Available online at www.sciencedirect.com
ScienceDirect
Free Radical Biology & Medicine 44 (2008) 444–451
www.elsevier.com/locate/freeradbiomed

Original Contribution

Oxidative stress in hypercholesterolemic LDL (low-density lipoprotein) receptor knockout mice is associated with low content of mitochondrial NADP-linked substrates and is partially reversed by citrate replacement

Bruno A. Paim [a], Jesus A. Velho [a], Roger F. Castilho [a], Helena C.F. Oliveira [b], Aníbal 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, 13083-970, Campinas, SP, Brazil

Received 16 July 2007; revised 18 September 2007; accepted 8 October 2007
Available online 16 October 2007

Abstract

We have previously proposed that hypercholesterolemic LDL receptor knockout (k/o) mice mitochondria possess a lower antioxidant capacity due to a large consumption of reducing equivalents from NADPH to sustain high rates of lipogenesis. In this work, we tested the hypothesis that this k/o mice mitochondrial oxidative stress results from the depletion of NADPH-linked substrates. In addition, the oxidative stress was further characterized by showing a lower mitochondrial GSH/GSSG ratio and a higher liver content of protein carbonyls as compared to controls. The activity of the antioxidant enzyme system glutathione reductase/peroxidase did not differ in k/o and control mitochondria. The faster spontaneous oxidation of endogenous NADPH in the k/o mitochondria was prevented by the addition of exogenous catalase, indicating that this oxidation is mediated by mitochondrially generated H_2O_2. The higher rate of H_2O_2 production was also prevented by the addition of exogenous isocitrate that maintains NADP fully reduced. The hypothesis that high rates of lipogenesis in the k/o cells decrease mitochondrial NADPH/$NADP^+$ ratio due to consumption of NADPH-linked substrates was supported by two findings: (i) oxygen consumption supported by endogenous NAD(P)H-linked substrates was slower in k/o than in control mitochondria, but was similar in the presence of exogenous isocitrate; (ii) in vivo treatment of k/o mice with sodium citrate/citric acid drinking solution for 2 weeks partially restored both the rate of oxygen consumption supported by NAD(P)H-linked substrates and the mitochondrial capacity to sustain reduced NADPH. In conclusion, the data demonstrate that the mitochondrial oxidative stress in hypercholesterolemic LDL receptor knockout mice is the result of a low content of mitochondrial NADPH-linked substrates in the intact animal that can be, at least in part, replenished by oral administration of citrate.

Keywords: Hypercholesterolemia; LDL receptor; Mitochondria; Reactive oxygen species; Pyridine nucleotide oxidation

Introduction

Atherosclerosis is a leading cause of death in the western world and is causally linked to elevated plasma concentrations of low-density lipoproteins (LDL) [1–3]. One of the most consistent hypothesis to explain atherogenesis postulates that it is triggered by in vivo LDL oxidation caused by reactive oxygen species (ROS) from circulating and vascular wall cells [4–6]. Oxidized LDL stimulates the recruitment of circulating monocytes, induces their differentiation into macrophages, and is cytotoxic to endothelial and other vascular cells [7]. Several in vitro studies have demonstrated that oxidized LDL causes death of all cells involved in atherogenesis [8–10].

Aerobic cells can generate ROS during reduction of molecular oxygen by enzymatic reactions (NADH/NADPH oxidase, xanthine oxidase, lipoxygenase, and cyclooxygenase systems), by autoxidation of diverse substances, and by the mitochondrial electron transport chain [11]. Mitochondria represent a major intracellular source of ROS [12,13] and may mediate cellular oxidative stress leading to cell death [14].

We have recently provided evidence that mitochondria from various tissues from the hypercholesterolemic, atherosclerosis prone, LDL receptor knockout (LDLr k/o) mice generate more

Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; HE, hydroethidine; IDH, isocitrate dehydrogenase; LDL, low-density lipoproteins; LDLr k/o, LDL receptor knockout; MLM, mouse liver mitochondria; MPT, mitochondrial permeability transition; OPT, *ortho*-phthalaldehyde; ROS, reactive oxygen species; *tert*-BOOH, *tert*-butyl hydroperoxide; WT, wild type.

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

0891-5849/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.freeradbiomed.2007.10.005

ROS than the controls, thus suggesting that mitochondrial ROS may be involved in the early steps of atherogenesis in this model [15]. Mitochondria isolated from the low-fat-fed LDLr k/o mice tissues produced more ROS and had greater susceptibility of developing mitochondrial permeability transition (MPT), an initial step in the mitochondrial pathway of cell death either by apoptosis or necrosis [14,16,17]. The higher ROS generation by the LDLr k/o mice mitochondria was shown to be unrelated to differences in mitochondrial membrane fluidity, cholesterol content, or MnSOD activity [15]. In addition, we showed that the LDLr k/o mice present significantly higher liver lipogenesis and lipid secretion rates than the controls, in order to compensate for the lack of LDL cholesterol uptake [15]. We proposed that the increased cholesterol synthesis in LDLr k/o mice decreases the availability of reduced cellular NADPH [15]. This nucleotide is the major mitochondrial source of reducing equivalents for the antioxidant systems glutathione reductase/peroxidase and thioredoxine reductase/peroxidase and its deficiency leads to the condition of oxidative stress [14,18].

In the present work, we characterized the condition of oxidative stress of LDLr k/o mice mitochondria, linked it to the depletion of endogenous content of NADPH-linked substrates, and show that in vivo citrate administration can decrease ROS generation by the LDLr k/o mitochondria.

