Life Sciences 88 (2011) 343–349

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Mutation of mitochondrial ATP8 gene improves hepatic energy status in a murine model of acute endotoxemic liver failure

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ARTICLE INFO

Article history:
Received 12 May 2010
Accepted 6 December 2010
Available online 15 December 2010

Keywords:
Apoptosis
ATPase
Lipopolysaccharide
Necrosis
Reactive oxygen species
Septic shock

ABSTRACT

Aims: Mitochondria not only generate and modulate bioenergy but also serve as biosensors for oxidative stress, and eventually become effector organelles for cell viability. Therefore, the implications of mitochondrial (dys)function in the development of multiple organ failure are profound. We investigated whether a mutation in the ATPase subunit-8 gene affects the course of endotoxemic acute liver failure.
Main methods: C57BL/6J (*ATP8* wild type) and C57BL/6J-mt$^{FVB/N}$ (*ATP8* mutant) mice were challenged with D-galactosamine (GalN) and *Escherichia coli* lipopolysaccharide (LPS) for induction of acute liver failure, and studied 6 h thereafter. Control mice received physiological saline only. Analysis included in vivo fluorescence microscopy of hepatic microcirculation and levels of hepatocellular apoptosis, hepatic adenosine nucleotides and oxidative stress. Additionally, survival rates were assessed.
Key findings: Induction of endotoxemic liver failure provoked marked liver damage, which was coexistent with a drop of total adenosine nucleotide levels and increased oxidative stress. Of interest, oxidative stress was higher in the GalN/LPS challenged *ATP8* mutants compared to wild types. Concomitantly, adenosine triphosphate (ATP) levels in livers of mice carrying the *ATP8* mutation remained higher than those in wild type mice. As net result, *ATP8* mutants showed lower transaminase release and a tendency to better survival rate upon GalN/LPS exposure compared to wild types.
Significance: Our findings demonstrate that mutation in the ATPase subunit-8 partially protects mice against endotoxemic stress, most probably due to better hepatic energy status despite elevated oxidative stress. Thus, modulating mitochondrial function to preserve bioenergetic status may be an effective strategy to protect against sepsis-induced multiorgan dysfunction.

Introduction

Mitochondrial (dys)function plays a critical role in the development of multiple organ failure (Singer et al., 2004), due to the fact that mitochondria are (i) the main source of cellular ATP generation, (ii) one of the major producers of reactive oxygen species (ROS) and (iii) a central player in cellular apoptosis (Kozlov et al., 2006; Power et al., 2002). ROS are mainly generated as by-products of oxidative phosphorylation or by immune cells (Kozlov et al., 2006). They are powerful cellular molecules, which are used both in host defense against bacterial infection and for the controlled activation of many intracellular signaling pathways (De Minicis et al., 2006). Because of their harmful effects cells have different antioxidant systems to remove ROS for maintaining cell homeostasis (De Minicis et al., 2006). Further, ROS are major mediators in the apoptotic signaling pathway (Power et al., 2002). Cell death in form of apoptosis and necrosis is a characteristic feature of many liver diseases (Malhi and Gores, 2008). It is thought, that both apoptosis and necrosis share – at least in part – common initiating factors and signaling pathways (Malhi and Gores, 2008), and that an overwhelming and dysregulated apoptosis can lead to necrosis in the liver (Malhi et al., 2006). In this context ATP depletion in hepatocytes may switch the mode of cell death from apoptosis, which is highly energy dependent, to necrosis (Le Minh et al., 2009; Miyoshi et al., 2006).

The present study aimed to expand our knowledge of mitochondrial dysfunction in endotoxemic liver failure by using mitochondrial-genome mutant mice. We utilized C57BL/6J-mt$^{FVB/N}$ mice, a mouse conplastic strain carrying the mtDNA of FVB/N mice with a mutation in the ATPase subunit-8 on C57BL/6J genetic background (Gimsa et al., 2009; Yu et al., 2009a). Studies in yeast demonstrated that the N-terminal domain of *ATP8* is located in the intermembrane space of

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0024-3205/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2010.12.011

mitochondria and is part of the F_0 subunit (Devenish et al., 1992; Stephens et al., 2000). The mutation in the *ATP8* gene alters mitochondrial performance, increasing H_2O_2 production and affecting mitochondrial structure (Yu et al., 2009b), but do not affect the cellular ATP level under normal metabolic conditions (data unpublished). Mice carrying the *ATP8* mutation show higher anxiety and stress reactivity, particularly to recurring stress and the polymorphism was accompanied by reduced corticosterone response to physiological stress (Gimsa et al., 2009). Moreover, *ATP8* polymorphism increases the susceptibility to multiple autoimmune diseases (Yu et al., 2009b).

To elucidate a potential impact of the *ATP8* variation of F_0 subunit of ATP-synthase on the development and course of endotoxemic acute liver failure (ALF), C57BL/6J-mt$^{FVB/N}$ and C57BL/6J mice were challenged with D-galactosamine (GalN) and lipopolysaccharide (LPS). This model is accompanied by an enormously increase of caspase-3 activity 4–5 h after stimulation, so that approximately 30% of the hepatocytes are apoptotic (Tapalaga et al., 2002). Parameters of acute liver failure, such as systemic and local inflammatory reaction, hepatic microcirculatory dysfunction, hepatocyte cell death as well as hepatic energy charge and ROS production, were examined.

