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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.
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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′.

GSTM1: FW: 5′-GTG ACG CTC CCG ACT TTG ACA G-3′; RV: 5′-TTA CTC CAG TGG GCC ATC TTT GAA-3′.

GSTP1: FW: 5′-ATG CCA CCA TAC ACC ATT GT-3′; RV: 5′-GTC CAG CAA GTT GTA ATC GG-3′.

ACTIN: FW: 5′-AGT ACT TGC GCT CAG GAG GA-3′; RV: 5′-TCC TCC CTG GAG AAG AGC TA-3′.

Western blot analysis

Proteins (50–80 μg) from different treatments were separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, incubated with primary antibodies against apoptosis inducting factor® (AIF) (13116; Santa Cruz Biotechnology, Santa Cruz, CA, USA), X-linked Inhibitor of Apoptosis Protein® (XIAP) (M044-3, MBL, Nagoya, Japan) or actin® (A2668; Sigma–Aldrich, St. Louis, MO, USA), washed, and incubated with horseradish peroxidase-linked secondary antibodies. The membranes were washed, and the proteins were detected using the enhanced chemiluminescence® method (Amersham, Piscataway, NJ, USA). Furthermore, the membranes were incubated with primary antibodies against FAS® (H-300; Santa Cruz Biotechnology), GSTP1® (311; MBL) and caspase-3® (H-277; Santa Cruz Biotechnology), washed and incubated with alkaline phosphatase secondary antibodies to reveal bands (Sanllorenti et al., 1992). Densitometric analyses were performed with the computer image analyzer system ImageQuant TL v2005 (Nonlinaer Dynamics Ltd., Durham, USA).

Analysis of caspase-3 activity

The caspase-3 activity was evaluated by measuring the proteolytic cleavage of its fluorogenic substrate, Ac-DEVD-AFC (311274; Santa Cruz Biotechnology, Santa Cruz, CA, USA), according to the manufacturer's suggestions. Briefly, the cytosolic fraction (20 μg of protein) was added to a reaction buffer containing 20 mM HEPES (pH 7.5), 10% glycerol, 2 mM DTT, and 13.3 μg/ml caspase substrate. The reaction mixture was incubated at 37 °C for 1 h. Subsequently, the activity was measured in a Fluoroskan Ascent FL 100–240 V (Thermo Fisher Scientific, MA, USA) at 405 nm/525 nm (excitation/emission wavelengths, respectively).

Analysis of SOD and CAT activities

The catalase activity (CAT) was assessed by the disappearance of H_2O_2 at 240 nm according to Aebi (1984). The reaction mixture contained 50 mM phosphate buffer pH 7.4, 10 mM H_2O_2 and cytosol. The enzyme unit was defined as Δ0.05 Abs. 240 nm: 1 U/ml/mg protein.

The superoxide dismutase (SOD) activity was determined from the cytosolic extract by the inhibition of NBT reduction method (Beauchamp and Fridovich, 1971). The reaction medium contained 0.4 ml of buffer with cytosol and 0.6 ml of the following solution: 50 mM HEPES pH 7.6, 0.1 mM EDTA, 50 mM $NaHCO_3$, 13 mM Met 0.025% (v/v) Triton X-100, 75 mM NBT and 2 μM riboflavin. The reaction was carried out for 4 min in a water bath at 30 °C illuminated with a 22 W fluorescent lamp (Philips); the absorbance was then measured at 560 nm. An enzymatic unit was defined as the amount of cytosol required to inhibit 50% of the reaction without enzyme.

Measurement of protein carbonyl content

The reactive carbonyl content of liver proteins was measured according to Ronchi et al. (2010). Briefly, livers were homogenized at 4 °C in 10 ml/g fresh weight of cold 0.15 M NaCl, 1 mM EDTA, and 20 mM Tris–HCl, pH 7.4. Two 0.75 ml aliquots per sample containing nearly 1 mg protein were mixed with 0.75 ml of 20% (w/v) trichloroacetic acid (TCA). The pellets were separated by centrifugation at 6000×*g* for 5 min, mixed with either 0.75 ml of 2 N HCl (blank) or 0.75 ml of 2 N HCl containing 0.2% (w/v) dinitrophenylhydrazine (DNPH) and agitated for 1 h at 25 °C in the dark. They were then re-precipitated with 0.75 ml of 20% (w/v) TCA, washed three times with ethanol: ethyl acetate (1:1, v/v), dried, and mixed with 0.75 ml of 6 M guanidine HCl at 25 °C. After removing the debris by centrifugation, the absorbance of DNPH-treated samples was assessed at 370 nm after the subtraction of blanks. This value was used to calculate the nanomoles of incorporated DNPH/mg of protein based on an average absorption of 21 mM^{-1} cm^{-1} for aliphatic hydrazones (Jones et al., 1956).

