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Oxidative stress in atherosclerosis-prone mouse is due to low antioxidant capacity of mitochondria

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SPECIFIC AIMS

An elevated plasma concentration of low density lipoprotein (LDL) is involved in the development of atherosclerosis, a leading cause of death in the Western world. One of the most consistent hypotheses for atherogenesis postulates that this disease is triggered by the LDL oxidation caused by reactive oxygen species (ROS) from vascular wall cells, but it is unclear where and how the oxidative stress condition is established. Mitochondria are the main intracellular sites of ROS generation and are also targets for oxidative damage. Increased mitochondrial release of ROS leads to nonspecific permeabilization of the inner membrane, a phenomenon known as the mitochondrial permeability transition (MPT). This process results in impairment of mitochondrial function and extensive organelle swelling with consequent outer membrane rupture and release of intermembrane components, including apoptogenic signal molecules. The MPT appears to be formed by the assembly of membrane proteins via thiol cross-linking produced by the attack of ROS generated by the respiratory chain, and is favored by the oxidized state of mitochondrial pyridine nucleotides, mainly NADPH, the major source of mitochondrial reducing equivalents for the antioxidant systems glutathione reductase/peroxidase and thioredoxine reductase/peroxidase. In this work, we examined the mitochondrial function and production of ROS in the atherosclerosis-susceptible, hypercholesterolemic LDL receptor knockout mice, which models hypercholesterolemia, a major lipid disorder contributing to increase atherosclerosis risk. The hypothesis tested here was that mitochondria from LDL receptor defective cells might be involved in the establishment of an oxidative stress condition, which precedes the development of atherosclerosis in this genetic disorder.

PRINCIPAL FINDINGS

1. Mitochondrial cholesterol content, membrane fluidity, respiratory control, and phosphorylation efficiency were not different in control and LDL receptor knockout mice

Healthy, chow-fed LDL receptor knockout mice presented blood triglyceride, free fatty acid, and glucose levels similar to control mice. The plasma total cholesterol concentration was 4-fold higher in knockout than in the control mice (314±53 vs. 72±20 mg/dL, $P<0.0001$), but no significant differences were observed in cholesterol content (1.12±0.13 and 1.11±0.14 μg/mg protein) or anisotropy of polar (0.282±0.004 and 0.290±0.009) and nonpolar (0.138±0.009 and 0.142±0.007) membrane regions in LDL receptor knockout and control mitochondria. Mitochondrial respiratory control (phosphorylating/resting respiration ratio: 4.34±0.79 and 4.60±0.81) and phosphorylation efficiency (ADP/O consumption ratio: 2.56±0.24 and 2.51±0.34) measured in calcium-free media containing NAD-linked substrates (malate+glutamate+α-ketoglutarate+pyruvate) were essentially similar in knockout and control mouse liver mitochondria.

2. Mitochondria isolated from the LDL knockout mice produced more reactive oxygen species (ROS) and were more susceptible to Ca^{2+}-induced membrane permeability transition (MPT) than those isolated from control mice

Liver mitochondrial ROS generation was determined by oxidation of the membrane-permeable probe H_2-DCFDA to DCF, which is fluorescent (**Fig. 1**). In the

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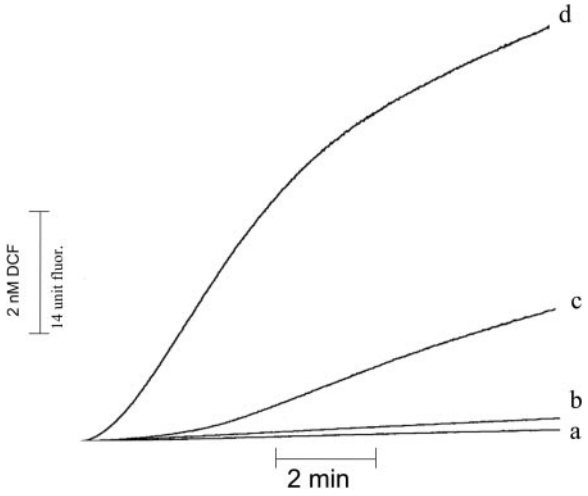

Figure 1. Generation of reactive oxygen species by control (*a, c*) and LDLR$^{-/-}$ (*b, d*) mouse liver mitochondria. The mitochondria were added to the standard reaction medium containing H_2-DCFDA in the presence (*a, b*) or absence (*c, d*) of 100 μM EGTA. Representative of $n = 8$. Maximum rates (slopes) of DCF production (nmol/mg/min): with EGTA, *a)* control 0.096 ± 0.034 vs. *b)* knockout 0.152 ± 0.043, $P > 0.05$; without EGTA, *c)* control 0.72 ± 0.18 vs. *d)* knockout 2.70 ± 0.27, $P < 0.0001$.

absence of Ca^{2+} ions (100 μM EGTA present), the fluorescence due to DCF formation increased slowly with time (lines *a, b*). In contrast, in the presence of ~10 μM Ca^{2+} (EGTA absent), an increase in fluorescence by ROS-induced DCF formation was much larger for mitochondria isolated from knockout (*d*) than from control (*c*) mice. ROS production was also higher in other tissues from hypercholesterolemic knockout mice, including heart and brain, and in whole mononuclear cells isolated from spleen.