Materials and methods

Animal model

The inbred strains C57BL/6J and FVB/N were obtained from the Jackson Laboratory (Bar Harbor, MA). To generate conplastic strains, we crossed FVB/N donor strain female mice with male C57BL/6J mice and then backcrossed the female offspring to male C57BL/6J mice for 10 subsequent generations. The offspring of the 10th generation was regarded as the conplastic strains C57BL/6J-mt$^{FVB/N}$ that carried the nuclear genome from C57BL/6J and the mitochondrial genome from the donor strain (Gimsa et al., 2009). Male mice were used at 8 to 12 weeks of age and were kept on water and standard laboratory chow ad libitum. All animals received humane care according to the German legislation on protection of animals and the study has been carried out in accordance with the directive 86/609/EEC on the protection of animals used for experimental purposes.

Model of acute endotoxemic liver failure

For induction of ALF, C57BL/6J (*ATP8* wild type) and C57BL/6J-mt$^{FVB/N}$ mice (*ATP8* mutant) were injected with GalN (720 mg/kg body weight i.p.; Sigma-Aldrich, Taufkirchen, Germany) and *E. coli* LPS (10 μg/kg body weight i.p.; serotype 0128:B12, Sigma-Aldrich). Concentrations of GalN and LPS as well as time point of analysis were chosen in accordance with previously published work of our and other groups (Klintman et al., 2004; Le Minh et al., 2007; Morikawa et al., 1996). Wild type and mutant animals without induction of ALF received only isotonic saline and served as sham controls. Six hours after GalN/LPS exposure, in vivo fluorescence microscopic analysis of the hepatic microcirculation as well as blood and tissue sampling was performed in the four groups mentioned above.

Intravital fluorescence microscopy

For in vivo analysis of the hepatic microcirculation 6 h after GalN/LPS exposure the left liver lobe of ketamine/xylazine anesthetized, laparotomized animals was exteriorized and covered with a glass slide for intravital fluorescence microscopy (n = 6–8 per group). A polyethylene catheter in the left jugular vein served for injection of fluorescent dyes. Using a Zeiss fluorescence microscope equipped with a 100 W mercury lamp and different filter sets for blue and green epi-illumination (Axiotech Vario; Zeiss, Jena, Germany), microscopic images were taken by a water immersion objective (×20/0.50 W; Zeiss), televised using a charge-coupled device video camera (FK 6990A-IQ; Pieper, Berlin, Germany), and recorded on videotape for subsequent off-line evaluation. Blood perfusion within individual sinusoids was studied after tissue contrast enhancement by sodium fluorescein (2 μmol/kg body weight i.v.; Merck, Darmstadt, Germany) and blue light epi-illumination (450 to 490/>520 nm, excitation/emission wavelength). In vivo labeling of leukocytes with rhodamine-6G (1 μmol/kg body weight i.v.; Merck) and green light epi-illumination (530 to 560/>580 nm) enabled quantitative analysis of intrahepatic leukocyte flow behavior.

Assessment of hepatic microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images at a magnification of 424-fold, using a computer-assisted image analysis system with a 19″ monitor (CapImage; Zeintl, Heidelberg, Germany). Within 10 acini per animal, microcirculatory analysis included: (i) the determination of the sinusoidal perfusion, representing the number of perfused sinusoids in percentage of all sinusoids crossing a 200 μm raster line, (ii) the number of adherent leukocytes, located within 10 postsinusoidal venules and not moving or detaching from the endothelial lining during an observation period of 20 s (given as cells/mm^2 endothelial surface, calculated from diameter and length of the vessel segment studied assuming cylindrical geometry), and (iii) the number of rolling leukocytes within 10 postsinusoidal venules moving with less than two-fifth of the centerline velocity, calculated within an observation period of 20 s and given in percent of all visible non-adherent leukocytes.

Sampling and assays

After in vivo microscopy, animals were exsanguinated by puncture of the vena cava inferior for immediate separation of ethylenediaminetetraacetic acid (EDTA) plasma. Alanine aminotransferase (ALT) activity was measured spectrophotometrically as an indicator of hepatocellular disintegration and necrosis. EDTA plasma further served for measurement of malondialdehyde (MDA) as an indicator of oxidative stress-induced lipid peroxidation using the MDA-586-method (OxisResearchTM, Portland, OR, USA) according to manufacturer's instructions. Liver tissue was sampled for subsequent Western blot protein analysis and immunohistochemistry.

For determination of hepatic metabolic status, an additional series of experiments with n = 6–9 animals per group was performed. Tissue extraction and assay of high energy phosphates were done as described in detail before (Minor et al., 2009). Briefly, livers were freeze-clamped using clamps precooled in liquid nitrogen and tissue was stored at −80 °C until later analysis. Specimens were weighed and preserved for at least 5 days in a vacuum freezer (−45 °C, <0.001 atm). After tissue water was evaporated during freeze drying, the samples were weighed again to determine total dry weight. The samples were then homogenized and deproteinized in the cold with 0.33 M $HClO_4$ by means of an Ultra-Turrax (Janke&Kunkel KG, Staufen i.Br., Germany). After centrifugation and neutralization of the protein free supernatant, tissue contents of high energy phosphates were determined from the extract by standard enzymatic tests using the hexokinase and glucose-6-phosphate dehydrogenase reactions for the ATP assay. The results were corrected for the respective dry to wet weight ratio of the tissue samples and expressed as μmol/g dry weight.

Histology and immunohistochemistry

Liver tissue was fixed in 4% phosphate-buffered formalin for 2 to 3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 4 μm sections were cut and incubated overnight at 4 °C with a rabbit polyclonal cleaved caspase 3 antibody (1:500, Cell Signaling Technology, Frankfurt, Germany). This antibody detects endogenous levels of the large fragment (17/19 kD) of activated caspase 3 but not full-length caspase 3. For the development of cleaved caspase 3, a horseradish peroxidase-(HRP)-conjugated goat anti-rabbit antibody was used as a secondary antibody (1:20,