Histological studies and immunohistochemistry

The livers of mice were fixed in 10% (v/v) neutral formaldehyde solution, dehydrated in an ascending ethanol series, cleared in xylene and embedded in paraffin wax (routine histology). The blocks were then cut into 5 μm thick sections on five slides. Three slides were used for histological examination after routine hematoxylin and eosin (H&E) staining, periodic acid–Schiff reaction (Pizer et al., 1998) and Masson's trichrome procedure, and the other two were used for the immunohistochemistry.

For the morphological observation according to the standard procedure by light microscopy, the structures of liver lobules, hepatocytes, portal tracts, and sinusoidal and perisinusoidal acini were analyzed.

Caspase-3® (Santa Cruz) and GSTP1® (MBL) antibodies were used for the immunohistochemistry. The liver sections were washed with PBS, and the slides were then incubated in 3% hydrogen peroxide for 5 min. After again washing with PBS, non-specific staining was blocked by treating the slides with 5% (w/v) milk. The sections were then separately incubated with anti caspase-3 or anti-GSTP1 antibody at 1:1,000 dilutions for 2 h in a humidified chamber at room temperature, followed by incubation with biotinylated secondary antibody. The samples were washed with PBS and treated with ABC reagent. DAB was used as the chromogen. After 10 min, the slides were counterstained with hematoxylin, and the brown color that represented the presence of antigen bound to antibody was detected by light microscopy. The specificity of staining was controlled with the omission of primary antibody (primary control) and secondary antibody (secondary control), which prevented staining. The results are expressed as the qualitative presence of immunopositive cells.

Digital photographs were taken using a system consisting of a trinocular microscope Leica DM4000B LED® and Leica DCC-380X® camera with the digital support of the LASZ Leica capture programme® (all manufactured by Leica Microsystems, Wetzlar, German).

Statistical analysis

All data are presented as the means ± SEM. The data were compared using the ANOVA test (InStat, Graph Pad software, La Jolla, CA, USA). *P*-values lower than 0.05 were considered significant. The data analyses include at least three separate experiments with $n > 5$.

Results

Body weight and liver composition

Compared with the controls fed a CD throughout, the body weight of mice fed either 3PFD-CD or 3PFD + Met-CD decreased by approximately 20%. The livers of mice fed CD, 3PFD-CD, and 3PFD + Met-CD weighed 1.03 ± 0.03, 0.94 ± 0.04*, 0.89 ± 0.03* g, respectively. The nucleic acid and protein contents of the CD,

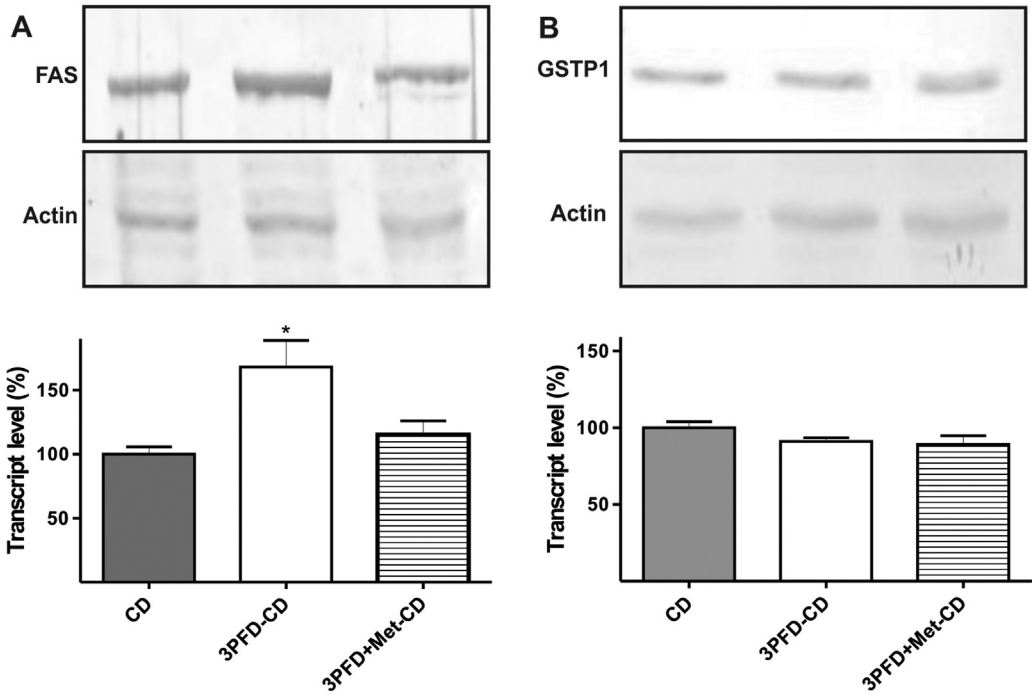

Fig. 1. Analysis the effects of 3PFD-CD and 3PFD + Met-CD on levels of cytosolic proteins FAS and GSTP1. Western blot determination with specific antibodies to: panel A, FAS and B, GSTP1. Data are expressed as the density relative of bands in gel (%) respect to CD group in three independent experiments ($n = 6$ each experiments). Values significantly different from the CD condition $^{*}P < 0.05$.