Mitochondria isolated from knockout mice developed MPT as estimated by cyclosporin A-sensitive irreversible drop in the transmembrane electrical potential ($\Delta\psi$) and extensive swelling when incubated in the standard medium containing Ca^{2+}. Mitochondria from control mice fully recovered from a transient Ca^{2+}-induced drop in $\Delta\psi$ and did not swell under these same experimental conditions.

3. Pyridine nucleotides (mainly NADPH), the major source of mitochondrial reducing equivalents for the antioxidant systems, were more oxidized in LDL receptor knockout mouse than in control mitochondria

A smaller NADPH mitochondrial pool is sufficient to cause oxidative stress, since it is a limiting step and the ultimate reducing power for mitochondrial enzymatic antioxidant system. MPT is favored by the oxidized state of mitochondrial pyridine nucleotides, mainly NADP. To ascertain whether the higher release of ROS and greater susceptibility to MPT in knockout mice could be the consequence of a lower capacity of their antioxidant defense system, we examined the redox state of pyridine nucleotides (**Fig. 2**). In contrast to control mitochondria, NAD(P)H fluorescence of knockout mitochondria decreased with time and returned to the control level upon addition of isocitrate, which reduces $NADP^+$. Isocitrate had no effect on control mitochondria fluorescence, indicating a fully reduced state of NADPH in these mitochondria. Addition of β-hydroxybutyrate, a specific NAD^+ reductant, did not change pyridine nucleotide fluorescence in either mitochondria preparation. Addition of diamide, a thiol oxidant that oxidizes glutathione, quickly exhausted NAD(P)H in knockout mitochondria through the activities of glutathione reductase and the NADP transhydrogenase. In control mitochondria, this oxidation was biphasic, with a slow second phase that took longer to consume NAD(P)H, indicating these mitochondria have a larger pool of reducing equivalents than knockout mitochondria. Thus, the experiments indicate that greater ROS production and susceptibility to MPT in knockout mice were associated with a more oxidized state of mitochondrial NADP. Indeed, additional experiments showed that isocitrate prevented the Ca^{2+}-induced decrease in $\Delta\Psi$ in knockout mitochondria.

4. The livers of hypercholesterolemic LDL receptor knockout mice secreted and synthesized more triglycerides and cholesterol than those of control mice

We hypothesized that the lower content of reduced nucleotides in the knockout hepatocytes, which do not take up LDL cholesterol, could be the result of higher rates of lipogenesis. The latter would consume large

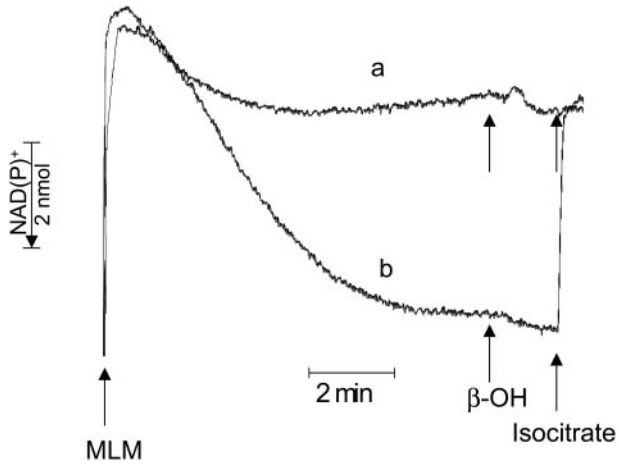

Figure 2. Spontaneous oxidation of endogenous pyridine nucleotides by liver mitochondria from control (*a*) and LDLR$^{-/-}$ (*b*) mice. Mouse liver mitochondria (MLM) were added to the standard reaction medium containing 500 μM EGTA; β-hydroxybutyrate (β-OH, 5 mM) and 1 mM isocitrate were added. Representative of $n = 8$. The extent of pyridine nucleotide oxidation was calculated as a function of fluorescence increase induced by isocitrate addition. Internal calibration was done with known concentrations of NADH. Amounts (nmol) of NADP+ reduced by isocitrate addition: *a)* control: none vs. *b)* knockout: 4.13 ± 0.20.

