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Liver damage and caspase-dependent apoptosis is related to protein malnutrition in mice: Effect of methionine

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ABSTRACT

This study aimed to determine whether the effects on the mouse liver caused by three periods of feeding a protein-free diet for 5 days followed by a normal complete diet for 5 days (3PFD-CD) are prevented by a constant methionine supply (3PFD + Met-CD). The expressions of carbonic anhydrase III (CAIII), fatty acid synthase (FAS), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glutathione S-transferase P1 (GSTP1) were assessed by proteomics and reverse transcriptase-polymerase chain reactions. The liver redox status was examined by measuring the activities of superoxide dismutase (SOD) and catalase (CAT), as well as protein carbonylation. Because oxidative stress can result in apoptosis, the activity and content of caspase-3, as well as the x-linked inhibitor of the apoptosis protein (XIAP) and mitochondrial caspase-independent apoptosis inducing factor (AIF) contents were assessed. In addition, the liver histomorphology was examined. Compared to the controls fed a normal complete diet throughout, feeding with 3PFD-CD increased the FAS content, decreased the CAIII content, decreased both the SOD and CAT activities, and increased protein carbonylation. It also activated caspase-3, decreased the XIAP content, decreased the AIF content, increased the number of GSTP1-positive foci and caspase-3-positive cells, and caused fatty livers. Conversely, the changes were lessened to varying degrees in mice fed 3PFD + Met-CD. The present results indicate that a regular Met supply lessens the biochemical changes, damage, and caspase-dependent apoptosis provoked by recurrent dietary amino acid deprivation in the mouse liver.

Introduction

Protein malnutrition leads to altered liver biochemical characteristics and histology (Aebi, 1984; Caballero et al., 2011; Ronchi et al., 2004; Ronchi et al., 2010). Consuming a protein-free diet (PFD) for 5 days changes the mouse liver proteome (Conde and Scornik, 1976; Pucciarelli and Conde, 1984; Ronchi et al., 2004; Ronchi et al., 2010; Sanllorenti et al., 1992; Sanllorenti et al., 2001). These changes are reversed after the intake of a normal complete diet (CD). However, they prevail with recurrent amino acid deficiency such as after 5 days of PFD followed by 5 days of CD repeated three times (3PFD-CD) (Caballero et al., 2011). Several of the affected proteins are recognized as precancerous, cancerous, and senescence markers, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutathione S-transferases (GSTs), carbonic anhydrase III (CAIII), and fatty acid synthase (FAS). One common aspect among GSTs and CAIII is their involvement in cellular detoxification and antioxidant defense, such as CuZn-superoxide dismutase (CuZn-SOD). Poor consumption of amino acids may lead to oxidative stress (Caballero et al., 2011; Ronchi et al., 2010). When antioxidant responses are overwhelmed, reactive oxygen species (ROS) injure cells in a way that can lead to their death by either necrosis or apoptosis (England and Cotter, 2005). The main biochemical mediators of apoptosis are caspases that are activated in various death triggers (Cryns and Yuan, 1998;

Abbreviations: 3PFD-CD, three periods of feeding a protein-free diet for 5 days followed by a complete diet for 5 days; 3PFD + Met-CD, three periods of feeding a protein-free supply with methionine diet for 5 days followed by a complete diet for 5 days; AIF, apoptosis inducing factor; CAIII, carbonic anhydrase III; CAT, catalase; CD, complete diet; CuZn-SOD, CuZn-superoxide dismutase; FAS, fatty acid synthase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSTP1, glutathione S-transferase P1; Met, methionine; PFD, protein-free diet; PDF + Met, protein-free diet supplemented with methionine; ROS, reactive oxygen species; SOD, superoxide dismutase; XIAP, x-linked inhibitor of apoptosis protein.

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Thornberry and Lazebnik, 1998). In addition, several molecules that control caspase activity belong to the inhibitors of apoptosis (IAPs) family (Deveraux and Reed, 1999; Holcik and Korneluk, 2001). Among them, the X-linked inhibitor of apoptosis (XIAP) is the most potent (Deveraux and Reed, 1999; Duckett et al., 1996; Harlin et al., 2001). Conversely, the apoptosis-inducing factor (AIF), described as a caspase-independent mediator of apoptosis, is needed for cell survival, proliferation and mitochondrial integrity (Hangen et al., 2010).

Despite the progress reported so far, knowledge of the mechanisms and pathogenesis of hepatocellular injuries of eating disorders is incomplete. In this study, we aim to show that the presence of methionine in a 3PFD + Met-CD treatment prevents the metabolic and structural changes caused by 3PFD-CD by reducing oxidative stress in liver and inhibits cell death by either necrosis or apoptosis. We examined the following features: FAS, CAIII, GSTP1 and GAPDH protein and mRNA contents; superoxide dismutase (SOD) and catalase (CAT) activities; total carbonyl groups, XIAP and AIF protein contents. Also, we examined the liver histology, including immuno-histochemical analyses for GSTP1 and caspase-3.

Our results show that a regular Met supply lessens the biochemical changes, damage, and caspase-dependent apoptosis provoked by recurrent dietary amino acid deprivation in the mouse liver.

Materials and methods

Animals

Two-month old female Balb/c mice (body weight 20–25 g) from IIB, UNMdP-CONICET, Mar del Plata, Argentina, were housed in a room at 22 °C illuminated from 07:00 to 19:00 h. They had *ad libitum* access to a complete diet and water and were housed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The local ethical committee for animal research approved the protocols used in this study.