3PFD-CD, and 3PFD + Met-CD livers were, respectively, measured as follows: RNA, 4.47 ± 0.43, $5.32 \pm 0.31^{*}$, 4.77 ± 0.16 mg; DNA, 1.95 ± 0.16, 2.09 ± 0.14, 2.01 ± 0.12 mg; Protein, 144.12 ± 9.30, $177.26 \pm 49.53^{*}$, and 142.44 ± 21.14 mg ($P^{*} < 0.05$).

FAS, GSTP1, CAIII, and GAPDH contents

According to the Western blot tests, the liver FAS content increased by 45% in response to 3PFD-CD but not in response to 3PFD + Met-CD (Fig. 1A). The GSTP1 content did not significantly change for either diet (Fig. 1B). The SDS-PAGE tests showed that the content of CAIII decreased by 50% in response to 3PFD-CD, but not for 3PFD + Met-CD. Conversely, the GAPDH content did not change for any diet (Fig. 2).

FAS, GSTP1, CAIII, and GAPDH mRNA contents

The relationship between the FAS, GSTP1, CAIII, and GAPDH contents and their mRNA levels was examined with RT-PCR tests (Fig. 3). The levels of FAS mRNA increased by 45% for the 3PFD-CD group, but not for the 3PFD + Met-CD group. The levels of GSTP1 mRNA increased by 35% for the 3PFD-CD group, but not for the 3PFD + Met-CD group. The levels of CAIII mRNA decreased by 54 and 44% for the 3PFD-CD and 3PFD + Met-CD groups, respectively. The levels of GAPDH mRNA did not change significantly with any diet.

SOD and CAT activities

The activities of SOD decreased by 30% for the 3PFD-CD group, but not for the 3PFD + Met-CD group. Furthermore, the CAT activities decreased by 45% in the 3PFD-CD, but not in the 3PFD + Met-CD group (Table 1).

Carbonyl group in proteins

The carbonyl group content of the total liver protein was assessed by a reaction with DNHP. It increased by 70% in the 3PFD-CD, but not in the 3PFD + Met-CD group (Table 1).

Caspase-3 activities and contents

The activities of caspase-3 increased by 49% in the 3PFD-CD group, while they decreased by 43% in the 3PFD + Met-CD group (Fig. 4A). The Western blot tests showed that the level of procaspase-3 decreased in the 3PFD-CD, but not in the 3PFD + Met-CD group. Moreover, they show that caspase-3 predominates in 3PFD-CD livers (Fig. 4B).

AIF and XIAP contents

The content of the mitochondrial protein AIF, which is associated with caspase-independent apoptosis pathways, was assessed by Western blot. It decreased by 25% in the 3PFD-CD, but not significantly in the 3PFD + Met-CD group (Fig. 5A). The content of XIAP, a cytosolic inhibitor of caspase-3 and -7, was also assessed by Western blot. It decreased by 25% in the 3PFD-CD, but not significantly in the 3PFD + Met-CD group (Fig. 5B).

Liver histology

The H&E staining of CD livers showed normal hepatic parenchymal cells with granulated cytoplasm and small uniform nuclei arranged radially to the central vein (Fig. 6A). The 3PFD-CD group showed a loss of hepatocyte architecture and presence of large and irregularly shaped cells with pleomorphic and hyperchromatic nuclei (Fig. 6B). Furthermore, masses of acidophilic material were noticed next to the central vein and microvesicular vacuolization was noticed near the portal triad, which is shown in Figs. 6E and M with other staining. Conversely, the H&E staining in the

