride levels. The relevance of xanthine oxidase activity for lipid-related disorders was demonstrated experimentally when its inhibitor tungsten prevented the development of atherosclerosis in hyperlipidemic ApoE knockout mice fed a high-fat diet [47].

Elevation of NADPH oxidase activity in hyperlipidemic states has been implicated in vascular tissue oxidative stress and endothelial dysfunction [48]. Gene disruption of p47(phox), an essential component of NADPH oxidase, reduces vascular superoxide production but is not sufficient to prevent atherosclerosis in hyperlipidemic ApoE knockout mice [49]. Hyperlipidemic rodent models have increased expression of NADPH oxidase in heart [50], aorta and kidney [16], and adipose tissue [17]. Here we showed a significant increase in NADPH oxidase activity in the liver of hypertriglyceridemic mice. Accordingly, studies with human aorta vascular endothelial cells showed that lipolysis of TG-rich lipoproteins significantly induces NADPH oxidase-dependent production of ROS [51].

The present data indicate that the oxidant status in HTG livers is involved in the mechanism of mitoK$_{ATP}$ activation, because in vivo antioxidant treatment with NAC inhibited mitoK$_{ATP}$ opening in HTG liver mitochondria, without changing plasma TG concentrations. MitoK$_{ATP}$ opening has previously been shown to be regulated by thiol oxidation in vitro [52,53,23]. NAC is a well-established thiol antioxidant that, after uptake, deacylation, and conversion to glutathione, functions as both a redox buffer and a ROS intermediate scavenger (for review see [54]). Indeed, in vitro addition of NAC to isolated mitochondria was capable of preventing mitoK$_{ATP}$-related K^+transport in HTG mitochondrial suspensions (results not shown). Therefore, the effect of NAC repressing higher mitoK$_{ATP}$ activity could be either directly to reduce channel thiol redox status or indirectly to decrease extramitochondrially generated ROS levels in HTG liver.

In our previous work, we proposed that higher liver mitoK$_{ATP}$ activity increases whole-body energy metabolism in HTG mice [19]. Here, in vivo NAC treatment decreased mitoK$_{ATP}$ opening and restored mitochondrial resting respiration, liver O_2 consumption, and body CO_2 release. Therefore, our results strongly support the idea that elevated overall metabolic rates in these HTG mice are due to higher mitoK$_{ATP}$ activity.

In conclusion, the results presented here indicate that the hypertriglyceridemic state per se, without other components of metabolic syndrome, increases the activity of extramitochondrial ROS-producing systems in liver. The tissue oxidized state activates mitoK$_{ATP}$ channels, which increases resting respiration and decreases mitochondrial ROS generation, conserving the organelle in a more reduced state and avoiding amplification of overall oxidative stress. This mitochondrial adaptation to the hyperlipidemic environment increases liver and body energy metabolism but does not fully prevent liver oxidative damage (Fig. 8). Thus, we showed, for the first time in an in vivo model, a biological role for the mitoK$_{ATP}$ as a

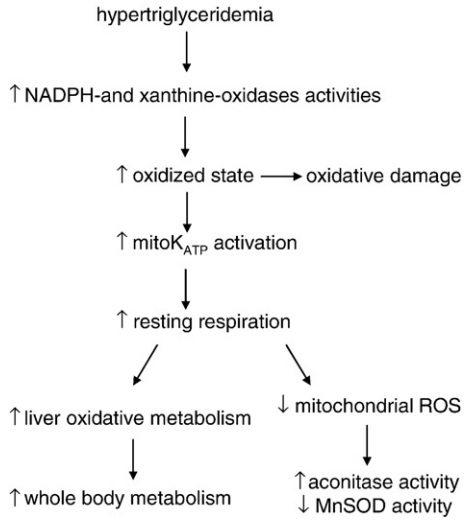

Fig. 8. Proposed model to explain how hypertriglyceridemia and mitoK$_{ATP}$ affect energy metabolism and cell redox state.

redox sensor allowing a cross talk between extra-and intramitochondrial compartments.

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