Materials and methods

Animals

LDLr k/o founders were purchased from the Jackson Laboratory (Bar Harbor, ME). Control wild-type C57Bl/6 mice (WT) were obtained from the breeding colony at the State University of Campinas (CEMIB/Unicamp). The experiments were approved by the Committee for Ethics in Animal Experimentation of the university (CEEA/Unicamp). The mice had free access to standard laboratory rodent low-fat (4%)-chow diet (Nuvital CR1, Paraná, Brazil) and water ad libitum and were housed at 22±1°C on a 12-h light:dark cycle. Male and female mice, 4 to 6 month old and weighing 20–26 g, were used in this study. Additional groups of LDLr k/o mice were provided with water or an autoclaved solution of 55 mM sodium citrate/67 mM citric acid [19] ad libitum for 2 weeks. When provided with water control and LDLr k/o mice had a fluid intake of 5.0±0.5 and 4.9±0.3 mL/day, respectively, while LDLr k/o mice provided with a sodium citrate/citric acid solution had a fluid intake of 3.5±0.3 mL/day. The average plasma cholesterol concentrations in k/o and control groups were 301±42 and 85±12 mg/dL, respectively. Two weeks of citrate supplementation did not change cholesterol levels in supplemented k/o mice (329±63 mg/dL).

Isolation of mouse liver mitochondria (MLM) and respiration measurements

MLM were isolated by conventional differential centrifugation from the livers of adult mice fasted overnight [20]. The protein concentration was determined by a modified Biuret assay. Oxygen consumption by the mitochondria (2 mg/mL) was measured in standard reaction medium (125 mM sucrose, 65 mM KCl, 2 mM inorganic phosphate, 1 mM magnesium chloride, and 10 mM Hepes buffer, pH 7.2) using a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, UK) in a 0.5-mL glass chamber equipped with a magnetic stirring. The experiments were done at 28°C.

Kinetics of pyridine nucleotide oxidation

The redox state of pyridine nucleotides in the mitochondrial suspension (1 mg/mL) was followed in a spectrofluorometer (Hitachi F-4500, Tokyo, Japan) using excitation and emission wavelengths of 366 and 450 nm, respectively, and slit widths of 5 nm. The extent of pyridine nucleotide oxidation was calculated as a function of fluorescence decrease. Internal calibration was done by the addition of known amounts of NADPH.

Reduced (GSH) and oxidized (GSSG) glutathione levels

Mitochondrial GSH and GSSG were assayed separately according to the fluorimetric *ortho*-phthalaldehyde (OPT) method of Hissin and Hilf [21]. This method is based on the principle that OPT reacts with GSH and GSSG, at pH 8.0 and pH 12, respectively, to yield a highly fluorescent product which can be activated at 350 nm with an emission peak of 420 nm. GSSG levels were determined after sample treatment with *N*-ethylmaleimide for removing GSH completely. The concentrations of GSH and GSSG in samples were calculated according to standard curves prepared with GSH and GSSG, respectively.

Activity of mitochondrial glutathione peroxidase/reductase system

The mitochondria were lysed by the addition of 0.1% Triton X-100 in standard medium reaction containing 500 μM GSH and 100 μM NADPH. The activity of the mitochondrial glutathione peroxidase/reductase system was estimated by the rate of NADPH oxidation after the addition of 0.5 mM *tert*-butyl hydroperoxide (*tert*-BOOH; an oxidant agent).

Reactive oxygen species production

H_2O_2 production in isolated mitochondria [22] was followed by measuring the conversion of Amplex red (Molecular Probes, Invitrogen, Carlsbad, CA), in the presence of extramitochondrial horseradish peroxidase, to highly fluorescent resorufin, with 1:1 stoichiometry [23]. Mitochondrial suspensions were incubated in the presence of 10 μM Amplex red and 1 U/mL horseradish peroxidase, and fluorescence was monitored over time using a temperature-controlled (28°C) spectrofluorometer (Hitachi F-4500, Tokyo, Japan) using excitation and emission wavelengths of 563 and 587 nm, respectively, and slit widths of 5 nm, with gentle continuous stirring. Under these conditions, a linear increment in fluorescence indicates the rate of H_2O_2 released from mitochondria, which reacts with the peroxidase.

Superoxide ($O_2^{\bullet -}$) production was measured using hydroethinine (HE) oxidation to fluorescent products [24,25] in mitochondrial suspensions supplemented with 5 μM hydroethinine. HE fluorescent products were measured using excitation and emission wavelengths of 470 and 590 nm, respectively, and slit widths of 5 nm, at 28°C.

Estimation of mitochondrial isocitrate content

Mitochondria suspension (10 mg/mL) was added to 0.5 mL buffer containing 40 mM Tris, 2 mM $MgCl_2$, 0.5% Triton X-100, and 2 mM $NADP^+$, pH 7.4. Then, 5 μg (0.03 U) of $NADP^+$-isocitrate dehydrogenase (IDH) was added. The isocitrate availability, i.e., isocitrate and its generation by Krebs cycle precursors, was estimated by the production of NADPH, at 28°C, in a Hitachi F-4500 spectrofluorometer using excitation and emission wavelengths of 366 and 450 nm, respectively, and slit widths of 2.5 nm. A calibration curve was obtained by adding known concentrations of isocitrate, and the results are expressed as nanomole isocitrate per milligram of protein. One unit of IDH activity is defined as the amount of enzyme catalyzing the production of 1 μmol of NADPH/min.

Estimation of protein carbonyls

Protein carbonyl content was estimated according to Reznick and Packer [26], as modifed by Schild et al. [27]. The samples (5 mg of protein from liver homogenate) were treated with 10 mM dinitrophenylhydrazine in 2.5 M HCl for 1 h at room temperature. The reaction was stopped by the addition of 20% tricholoracetic acid. The pellets were washed twice with absolute ethanol/ethylacetate (1/1) and once with 10% tricholoracetic acid. The protein pellets were finally dissolved in 6 M guanidine hydrochloride and the absorption at 370 nm was determined. Carbonyl content was calculated using the molar absorption coefficient of aliphatic hydrazones of 22,000 M^{-1} cm^{-1} and expressed as nanomole carbonyl per milligram of protein.

Data analysis

Data from the experiments were analyzed by one-way ANOVA followed by Turkey's post hoc test performed by Origin 7.5 software (OringinLab Corp., Northampton, MA). When one parameter was compared betwen two groups, Student's *t* test was used. Data are presented as mean ± SE of at least four experiments conducted with different preparations.