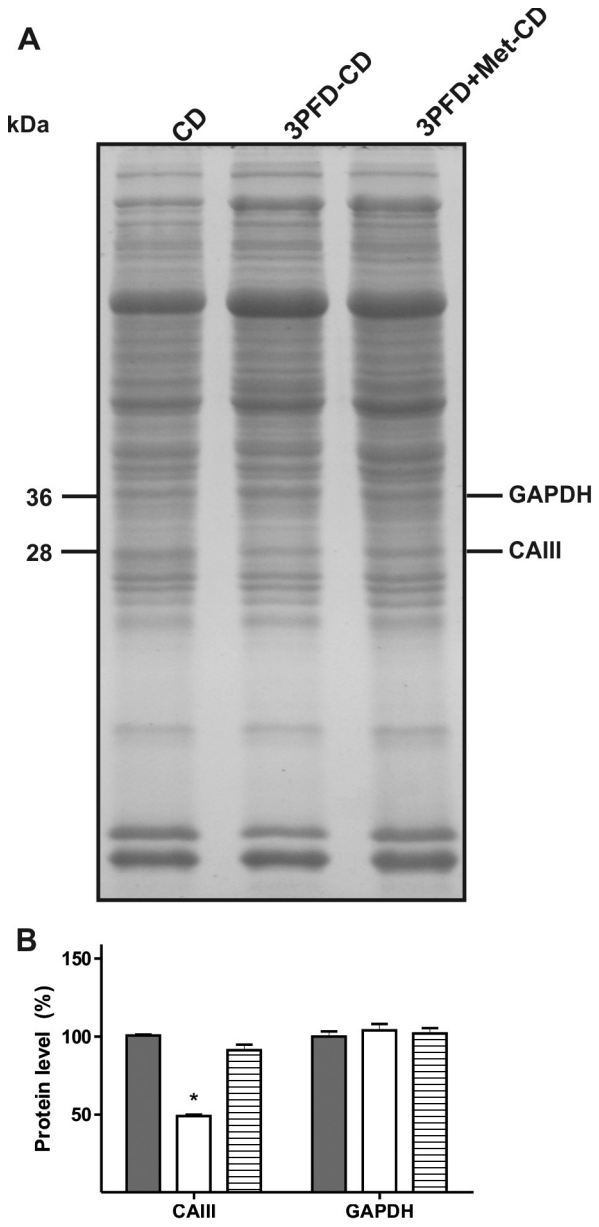

Fig. 2. Effects of 3PFD-CD and 3PFD + Met-CD on cytosolic protein GAPDH and CAIII. A) SDS-PAGE representative analysis of protein samples (25 μg) from CD, 3PFD-CD and 3PFD + Met-CD. Positions of size markers are signaled on the left while those of GAPDH and CAIII on the right. B) Average percentages of GAPDH and CAIII band densities in 3PFD-CD (□) and 3PFD + Met-CD (≡) respect to CD (■). Values represent the means ± SEM of three independent experiments ($n = 6$ each experiments). Values significantly different from the CD condition: $^{*}P < 0.05$.

3PFD + Met-CD group revealed normal parenchyma with hepatocytes radially arranged around the central vein and without microvesicular vacuolization (Fig. 6C).

Both the PAS and Masson's trichrome stains showed a normal liver appearance in the CD group (Fig. 6D and G). In the 3PFD-CD group, they revealed diverse glycogen distribution and prominent periportal and pericellular collagen deposition with moderate hyperemia and hemorrhage near the central vein (Fig. 6E and H). In the 3PFD + Met-CD group, PAS revealed a periportal gradient distribution of glycogen and some cells with vacuolization. Furthermore, some cells showed differing glycogen contents (Fig. 6F). Masson staining showed a normal parenchyma with a slight thickening of vessel walls. Furthermore, it revealed an increased mass of connective tissue near the central vein and slight hyperemia (Fig. 6I).

Few caspase-3- and GSTP1-positive cells were visible in the liver of CD mice (Fig. 6J and L). In the 3PFD-CD group, caspase-3-positive cells and GSTP1-positive foci were identified near the central vein and middle lobular and near portal triad, respectively (Fig. 6K and N). Conversely, few caspase-3- and GSTP1-positive cells were observed in the 3PFD + Met-CD group (Fig. 6L and O).

Discussion

We previously reported that 5 days of PFD decreases the mouse liver levels of enzymes involved in ROS removal (Ronchi et al., 2010). These changes are in part prevented by adding of Met to PFD (Ronchi et al., 2004; Ronchi et al., 2010). Lately, we found that three cycles of feeding PFD for 5 days followed by CD for 5 days (3PFD-CD) produce irreversible changes in mouse liver protein composition and function (Caballero et al., 2011). Thus, this work was designed to determine whether feeding 3PFD + Met-CD can prevent the effects of 3PFD-CD.

The body weight loss (~20%) of mice fed 3PFD + Met-CD was similar to that seen with 3PFD-CD (Caballero et al., 2011). This finding agrees with a report that shows that several types of stress coupled with malnutrition can reduce body weight (Chaveroux et al., 2010). This effect likely accounts for the small decrease in liver weight noted in mice fed 3PFD + Met-CD and 3PFD-CD diets. The nucleic acid and protein contents of livers from mice fed 3PFD + Met-CD were similar to those of controls. However, the levels of RNA and protein in the 3PFD-CD livers increased, as previously reported by Caballero et al. (2011). These increases possibly reflect the overshoot caused by the last CD stage in 3PFD-CD (Caballero et al., 2011; Conde and Scornik, 1976; Conde and Franze-Fernandez, 1980). Furthermore, they often arise because of increased synthesis with decreased degradation (Blommaart et al., 1997; Conde and Scornik, 1976).

As previously described (Caballero et al., 2011), a 3PFD-CD diet increased the FAS content by elevating its mRNA level. This finding agrees with the increased FAS expression demonstrated in mammalian cells incubated in media lacking amino acids (Fafournoux et al., 2000). Furthermore, a FAS increase is associated with onset of hepatocellular carcinoma (Evert et al., 2005; Wakil et al., 1983; Yahagi et al., 2005). Therefore, Met likely plays a hepatoprotective role because the FAS did not change in response to a 3PFD + Met-CD diet.

