ELSEVIER

Journal of Hepatology 46 (2007) 858–868

Journal of Hepatology

www.elsevier.com/locate/jhep

High hepatic glutathione stores alleviate Fas-induced apoptosis in mice

Sophie Cazanave, Alain Berson, Delphine Haouzi, Nathalie Vadrot, Daniel Fau[†], Alain Grodet, Philippe Lettéron, Gérard Feldmann, Jamel El-Benna, Bernard Fromenty, Marie-Anne Robin, Dominique Pessayre[*]

INSERM, U773, Centre de Recherche Biomédicale Bichat Beaujon CRB3, BP 416, F-75018 Paris, France
Université Paris 7 Denis Diderot, Site Bichat, BP 416, F-75018 Paris, France

***Background/Aims*: The agonistic Jo2 anti-Fas antibody reproduces human fulminant hepatitis in mice. We tested the hypothesis that enhancing hepatic glutathione (GSH) stores may prevent Jo2-induced apoptosis.**

***Methods*: We fed mice with a normal diet or a sulfur amino acid-enriched (SAA^+) diet increasing hepatic GSH by 63%, and challenged these mice with Jo2.**

***Results*: The SAA^+ diet markedly attenuated the Jo2-mediated decrease in hepatic GSH and the increase in the oxidized glutathione (GSSG)/GSH ratio in cytosol and mitochondria. The SAA^+ diet prevented protein kinase Czeta (PKCζ) and $p47^{phox}$ phosphorylations, Yes activation, Fas-tyrosine phosphorylation, Bid truncation, Bax, and cytochrome *c* translocations, the mitochondrial membrane potential collapse, caspase activation, DNA fragmentation, hepatocyte apoptosis, and mouse lethality after Jo2 administration. The protective effect of the SAA^+ diet was abolished by a small dose of phorone decreasing hepatic GSH back to the levels observed in mice fed the normal diet. Conversely, administration of GSH monoethyl ester after Jo2 administration prevented hepatic GSH depletion and attenuated toxicity in mice fed with the normal diet.**

***Conclusions*: The SAA^+ diet preserves GSSG/GSH ratios, and prevents PKCζ and $p47^{phox}$ phosphorylations, Yes activation, Fas-tyrosine phosphorylation, mitochondrial permeabilization, and hepatic apoptosis after Fas stimulation. GSH monoethyl ester is also protective, suggesting possible clinical applications.**

***Keywords*: Apoptosis; Glutathione; Fas; Liver; Mitochondria; Therapeutics**

1. Introduction

In fulminant viral hepatitis, cytotoxic T lymphocytes kill hepatocytes by several mechanisms, including Fas ligand expression [1,2]. The agonistic, Jo2, anti-Fas antibody reproduces fulminant hepatitis in mice [3].

Fas engagement activates sphingomyelinases, which generate ceramide [4]. Ceramide binds to protein kinase Czeta (PKCζ) and triggers PKCζ phosphorylation on threonine 410, and PKCζ activation [4,5]. PKCζ phosphorylates $p47^{phox}$, and activates NADPH oxidase, which forms reactive oxygen species (ROS) [4]. ROS activate Yes, an Src family kinase, which triggers c-Jun N-terminal kinase (JNK) activation [6]. Yes also phosphorylates the epidermal growth factor receptor, which associates with Fas to phosphorylate Fas on tyrosine residues, leading to the translocation of Fas to the plasma membrane [4,6–8]. Fas associates with Fas-associated protein with death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC) [6–8]. DISC

Received 5 December 2005; received in revised form 9 October 2006; accepted 3 November 2006; available online 18 December 2006

[*] Corresponding author. Tel.: +33 1 44 85 61 93; fax: +33 1 44 85 61 38.
E-mail address: pessayre@bichat.inserm.fr (D. Pessayre).
[†] Present address: CNRS UMR 6174, Besançon, France.

Abbreviations: Bax, Bcl-2-associated x; Bid, BH3 interacting domain death agonist; DISC, death-inducing signaling complex; FADD, Fas-associated protein with death domain; GSH, reduced glutathione; GSSG, oxidized glutathione; MPT, mitochondrial permeability transition; JNK, c-Jun N-terminal kinase; PKCζ, protein kinase Czeta; ROS, reactive oxygen species; tBid, truncated Bid; SAA^+ diet, sulfur amino acid-enriched diet; SAA^+ mice, mice fed the SAA^+ diet.

0168-8278/$32.00
doi:10.1016/j.jhep.2006.11.015

oligomerization by Fas ligand or Jo2 activates caspase-8 [9]. Receptor-mediated caspase-8 activation is weak in hepatocytes, but is amplified by mitochondria [10]. Caspase-8 cuts BH3 interacting domain death agonist (Bid) into truncated bid (tBid) [11], which modifies the conformation of Bcl-2-associated x (Bax) [12]. Bax translocates to mitochondria [13], and associates with Bak [14], to permeabilize the outer mitochondrial membrane [15]. Mitochondrial permeability transition (MPT) occurs in some mitochondria, causing outer membrane rupture [16]. Mitochondrial cytochrome *c* translocates to the cytosol, and activates caspase-9 [17], which activates caspase-3 [1], which re-initiates the loop by activating caspase-8 [18] and directly cutting Bid [19]. After several turns of this mitochondrial loop, the extensive activation of effector caspases triggers apoptosis [10].