Results

In vivo treatment with citrate increases the content of liver mitochondrial NAD(P)H-linked substrates in hypercholesterolemic LDLr k/o mice

We have previously proposed that hypercholesterolemic LDLr k/o mice mitochondria have a lower antioxidant capacity due to a large consumption of reducing equivalents from NADPH to sustain high rates of lipogenesis [15]. In order to ascertain this proposition we measured both the content of endogenous isocitrate and the rates of respiration supported by endogenous NAD(P)H-linked substrates in liver mitochondria isolated from control and LDLr k/o mice. Fig. 1 shows that the content of endogenous isocitrate is significantly higher in the control (line a) than in LDLr k/o mitochondria (line b). Fig. 2A shows that the rate of oxygen consumption supported by endogenous NAD(P)H-linked substrates by LDLr k/o mice mitochondria (line b) is slower than by control mitochondria (line a). These data and those demonstrating that the k/o mice mitochondria have higher rates of lipogenesis [15] led to the hypothesis that the content of mitochondrial NAD(P)H-linked substrates could be replenished in k/o mice mitochondria by oral supplementation of LDLr k/o mice with citrate. Indeed, after they had drunk water containing 55 mM sodium citrate/67 mM citric acid for 2 weeks, the initial rate of mitochondrial oxygen consumption was significantly increased (Fig. 2A, line c), approaching that of the control mitochondria (line a). When 1 mM isocitrate was present in the incubation medium (Fig. 2B), the rates of oxygen consumption were similar under all conditions, showing that the activities of the respective dehydrogenases were similar and that the limiting factor in the LDLr k/o mitochondria was indeed the content of substrates. These experiments were done in the presence of ADP to release the inhibition of respiration by $\Delta\Psi$.

Accordingly, the capacity of citrate-treated LDLr k/o mice mitochondria to sustain reduced NADPH was also significantly improved compared to untreated LDLr k/o mitochondria. This is illustrated in Fig. 3, where the time-dependent decrease in NADPH fluorescence was significantly slower in citrate-treated k/o mice mitochondria (line c compared with line b). This reflects the shift of NADPH to a more oxidized state in a manner reversed by isocitrate [28]. Re-reduction by isocitrate but not by β-hydroxybutirate [15] confirms that the observed fluorescence decrease is mainly due to NADPH and not to NADH oxidation [28]. Line a represents an experiment using mitochondria isolated from control mice.

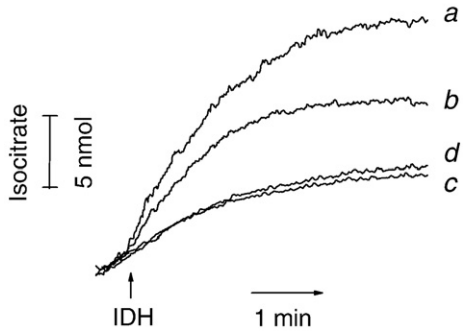

Fig. 1. Isocitrate content is lower in LDLr k/o than in wild-type mitochondria. MLM (10.0 mg/mL) from wild-type (WT) (line a) and LDLr k/o mice (line b) were added to 40 mM Tris buffer containing 0.5% Triton X-100, 2 mM $NADP^+$, and 2 mM $MgCl_2$. The arrow indicates the addition of 5 μg isocitrate dehydrogenase (IDH) (0.03 U). Lines c and d represent experiments with MLM from WT and LDLr k/o mice, respectively, without the addition of IDH. The isocitrate contents (nmol/mg) are as follows: 2.35 ± 0.17 for WT vs 1.40 ± 0.19 for LDLr k/o, $P < 0.001$. Lines are representative of six independent experiments.

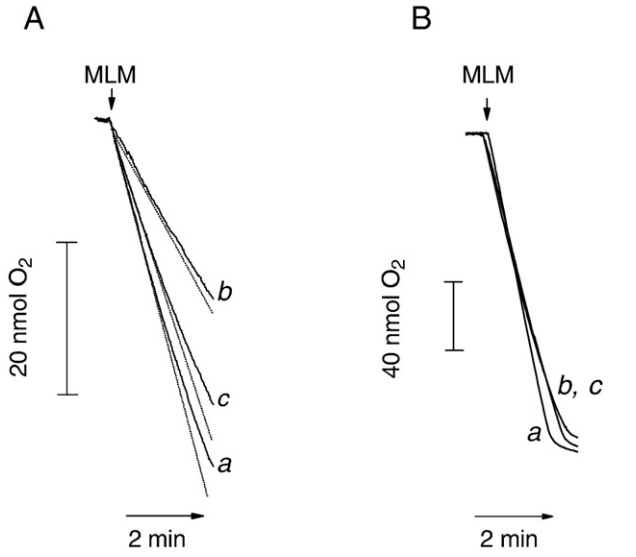

Fig. 2. Effects of in vivo citrate (A) and in vitro isocitrate (B) treatment on the oxygen consumption in mitochondria isolated from WT and LDLr k/o mice. (A) MLM (2.0 mg/mL) from WT (line a), LDLr k/o (line b), and citrate-treated LDLr k/o (line c) were added to standard reaction medium in the presence of 100 μM EGTA and 1 mM ADP. The rates of O_2 consumption (nmol O_2 /mg protein/min) supported by endogenous substrates were as follows: 5.40±0.27 for WT (line a), 3.20±0.15 for LDLr k/o (line b), and 4.89±0.19 for citrate-treated LDLr k/o (line c); $P<0.001$ for LDLr k/o vs WT and for LDLr k/o vs citrate-treated LDLr k/o. (B) Oxygen consumption in the presence of 1 mM exogenous isocitrate. The rates of O_2 consumption (nmol O_2 /mg protein/min) are as follows: 43.80±2.85 for WT (line a), 40.55±2.07 for LDLr k/o (line b), and 45.00±1.45 for citrate-treated LDLr k/o (line c). Lines are representative of five independent experiments.