CAIII integrates a set of proteins whose content is regulated by protein depletion and, principally, by sulfur amino acids (Ronchi et al., 2004; Ronchi et al., 2010; Ishii et al., 2005). As well as preserving the acid-base balance, they protect cells from oxidative stress by

Table 1
Activities of liver cytosolic enzymes and carbonyl group after dietary treatments.

	SOD (U/ml cytosol/mg protein)	CAT (U/ml citosol/mg protein)	Carbonyl group (nmol/mg protein)
CD	1.24 ± 0.08	8.07 ± 0.38	6.11 ± 0.96
3PFD-CD	0.84 ± 0.01*	4.52 ± 1.12*	10.76 ± 0.75*
3PFD + Met-CD	1.25 ± 0.14	8.69 ± 1.07	5.53 ± 0.13

Values are means ± SEM of three independent experiments ($n = 4$ each experiments). Values significantly different from CD group: $^{*}P < 0.05$.

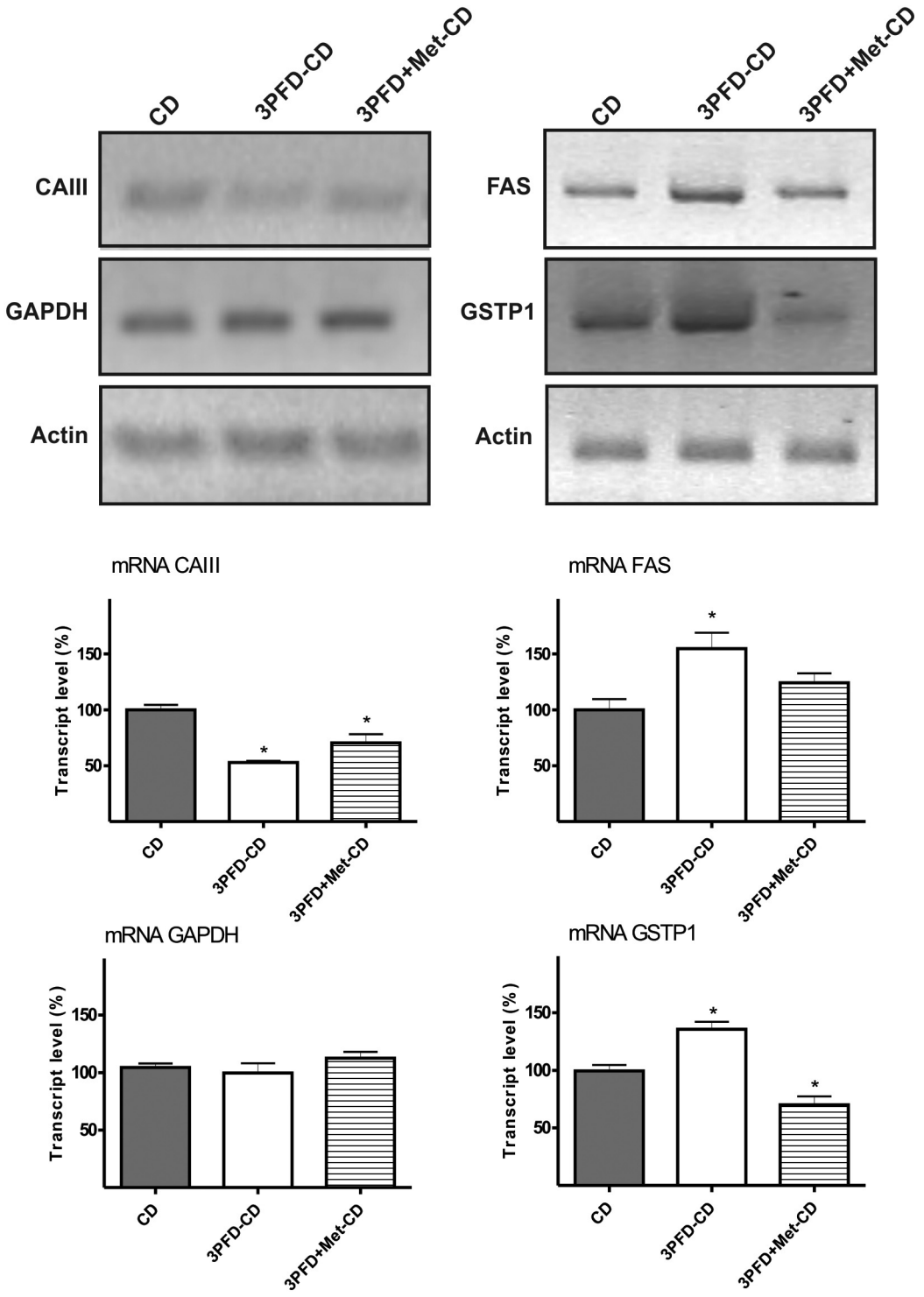

Fig. 3. Effects of 3PFD-CD and 3PFD + Met-CD on mRNA content. RT-PCR analysis of ACIII, GAPDH, FAS and GSTP1 mRNA. Relative changes in mRNA content were assessed by scanning densitometry of RT-PCR bands. Data are expressed as the intensity of bands determined by densitometry and related to the CD group. 3PFD-CD (□) and 3PFD + Met-CD (〓) and CD (■). Actin content was used for RNA normalization. Values represent the means ± SEM of three independent experiments ($n = 6$ each experiments). Values significantly different from CD condition: $^{*}P < 0.05$.

removing oxygen radicals. As previously reported (Caballero et al., 2011), the CAIII content decreased by 50% after feeding 3PFD-CD. In contrast, it remained within normal values in mice subjected to 3PFD + Met-CD. Thus, a regular Met supply likely protects the liver against oxidative stress. Therefore, the major CAIII content difference between the 3PFD + Met-CD and the 3PFD-CD group could be attributed to increased synthesis coupled to decreased breakdown. These results suggest that liver cells were minimally exposed to oxidative damage in the 3PFD + Met-CD group.