Fas stimulation triggers a cellular efflux of reduced glutathione (GSH), decreasing GSH stores [20]. GSH extrusion inhibitors prevent GSH depletion and attenuate apoptosis, suggesting that Fas-mediated GSH depletion self-aggravates apoptosis [21]. In contrast, pre-existing GSH depletion had divergent effects. Whereas acute, severe GSH depletion prevented Fas-mediated apoptosis by impairing caspase-8 activation at the DISC [22,23], prolonged GSH depletion instead worsened Fas-mediated apoptosis [24].

It therefore seemed difficult to predict what would be the effects of enhancing GSH stores before Fas stimulation. We report that a diet enriched in sulfur amino acids (SAA^+ diet) increased hepatic GSH and attenuated Jo2-mediated hepatic apoptosis in mice.

2. Materials and methods

2.1. Animals, diets, and treatments

Male CR1:CD1-ICR BR Swiss mice (30–32 g) from Charles River (L'Arbresle, France) were allowed water and food ad libitum. Ten days before the experiments, SAA^+ mice were switched from a normal, standard diet (AO4-biscuits, UAR, Villemoisson-sur-Orge, France) to a semi-synthetic diet (sucrose, peanut oil, mineral mix, vitamin mix, cellulose, starch, and 12% lactic casein) [24] supplemented with 1% L-cystine and 1% GSH.

Mice received saline (150 μL) or 8 μg/mouse of the agonistic Jo2 hamster monoclonal anti-mouse Fas antibody (BD Pharmingen, Heidelberg, Germany) intraperitoneally. Some mice were pre-treated with phorone (10 mg/kg) in corn oil (100 μL), or the c-Jun N-terminal kinase (JNK) inhibitor [25], SP600125 (10 mg/kg) in dimethylsulfoxide (DMSO; 75 μL). Other mice were post-treated with GSH monoethyl ester (3 g/kg) in saline (500 μL).

Unless otherwise indicated, mice were killed 4 h after Jo2 administration.

2.2. Hepatic GSH, serum ALT, DNA fragmentation, and caspase activities

A whole liver homogenate, a cytosolic fraction and mitochondria were prepared [24]. We assessed GSH as non-protein sulfhydryls [24], and oxidized glutathione (GSSG) with the GSH/GSSG-412 kit (Tebu-bio, Le-Perray-en-Yvelines, France). Serum ALT activity was measured using a Biomérieux (Marcy-l'Etoile, France) commercial kit. To assess DNA fragmentation, DNA was extracted with QIAGEN columns (Hilden, Germany), and run on ethidium bromide-containing 2% agarose gels [26].

To assess caspases, livers were homogenized in 1 mM EDTA, 50 mM Hepes, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate, 5 mM dithiothreitol, 4 mg/mL leupeptin, and 4 mg/mL pepstatin, pH 7.4. After centrifugation at 14,000*g*, we measured caspase-9 activity in the supernatant with 25 μM AC-LEHD-AFC (Calbiochem, Darmstadt, Germany), and caspase-3 and caspase-8 with fluorescent kits (Biomol, Plymouth Meeting, PA).

2.3. Morphologic studies

A fragment for light microscopy was fixed in PBS-buffered 10% formalin, dehydrated, placed in toluene baths, and embedded in paraffin. Sections were stained with haematoxylin and eosin, and the percentage of apoptotic nuclei and/or apoptotic bodies was quantified [24].

Three blocks per animal were processed for electron microscopy [16], and 10 hepatocyte cytoplasmic fields per block were taken randomly, and photographed at 15,000× magnification. We counted the percentage of mitochondria exhibiting outer mitochondrial membrane rupture, matrix herniation through the outer membrane gap, and disappearance of cristae in the herniated portion [16].

For in situ caspase-3 detection, liver fragments were placed in cold isopentane. Slices were fixed in 3% paraformaldehyde, permeabilized with 0.1% Triton, and exposed to a rabbit polyclonal antibody against mouse-activated caspase-3 (Cell Signaling Technology, Beverly, MA) and a fluorescein-conjugated goat antibody against rabbit immunoglobulins (Molecular Probes, Eugene, OR). Nuclei were counterstained with TO-PRO-3 blue fluorescent cyanine dye monomers (Molecular Probes).

2.4. Assessment of Fas, FADD, and procaspase-8 mRNAs

Liver RNA was isolated with the TRIZOL reagent (Invitrogen, Carlsbad, CA), and RNase protection assays were performed with the mouse mAPO-3 multiprobe template set, and the in vitro Transcription Kit of BD Biosciences Pharmingen (San Diego, CA). Protected bands were quantified by phosphorimaging, and ratios to GAPDH mRNA were calculated.

2.5. Detection of phosphorylated $p47^{phox}$

Livers were homogenized in 250 mM sucrose, 10 mM Tris, 1 mM $MgCl_2$, 20 mM sodium fluoride, 1 mM trisodium orthovanadate, and 1 mM sodium pyrophosphate, pH 7.4, with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche

Table 1
Effects of the SAA^+ diet on hepatic GSH[a]

	Total GSH (μmol/g liver)	Cytosolic GSH (nmol/mg protein)	Mitochondrial GSH (nmol/mg protein)
Normal diet	5.4 ± 0.3	35 ± 2	4.2 ± 0.2
SAA^+ diet	8.8 ± 0.3[b]	61 ± 4[b]	7.2 ± 0.4[b]

[a] We fed mice with a normal diet or the SAA^+ diet for 10 days, prepared a whole liver tissue homogenate, a cytosolic hepatic fraction and a mitochondrial fraction, and measured GSH in these fractions. Results are means ± SEM in at least 8 mice.
[b] Different from normal diet, $P < 0.001$.