Low mitochondrial GSH/GSSG ratio and high content of protein carbonyls in the liver of k/o mice

In order to further characterize the condition of oxidative stress in the livers of the hypercholesterolemic k/o mice we measured the levels of reduced and oxidized mitochondrial glutathione and the content of tissue protein carbonyls. Fig. 4 shows that the ratio GSH/GSSG was 30% lower in the k/o than in the control mitochondria and that this was totally reversed when the mitochondrial suspension was preincubated with isocitrate prior to the glutathione determination. The occurrence of protein oxidation in the livers of these mice was assessed by measuring their contents of protein carbonyls, which were significantly higher (12%) in the k/o than in the control liver (Fig. 5). These results strongly support the association between low content of mitochondrial NADPH and oxidative stress in these mice.

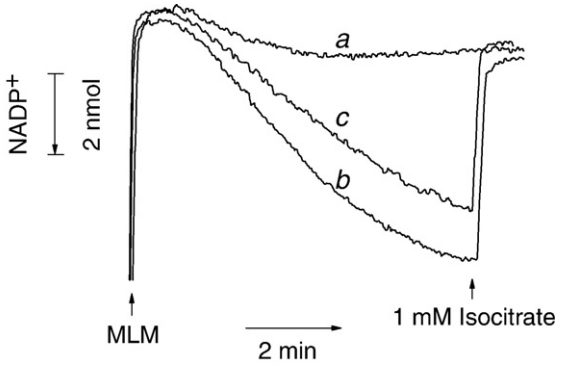

Fig. 3. Spontaneous oxidation of mitochondrial pyridine nucleotides was partially inhibited in LDLr k/o mice treated with citrate. MLM (1 mg/mL) from WT (line a), LDLr k/o (line b), and citrate-treated LDLr k/o (line c) were added to standard reaction medium in the presence of 100 μM EGTA, 5 mM succinate, and 5 μM rotenone. Isocitrate (1 mM) was added as indicated. Extent of pyridine nucleotide oxidation (nmol/mg/min) is as follows: none for WT, 0.8±0.06 for LDLr k/o, and 0.57±0.07 for citrate-treated LDLr k/o. Lines are representative of five independent experiments. $P<0.001$ for WT vs LDLr k/o or citrate-treated LDLr k/o; $P<0.05$ for LDLr k/o vs citrate-treated LDLr k/o.

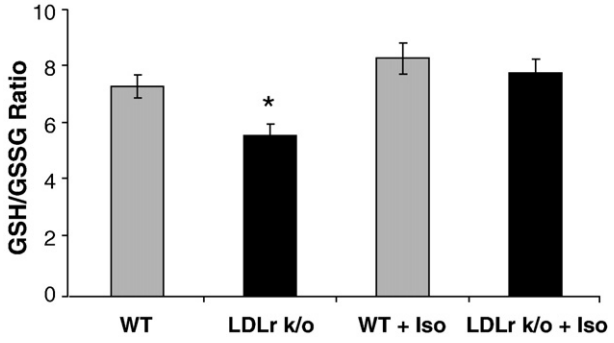

Fig. 4. GSH/GSSG ratio is lower in LDLr k/o than in WT mice mitochondria. MLM (0.5 mg/mL) from WT or LDLr k/o were added to standard reaction medium containing 5 mM succinate and 5 μM rotenone in the absence or presence of 1 mM isocitrate ("+ Iso"). After 10 min of incubation, aliquots (100 μL) were removed and assayed for GSH and GSSG as described under Materials and methods. Bars represent the mean±SE of five independent experiments, *$P<0.05$ vs WT.

Normal activity of the glutathione reductase/peroxidase system in LDLr k/o mice

A possible dysfunction of the glutathione reductase/peroxidase system in the k/o mice liver was ruled out by experiments measuring its activity in mitochondria treated with 0.1% Triton X-100 to expose the enzymes to their substrates. Fig. 6 shows that the addition of either control (line a) or LDLr k/o (line b) mitochondrial lysates promoted NADPH oxidation (fluorescence decrease)

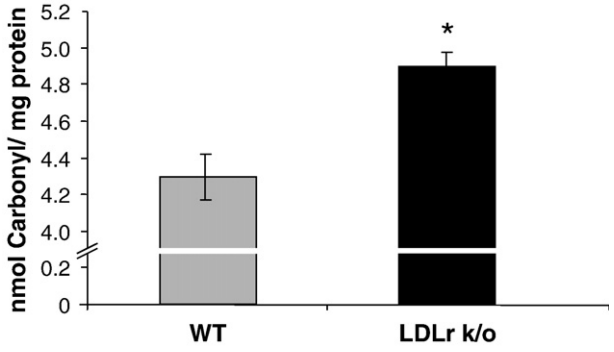

Fig. 5. Levels of protein oxidation estimated as carbonyl compounds were higher in LDLr k/o than in WT mice. Carbonyl contents were measured in liver homogenates (5 mg protein/mL) as described under Materials and methods. Bars represent the mean±SE of seven independent experiments; *$P<0.01$ vs WT.

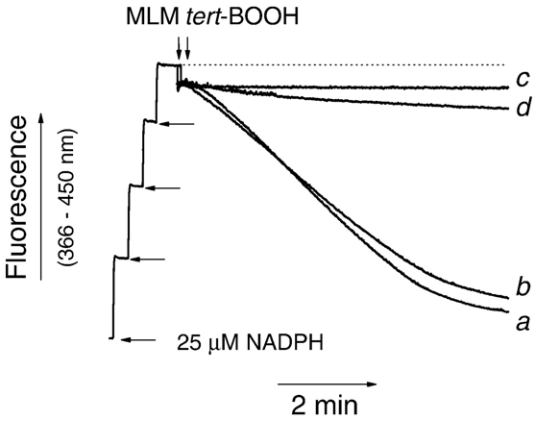

Fig. 6. Activity of glutathione peroxidase/glutathione reductase is similar in LDLr k/o and wild-type liver mitochondria. MLM (1.0 mg/mL) from WT (lines a, c, d) and LDLr k/o (line b) were lysed by the addition of 0.1% Triton X-100. Standard reaction media contained 500 μM GSH and 100 μM NADPH. The reaction started after the addition of 0.5 mM *tert*-BOOH. Lines c, d, and dashed represent control experiments conducted in the absence of *tert*-BOOH, GSH, and mitochondria, respectively. The rates of NADPH oxidation (nmol NADPH/mg protein/min) were as follows: 34.54±1.58 for WT and 30.98±2.52 for LDLr k/o. Lines are representative of five independent experiments.

at similar rates, demonstrating that both mitochondria have activities similar to those of the glutathione reductase/peroxidase enzyme system. Control experiments show that in the absence of mitochondria (dashed line) or when the mitocondrial lysates were added in the absence of either *tert*-BOOH (line c) or GSH (line d) only a very slow rate of fluorescence decrease was observed.