As previously reported (Caballero et al., 2011), the GSTP1 content did not change in response to 3PFD-CD treatment because of its increased mRNA levels. This result agrees with the response of rat hepatocytes to sulfur amino acids restriction (Tsai et al., 2005). Furthermore, the GSTP1 content did not change in the

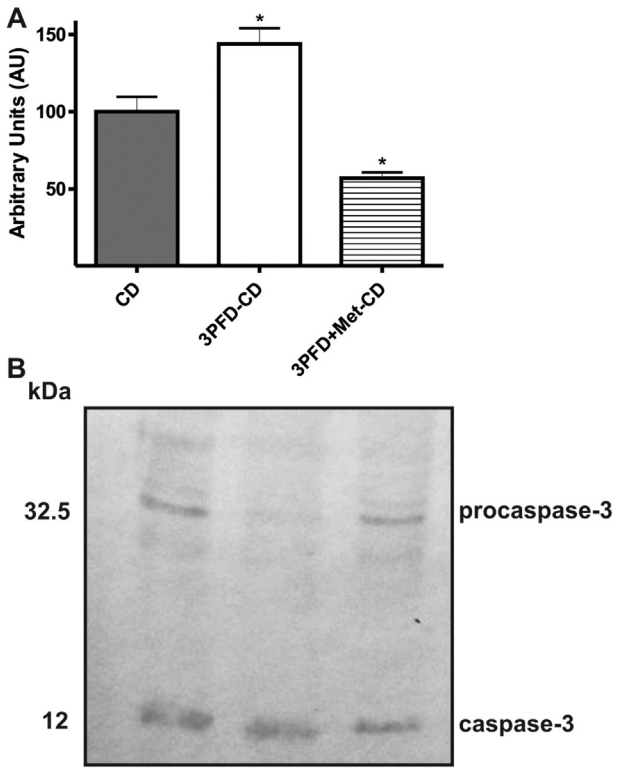

Fig. 4. Caspase-3 analysis. Study of effect 3PFD-CD and 3PFD + Met-CD on caspase-3 activity and their relation with procaspase-3. (A) Activity of caspase-3 in cytosolic extract evaluated by measuring the cleavage of their substrate Ac-DEVD-AFC. Data are expressed as arbitrary units (AU) of 3PFD-CD (□) and 3PFD + Met-CD (▤) respect to CD (■). Values significantly different from the CD condition $^{*}P < 0.05$. (B) Figure representative of Western blot with specific antibodies of caspase-3 and procaspase-3.

3PFD + Met-CD group, which agrees with the behavior of rat hepatocytes in the presence of Cys (Cho et al., 2000).

Feeding either 3PFD + Met-CD or 3PFD-CD did not change either the GAPDH or GAPDH mRNA content compared with CD. These responses are attributed to the final feeding with CD, which cancels the GAPDH increase caused by PFD (Caballero et al., 2011; Sanllorenti et al., 1992). In fact, a normal amino acid supply, but no particular amino acid, controls the GAPDH content (Ronchi et al., 2004).

As an indicator of the oxidative stress in the liver, the activities of SOD and CAT decreased as protein carbonylation increased in the 3PFD-CD group. In contrast, these features did not change in the 3PFD + Met-CD, which agrees with the hepatoprotective action of Met (Ronchi et al., 2004).