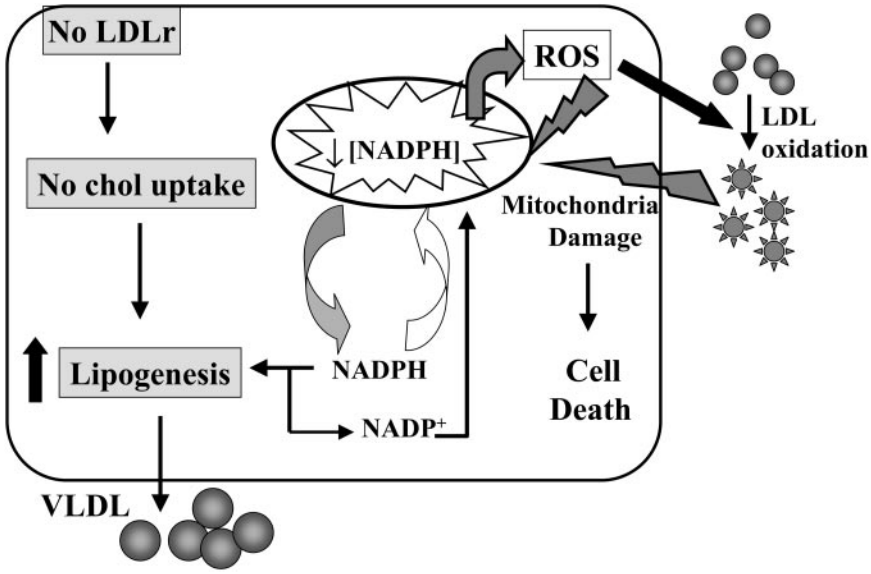

Figure 3. Diagram of the proposed model to explain increased oxidative stress in LDL receptor defective cells. chol: cholesterol; LDLr: LDL receptor; LDL: low density lipoprotein; VLDL: very low density lipoproteins; ROS: reactive oxygen species.

amounts of reducing equivalents from NADPH. To assess this, the hepatic secretion of triglycerides (TG) and cholesterol (chol) in vivo was investigated. Livers of hypercholesterolemic knockout mice secreted about twice as much TG and chol as those of control mice (in mg dL^{-1} min^{-1}): TG: 4.49 ± 0.23 vs. 2.46 ± 0.19 ($P<0.0001$) and chol: 0.29 ± 0.05 vs. 0.14 ± 0.05 ($P=0.054$). Therefore, the lower content of reduced nucleotides in knockout mitochondria probably reflected the higher output and/or lower input of reducing equivalents between mitochondria and the cytosol via substrate shuttling mechanisms. Direct evidence supporting our proposal was obtained by measuring de novo synthesis of cholesterol and other lipids by the ^3H_2O incorporation technique. Total lipid and cholesterol synthesis (μmol ^3H_2O/g/h) were significantly increased in the livers of knockout compared with control mice (total lipids: 632±80 vs. 441±35, $P<0.05$; cholesterol: 201±26 vs. 127±11, $P=0.014$), thus necessarily consuming more NADPH than the control livers. The biosynthesis of 1 mol of cholesterol oxidizes 24 mol of NADPH. Although this may not be the only causative mechanism, it certainly contributes to reduce the NADPH pool in the knockout mitochondria.

CONCLUSIONS AND SIGNIFICANCE

Our results indicate that mitochondria from LDL receptor knockout mice produced more ROS and had greater susceptibility to MPT. The latter effect was illustrated by the lower resistance to Ca^{2+}-induced, CsA-sensitive elimination of $\Delta\psi$ and organelle swelling. The faster net generation of ROS by mitochondria from knockout mice was attested by the faster rate of H_2-DCFDA oxidation in four distinct tissues. The differences in ROS generation were unrelated to mitochondrial membrane fluidity, cholesterol content or MnSOD activity, but rather reflected the lower capacity of the knockout mitochondria to sustain reduced NADPH. Higher rates of triglyceride and cholesterol synthesis may explain the lower content of reduced nucleotides in mitochondria from LDL receptor knockout mice.

The present findings suggest that LDL receptor knockout cells are exposed to more oxidative stress and thus are more susceptible to cell death because of a less effective mitochondrial antioxidant defense system and a greater susceptibility to MPT. The proposed model is shown in **Fig. 3**. Thus, the LDL receptor defect leads to two important atherogenic effects that can be observed before the initiation of the disease: increased extracellular levels of oxidizable substrate (LDL) and an imbalance in cell redox processes. As shown for mononuclear cells, the latter phenomenon probably occurs in the vascular wall, where it is responsible for the local oxidative stress, triggering lipoprotein oxidation, cell death, and atherogenesis in hypercholesterolemia caused by the lack of LDL receptor. FJ