H_2O_2 generation is faster in k/o mice than in control mice mitochondria

The preceding results agree with data indicating that the condition of oxidative stress in the LDLr k/o mice mitochondria is related to lack of reducing power instead of defects in their antioxidant machinery. To confirm this proposition, we estimated the rates of $O_2^{\bullet -}$ and H_2O_2 production by control and k/o mice mitochondria. Fig. 7 shows that the rates of $O_2^{\bullet -}$ generation did not differ in either mitochondria. In contrast, Fig. 8 shows that H_2O_2 is produced at a faster rate in the LDLr k/o (line b) than in control (line a) mitochondria. However, preincubation of both mitochondria with isocitrate eliminated

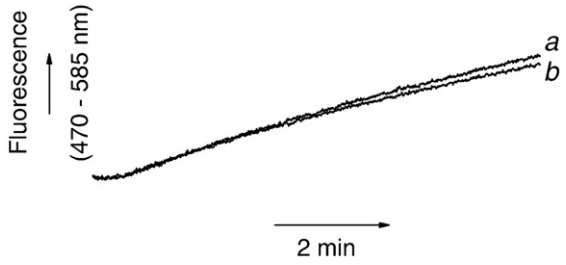

Fig. 7. Superoxide generation is similar in LDLr k/o and WT liver mitochondria. MLM (0.5 mg/mL) were added to standard reaction medium containing 5 mM succinate and 5 μM hydroethidine. Lines a and b represent WT and LDLr k/o mitochondria, respectively. Traces are representative of five independent experiments. $P>0.05$ for LDLr k/o vs WT.

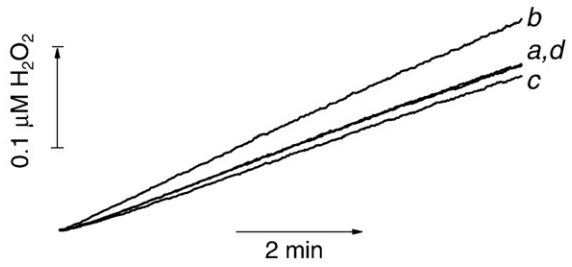

Fig. 8. H_2O_2 release is higher in LDLr k/o than in WT liver mitochondria. MLM (0.5 mg/mL) from WT (lines a, c) and LDLr k/o (lines b, d) were added to a standard reaction medium containing 5 mM succinate, 10 μM Amplex red, and 1 U/mL HRP in the presence of 100 μM EGTA. The experiments represented by lines c and d were conducted in the presence of 1 mM isocitrate and 1 μM rotenone. Maximum rates (slopes) of H_2O_2 production (μM/mg/min) are as follows: 0.12±0.01 for WT, 0.15±0.01 for LDLr k/o, 0.09±0.01 for WT+1 mM isocitrate and 5 μM rotenone, and 0.10±0.01 for LDLr k/o+1 mM isocitrate and 5 μM rotenone. Lines are representative of six independent experiments. $P<0.05$ for LDLr k/o vs WT, $P<0.01$ for LDLr k/o vs LDLr k/o+isocitrate and rotenone.

the differences in the rates of H_2O_2 generation (lines c and d), both being similar to the rate of H_2O_2 generation under the control conditions (line a). This faster generation of H_2O_2 in k/o mice mitochondria raises the possibility that its accumulation may be responsible for the spontaneous NADPH oxidation that occurs when k/o mice mitochondria are incubated in medium containing rotenone that inhibits NAD(P)H oxidation by the electron transport chain [15]. If this is the case, then the presence of exogenous catalase could prevent this NADPH oxidation, as we have observed under other experimental conditions [29]. It is known that exogenous catalase prevents H_2O_2 accumulation in the matrix, given its fast movement across biological membranes [30]. In fact, Fig. 9 shows that, in contrast to the spontaneous oxidation (line b), the LDLr k/o mice mitochondria sustain NADPH reduced in the presence of exogenous

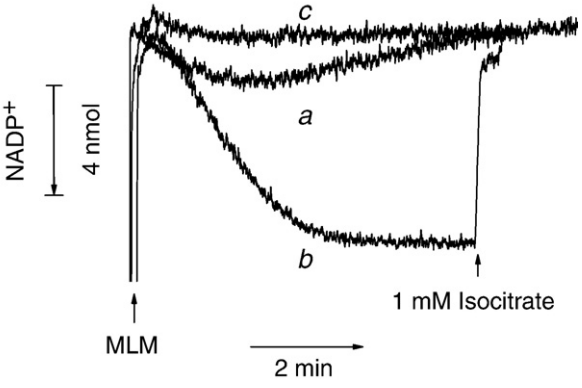

Fig. 9. Exogenous catalase prevents endogenous pyridine nucleotide oxidation in liver mitochondria isolated from the LDLr k/o mice. MLM (0.5 mg/mL) from WT (line a) or LDLr k/o (lines b, c) were added to a standard reaction medium in the presence of 100 μM EGTA, 5 mM succinate, and 5 μM rotenone. Line c represents an experiment conducted in the presence of 2 μM catalase. Extent of pyridine nucleotide oxidation rates (nmol/mg/min) are as follows: none for WT, 1.05±0.08 for LDLr k/o, and none for LDLr k/o+catalase. Lines are representative of five independent experiments. $P<0.001$ for LDLr k/o vs WT or LDLr k/o+catalase.