Oxidative stress may either cause direct cell death by necrosis or apoptosis (Resch et al., 2008; Ronchi et al., 2010). 3PFD-CD feeding activated caspase-3, which was accompanied by an increase in caspase-3-positive liver cells. This caspase-dependent apoptosis then initiated a defense response, which prevented the uncontrolled development of the survival of initiated cells (Bursch et al., 2005). Conversely, 3PFD + Met-CD did not allow caspase-3 activation and decreased the number of caspase-3-positive cells, which suggested the restraint of caspase-dependent apoptosis. In agreement with these results, the XIAP protein content (a potent member of the IAPs family for caspase restraint and apoptosis suppression) decreased in the 3PFD-CD group, and this decrease was less pronounced in the 3PFD + Met-CD group (Chai et al., 2000; Liu et al., 2000; Srinivasula et al., 2001).

Organelles, in particular mitochondria, are essential in controlling the cell response to protein oxidation, and therefore cell

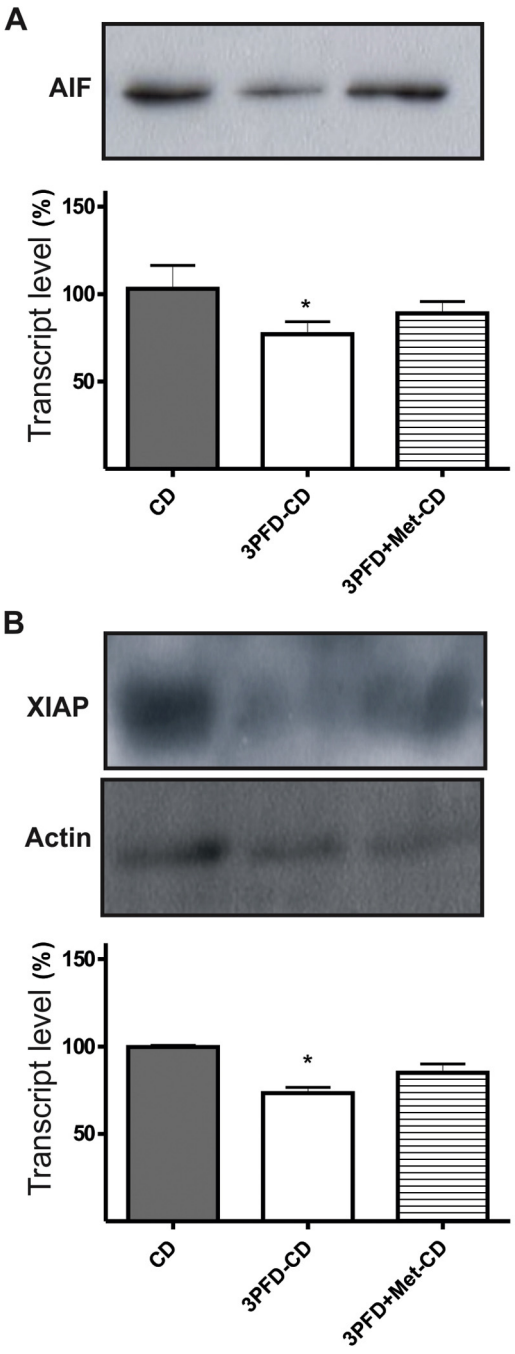

Fig. 5. Analysis of effect of 3PFD-CD and 3PFD + Met-CD on AIF and XIAP content. Western blot of mitochondrial fraction were used for detection of AIF (panel A) and cytosolic fraction were used for detection of XIAP (panel B). 60 μg of protein were separated by SDS-PAGE and transferred to polyvinylidene. Then, membranes were incubated with specific antibodies, revealed and quantified. Data are expressed as the density relative of bands in gel (%) respect to CD group, values significantly different from the CD condition $^{*}P < 0.05$.

fate (England and Cotter, 2005). Mitochondrial AIF loss leads to the dissipation of membrane potential and ROS content increase (Apostolova et al., 2006; Gujral et al., 2002; Kon et al., 2004; Kon et al., 2007). The mitochondrial AIF content decrease caused by 3PFD-CD then leads to oxidative stress and subsequent cellular damage (Apostolova et al., 2006). In contrast, the presence of Met in 3PFD + Met-CD preserved a normal AIF content, which demonstrates the mitochondrial membrane integrity.

The hepatic lobular architecture was clear and intact without any abnormalities in the liver section of CD mice. Conversely,