Diets

All diets used were based on the protein free diet (PFD) outlined by the USP XV Pharmacopeia (The Pharmacopeia USA, 1955) and the amino acid composition of bovine β-casein (Ribadeau Dumas et al., 1972). A normal or complete diet (CD) containing 23% (w/w) β-casein and protein-free diet supplemented with Met (PFD + Met) were prepared as previously described (Ronchi et al., 2010). Because carbohydrates replaced protein and amino acids, all diets were isocaloric.

The test group was subjected to three cycles of 5 days of PFD or PFD + Met followed by 5 days of CD (3PFD-CD and 3PFD + Met-CD). The control group was fed a CD. After treatments, the mice were killed by cervical dislocation. Their livers were rapidly removed, weighed and placed in buffer A (0.15 M NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, and 20 mM Tris–HCl buffer pH 7.4).

Cytosol preparation

The livers were homogenized in 5 ml/g fresh weight of cold buffer A and centrifuged at 100,000×*g* for 60 min. The obtained supernatant represents the cytosol (Sanllorenti et al., 2001).

Preparation of mitochondria-enriched fractions and cytosolic extracts

To obtain a mitochondrial fraction, the livers were homogenized in 4 ml/g fresh cold buffer B (450 mM sucrose, 1 mM EDTA, 10 mM Tris–HCl pH 7.5, 1 mM DTT, 6 g/l PVP 40, 1 mM PMSF, and 2 g/l BSA). The homogenate was then centrifuged at 1500×*g* for 10 min, and the resulting supernatant was further centrifuged at 7000×*g* for 10 min. The pellet was washed in 5 ml of buffer B, centrifuged at 7000×*g* 10 min, and re-suspended in 1 ml of buffer C (300 mM sucrose, 1 mM EGTA, 0.2 mM PMSF, and 10 mM Tris–HCl pH 7.5). When indicated, this supernatant was further centrifuged at 100,000×*g* for 60 min to obtain the cytosol (Frezza et al., 2007; Garcia-Mata et al., 1997).

The enrichment of the cytosolic and mitochondrial fractions was determined by measuring the lactate dehydrogenase and succinate cytochrome C reductase activities, respectively (Evans, 1987). The specific activity of lactate dehydrogenase in the cytosol was 36 ± 2-fold higher than in the mitochondrial fraction, whereas that of succinate cytochrome C reductase in mitochondria was 10 ± 2-fold higher than in the cytosol.

Protein and nucleic acid determination

The protein concentration was determined according to Bradford (1976) using bovine serum albumin as a standard. The nucleic acid concentrations were determined according to the method described by Fleck and Munro (1962).

Polyacrylamide gel electrophoresis

The cytosols were subjected to SDS-PAGE in 12% acrylamide under reducing conditions (Laemmli, 1970) and stained with Coomassie Blue (Meyer and Lamberts, 1965). The resulting protein patterns were photographed and analyzed with the computer ImageQuant TL v2005 system (Nonlinaer Dynamics Ltd., Durham, USA). The CAIII, FAS and GSTP1 bands were previously identified and quantified by mass spectroscopy combined with a sequence analysis of peptides produced by in-gel trypsin digestion (Garcia-Mata et al., 1997; Ronchi et al., 2004). In addition, the GAPDH band was identified and quantified by both a Western blot test and N-term amino acid sequence (Sanllorenti et al., 1992).

Reverse transcriptase-polymerase chain reaction

The total liver RNA was extracted with Trizol® (Invitrogen, Gaitherburg, MD, USA). The extracts were treated with RQ1 RNase-Free DNase® (Promega), quantified by measuring the absorbance at 260 nm/280 nm, and tested for quality with agarose 1% native gel electrophoresis. First-strand cDNA synthesis was carried out with M-MLV reverse transcriptase® (Invitrogen) and oligo dT® (Sigma–Aldrich) primers and used for RT-PCR. The products were obtained after 30–35 cycles of amplification considering the exponential phase for each probe and 55–65 °C annealing temperatures. The RT-PCR products were separated on 2% agarose gels. Electrophoresis was carried out at 100 V for 60 min in TBE 1X buffer (0.89 M Tris, 0.89 M borate, 2 mM EDTA). The gels were stained with SYBR Gold® nucleic acid stain (Molecular Probes, Eugene, OR, USA). The bands were detected with a Scanner Storm Amersham Bioscience (Pittsburgh, USA) and quantified using ImageQuant TL v2005. All band intensities were related to actin.

The mouse-specific primer sequences for the PCR reaction were defined as follows: *(Probe: Primer Sequence FW,RV)*

FAS: FW: 5′-TGC GCC CAG CCT CCT AAG GC-3′; RV: 5′-ATC ACA CGC CGG CAA CAC CTA TCC-3′.

CAIII: FW: 5′-TGC CAA AGG GGACAA CCA GT-3′; RV: 5′-GCA CCG GGG GCTCAT TCT C-3′.

GAPDH: FW: 5′-ACG GCA AAT TCA ACG GCA CAG TCA-3′; RV: 5′-CAT TGG GGG TAG GAA CAC GGA AGG-3′.

GSTA3: FW: 5′-GCG GGG AAG CCA GTC CTT CAT T-3′; RV: 5′-CCT CTG GCT GCC AGG TTG AA-3′.

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