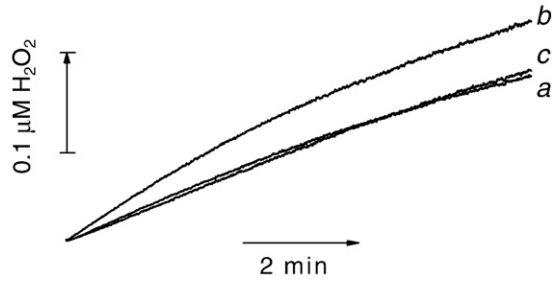

Fig. 10. In vivo citrate treatment reduces H_2O_2 release by the LDLr k/o mitochondria. MLM (0.5 mg/mL) from WT (line a), LDLr k/o (line b), and citrate-treated LDLr k/o (line c) were added to standard reaction medium containing 100 μM EGTA, 5 mM succinate, 10 μM Amplex red, and 1 U/mL HRP. Maximum rates (slopes) of H_2O_2 production (μM/mg/min) are as follows: 0.12±0.01 for WT, 0.14±0.01 for LDLr k/o, and 0.12±0.01 for citrate-treated LDLr k/o. Lines are representative of four independent experiments. $P<0.01$ for LDLr k/o vs WT or citrate-treated LDLr k/o.

catalase (line c). Line a shows that control mice mitochondria sustain NADPH reduced even in the absence of exogenous catalase.

In order to acertain that this higher rate of H_2O_2 generation is, in fact, related to a lower content of endogenous NADPH-linked substrates, we estimated H_2O_2 generation in LDLr k/o mitochondria isolated from mice treated with citrate. The results presented in Fig. 10 show that the H_2O_2 generation by the citrate-treated LDLr k/o mitochondria (line c) decreased to the levels of the control mitochondria (line a).

Discussion

Recent evidence has shown that mitochondrial dysfunction may play an important role in the initiation and development of atherosclerosis (for review, see [31,32]). Mitochondrial DNA mutations have been associated with early phases of atherosclerosis in man and mouse [33] and probably reflect increased production of ROS which attack lipids, proteins, and DNA. We have proposed that the cause of mitochondrial oxidative dysfunction in a proatherogenic mouse model (LDLr k/o) is metabolic [15]. Mitochondria from the LDLr k/o produced more ROS due to low content of pyridine nucleotides in the reduced state which were presumably consumed by augmented lipogenesis [15]. The present work provides evidence that, in addition to a more oxidized state of the mitochondrial pyridine nucleotides, LDLr k/o mice present lower mitochondrial contents of NADP-linked substrates, low GSH/GSSG ratios, and higher contents of liver protein carbonyls. This supports the idea that the condition of oxidative stress is not restricted to mitochondria, although it probably originated in these organelles.

The proposition that this condition of oxidative stress results from insufficient amount of reducing equivalents to sustain pyridine nucleotides in the reduced state was confirmed by a slower rate of oxygen consumption supported by endogenous NAD(P)H-linked substrates in the k/o than in control mitochondria. This difference was totally corrected by isocitrate addition under in vitro conditions (Fig. 2B) and partially corrected by in vivo supplementation of k/o mice with citrate (Fig. 2A). More importantly, mitochondria from citrate-treated k/o mice had higher ability to sustain reduced NADPH (Fig. 3) and generated less ROS (Fig. 10) than mitochondria from untreated k/o mice. Citrate replacements in vitro and in vivo were useful tools for demonstrating the biochemical pathway (substrate deficiency) underlying the decreased antioxidant capacity of the k/o mitochondria. However, its long-term use for the prevention of the mitochondrial oxidative stress and disease development would certainly not work, since it could further increase the already stimulated liver lipogenesis in the k/o mice. This would be the same as trying to block de novo synthesis of cholesterol by providing dietary cholesterol. In this regard, we tried to block endogenous cholesterol synthesis by treating the k/o mice with therapeutic doses of lovastatin. However, this compound had a direct effect of opening the mitochondrial permeability transition pore [34].

Our findings are in accordance with a previous work by Jo et al. [35] who demonstrated that decreased activity of isocitrate dehydrogenase (IDH) in NIH3T3 cells transfected with the IDH antisense cDNA markedly elevated the ROS generation, DNA fragmentation, lipid peroxidation, and mitochondrial damage. Conversely, overexpression of IDH efficiently protected the transfected cells from the ROS-induced damages. This protection was attributed to increased levels of NADPH needed for regeneration of glutathione in mitochondria [35].

We have recently shown that a distinct type of hyperlipidemia, named hypertriglyceridemia mediated by the overexpression of apolipoprotein CIII, induces a higher mitochondrial resting respiration rate [36]. This mitochondrial uncoupling was related to an elevation in the activity of the mitochondrial ATP-dependent potassium channel (mitoK_{ATP}) [36], a mechanism that may be involved in the control of the mitochondrial redox state [32]. In contrast to this mitochondrial response to hypertriglyceridemia, present and previous [15] results showing no alterations of mitochondrial respiratory rates in the hypercholesterolemic LDLr k/o mitochondria suggest that uncoupling mechanisms such as the mitoK_{ATP} channel or uncoupling protein (UCP) are not activated in the LDLr k/o mitochondria.

As previously shown for MnSOD [15], the glutathione reductase and peroxidase activities are similar in control and k/o mice mitochondria (Fig. 6). In addition, H_2O_2 but not $O_2^{\bullet -}$ production (Figs. 7 and 8) is faster in the LDLr k/o mice mitochondria, again supporting the idea that the LDLr k/o mice have deficient levels of mitochondrial substrates to support the antioxidant function of the glutathione and thioredoxin reductase/peroxidase system, i.e., H_2O_2 scavenging. The importance of the thioredoxin reductase/peroxidase system in the development of atherosclerosis was recently demonstrated by the use of endothelial cell-specific transgenesis of the mitochondrial form of the thioredoxin gene. These genetically modified cells showed increased capacity of scavenging ROS and transgenic mice presented improved endothelial cell function and reduced atherosclerosis in the apo E knockout background [37].