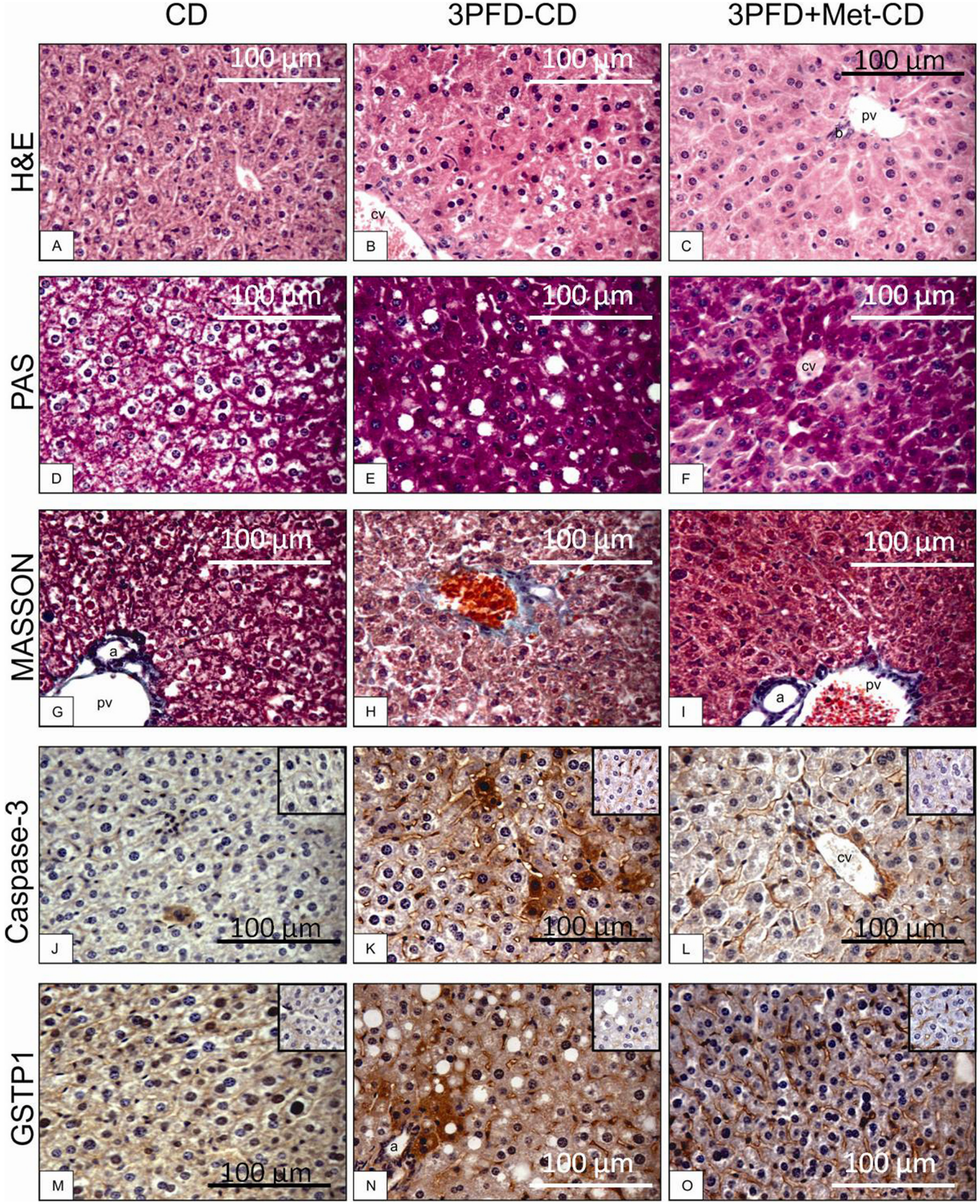

Fig. 6. Effect of chronic malnutrition and supplementation with Met on liver histology. Representative morphology of liver tissue from experimental animals ($n = 6$, magnification 400×). (A–C) Liver sections with H&E staining, (D–F) Liver sections with PAS staining, (G–I) Liver sections with Masson staining, (J–L) Liver sections with immunohistochemistry reveal caspase-3 positive cells, negative control is shown in lower panel in each picture. (M–O) Liver sections reveal Immunohistochemisty GSTP1-positive foci, negative control is shown in lower panel in each picture. In column CD, PFD-CD and PFD + Met-CD treatments. (cv) central vein, (a) arterial, (pv) portal vein.

apoptotic cells and pieces of hepatic necrosis with leukocyte infiltration were observed in 3PFD-CD livers. In addition, 3PFD-CD treatment produced a fatty liver, as seen by the large number of lipid vesicles. Recent studies show that deprivation of amino acids in the culture medium of hepatocytes could be responsible for the generation of pre-neoplastic stages in the liver (Chisari et al., 2012). The results presented here indicate that a 3PFD-CD diet also increases the number of GSTP1-positive cells, which are considered precursors of pre-neoplastic foci that often occur in the early stages of experimental carcinogenesis (Satoh and Hatayama, 2002). Also, the present results prove a non-toxic effect of 3PFD + Met-CD feeding on the liver. Indeed, the noted decline in both cell vacuolization and the number of GSTP1-positive cells suggests a hepatoprotective effect of Met.

In conclusion, this study shows that repeated periods of protein malnutrition produce oxidative stress followed by caspase-dependent apoptosis and cellular transformation symptoms. These changes are prevented by a permanent intake of Met. Although the main role of Met is to prevent oxidative stress (Caballero et al., 2011; Ishii et al., 2005; Ronchi et al., 2010) the mechanisms of action may differ for each of the considered proteins.

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