Since H_2O_2 is rapidly diffusible through membranes, the accumulation of H_2O_2 in the mitochondria of LDL receptor defective cells (Figs. 8 and 9) may initiate and progressively augment the oxidation of surrounding LDL. Oxidized LDL, in

its turn, induces mitochondrial damage and cell apoptosis through two distinct Ca^{2+}-dependent mitochondrial pathways (activation of calpain and release of cytochrome *c*) [38,39].

Mitochondrial ROS may lead to cell death mainly when the organelle is loaded with Ca^{2+}, a situation that results in mitochondrial permeability transition [14]. Unpublished data from our group (B.A. Paim, G.R. Degasperi, and A.E. Vercesi, unpublished results) show that mononuclear cells isolated from the spleen of LDLr k/o mice present cytosolic-free Ca^{2+} concentrations three times higher than those of the controls, a condition that favors Ca^{2+} accumulation by mitochondria. During the process of mononuclear cell activation both ROS generation and cytosolic-free Ca^{2+} concentrations increase [40], conditions that favor the occurrence of mitochondrial permeability transition. Since atherosclerosis is an inflammatory disease, these findings suggest that mitochondria are involved in mononuclear cell activation and death during atherosclerotic lesion formation in the LDLr k/o mice.

In summary, the oxidative stress in hypercholesterolemic LDLr k/o mice is associated with depletion of mitochondrial NADP-linked substrates and hence insufficient amounts of reducing equivalents to reconstitute the H_2O_2 scavenger function of the glutathione and thioredoxin reductase/peroxidase system. This oxidative process is likely to occur not only in steroidogenic tissues such as liver but also in vascular and immune system cells. The present data clarify additional biochemical mechanisms underlying the cell death susceptibility in LDL receptor defective cells.

Acknowledgments

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundo de Apoio ao Ensino, à Pesquisa e à Extensão (FAEPEX) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

[1] Brown, M. S.; Goldstein, J. L. A receptor-mediated pathway for cholesterol homeostasis. *Science* **232:**34–47; 1986.

[2] Stokes, J. III; Kannel, W. B.; Wolf, P. A.; Cupples, L. A.; D'Agostino, R. B. The relative importance of selected risk factors for various manifestations of cardiovascular disease among men and women from 35 to 64 years old: 30 years of follow-up in the Framingham Study. *Circulation* **75:**V65–V73; 1987.

[3] Ishibashi, S.; Brown, M. S.; Goldstein, J. L.; Gerard, R. D.; Hammer, R. E.; Herz, J. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92:**883–893; 1993.

[4] Steinberg, D.; Parthasarathy, S.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:**915–924; 1989.

[5] Witztum, J. L.; Steinberg, D. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88:**1785–1792; 1991.

[6] Chisolm, G. M.; Steinberg, D. The oxidative modification hypothesis of atherosclerosis: an overview. *Free Radic. Biol. Med.* **28:**1815–1826; 2000.

[7] Libby, P. Atherosclerosis: disease biology affecting the coronary vasculature. *Am. J. Cardiol.* **98:**3Q–9Q; 2006.

[8] Hessler, J. R.; Robertson, A. L. Jr.; Chisolm III, G. M. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis* **32:**213–229; 1979.

[9] Alcouffe, J.; Caspar-Bauguil, S.; Garcia, V.; Salvayre, R.; Thomsen, M.; Benoist, H. Oxidized low density lipoproteins induce apoptosis in PHA-activated peripheral blood mononuclear cells and in the Jurkat T-cell line. *J. Lipid Res.* **40:**1200–1210; 1999.

[10] Marchant, C. E.; Law, N. S.; van der Veen, C.; Hardwick, S. J.; Carpenter, K. L.; Mitchinson, M. J. Oxidized low-density lipoprotein is cytotoxic to human monocyte-macrophages: protection with lipophilic antioxidants. *FEBS Lett.* **2:**175–178; 1995.

[11] Halliwell, B.; Gutteridge, J. M. C. The importance of free radicals and catalytic metal ions in human diseases. *Mol. Aspects Med.* **8:**89–193; 1985.

[12] Boveris, A. Mitochondrial production of superoxide radical and hydrogen peroxide. *Adv. Exp. Med. Biol.* **78:**67–82; 1977.

[13] St-Pierre, J.; Buckingham, J. A.; Roebuck, S. J.; Brand, M. D. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J. Biol. Chem.* **277:**44784–44790; 2002.

[14] Kowaltowski, A. J.; Castilho, R. F.; Vercesi, A. E. Mitochondrial permeability transition and oxidative stress. *FEBS Lett.* **495:**12–15; 2001.

[15] Oliveira, H. C. F.; Cosso, R. C.; Alberici, L. C.; Maciel, E. N.; Salerno, A. G.; Dorighello, G. G.; Velho, J. A.; Faria, E. C.; Vercesi, A. E. Oxidative stress in atherosclerosis-prone mouse is due to low antioxidant capacity of mitochondria. *FASEB J.* **19:**278–280; 2005.

[16] Crompton, M. The mitochondrial permeability transition pore and its role in cell death. *Biochem. J.* **341:**233–249; 1999.

[17] Kroemer, G.; Galluzzi, L.; Brenner, C. Mitochondrial membrane permeabilization in cell death. *Physiol. Rev.* **87:**99–163; 2007.

[18] Netto, L. E.; Kowaltowski, A. J.; Castilho, R. F.; Vercesi, A. E. Thiol enzymes protecting mitochondria against oxidative damage. *Methods Enzymol.* **348:**260–270; 2002.

[19] Tanner, G. A.; Vijayalakshmi, K.; Tanner, J. A. Effects of potassium citrate/citric acid intake in a mouse model of polycystic kidney disease. *Nephron* **84:**270–273; 2000.

[20] Kaplan, R. S.; Pedersen, P. L. Characterization of phosphate efflux pathways in rat liver mitochondria. *Biochem. J.* **212:**279–288; 1983.

[21] Hissin, P. J.; Hilf, R. A fluorometric method for determination of oxidized and reduced glutathione in tissues. *Anal. Biochem.* **74:**214–226; 1976.

[22] Ferranti, R.; da Silva, M. M.; Kowaltowski, A. J. Mitochondrial ATPsensitive K^+ channel opening decreases reactive oxygen species generation. *FEBS Lett.* **536:**51–55; 2003.

[23] Zhou, M.; Diwu, Z.; Panchuk-Voloshina, N.; Haugland, R. P. A stable nonfluorescent derivative of resorufin for the fluorometric determination of trace hydrogen peroxide: applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. *Anal. Biochem.* **15:**162–168; 1997.

[24] Benov, L.; Sztejnberg, L.; Fridovich, I. Critical evaluation of the use of hydroethidine as a measure of superoxide anion radical. *Free Radic. Biol. Med.* **25:**826–831; 1998.

[25] Han, D.; Antunes, F.; Canali, R.; Rettori, D.; Cadenas, E. Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J. Biol. Chem.* **278:**5557–5563; 2003.

[26] Reznick, A. Z.; Packer, L. Oxidative damage to proteins: spectrophotometric method for the carbonyl assay. *Methods Enzymol.* **233:**357–363; 1994.

[27] Schild, L.; Reinheckel, T.; Wiswedel, I.; Augustin, W. Short-term impairment of energy production in isolated rat liver mitochondria by hypoxia/reoxygenation: involvement of oxidative protein modification. *Biochem. J.* **15:**205–210; 1997.

[28] Vercesi, A. E. The participation of NADP, the transmembrane potential and the energy-linked NAD(P) transhydrogenase in the process of Ca2+ efflux from rat liver mitochondria. *Arch. Biochem. Biophys.* **252:**171–178; 1987.

[29] Zago, E. B.; Castilho, R. F.; Vercesi, A. E. The redox state of endogenous pyridine nucleotides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore. *FEBS Lett.* **428:**29–33; 2000.

[30] Valle, V. G.; Fagian, M. M.; Parentoni, L. S.; Meinicke, A. R.; Vercesi, A. E. The participation of reactive oxygen species and protein thiols in the mechanism of mitochondrial inner membrane permeabilization by calcium plus prooxidants. *Arch. Biochem. Biophys.* **307:**1–7; 1993.

[31] Nageswara, R. M.; Marschall, S. R. Mitochondrial dysfunction in atherosclerosis. *Circ. Res.* **100:**460–473; 2007.

[32] Vercesi, A. E.; Castilho, R. F.; Kowaltowski, A. J.; Oliveira, H. C. Mitochondrial energy metabolism and redox state in dyslipidemias. *IUBMB Life* **59:**263–268; 2007.

[33] Ballinger, S. W.; Patterson, C.; Knight-Lozano, C. A.; Burow, D. L.; Conklin, C. A.; Hu, Z.; Reuf, J.; Horaist, C.; Lebovitz, R.; Hunter, G. C.; McIntyre, K.; Runge, M. S. Mitochondrial integrity and function in atherogenesis. *Circulation* **106:**544–549; 2002.

[34] Velho, J. A.; Okanobo, H.; Degasperi, G. R.; Matsumoto, M. Y.; Alberici, L. C.; Cosso, R. G.; Oliveira, H. C.; Vercesi, A. E. Statins induce calcium-dependent mitochondrial permeability transition. *Toxicology* **219:**124–132; 2005.

[35] Jo, S. H.; Son, M. K.; Koh, H. J.; Lee, S. M.; Song, I. H.; Kim, Y. O.; Lee, Y. S.; Jeong, K. S.; Kim, W. B.; Park, J. W.; Song, B. J.; Huh, T. L.; Huh, T. L. Control of mitochondrial redox balance and cellular defense against oxidative damage by mitochondrial $NADP^+$-dependent isocitrate dehydrogenase. *J. Biol. Chem.* **276:**16168–16176; 2001.

[36] Alberici, L. C.; Oliveira, H. C.; Patricio, P. R.; Kowaltowski, A. J.; Vercesi, A. E. Hyperlipidemic mice present enhanced catabolism and higher mitochondrial ATP-sensitive K^+ channel activity. *Gastroenterology* **131:**1228–1234; 2006.

[37] Zhang, H.; Luo, Y.; Zhang, W.; He, Y.; Daí, S.; Zhang, R.; Huang, Y.; Bernatchez, P.; Giordano, F. J.; Shadel, G.; Sessa, W. C.; Min, W. Endothelial-specific expression of mitochondrial thioredoxin improves endothelial cell function and reduces atherosclerotic lesions. *Am. J. Pathol.* **170:**1108–1120; 2007.

[38] Mabile, L.; Meilhac, O.; Escargueil-Blanc, I.; Troly, M.; Pieraggi, M. T.; Salvayre, R.; Negre-Salvayre, A. Mitochondrial function is involved in LDL oxidation mediated by human cultured endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **17:**1575–1582; 1997.

[39] Vindis, C.; Elbaz, M.; Escargueil-Blanc, I.; Auge, N.; Heniquez, A.; Thiers, J.-C.; Negre-Salvayre, A.; Salvayre, R. Two distinct calcium-dependent mitochondrial pathways are involved in oxidized LDL-Induced apoptosis. *Arterioscler. Thromb. Vasc. Biol.* **25:**639–645; 2005.

[40] Degasperi, G. R.; Velho, J. A.; Zecchin, K. G.; Souza, C. T.; Velloso, L. A.; Borecky, J.; Castilho, R. F.; Vercesi, A. E. Role of mitochondria in the immune response to cancer: a central role for Ca^{2+}. *J. Bioenerg. Biomembranes* **38:**1–10; 2006.