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Experimental Hyperprolinemia Induces Mild Oxidative Stress, Metabolic Changes, and Tissue Adaptation in Rat Liver

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ABSTRACT

The present study investigated the effects of chronic hyperprolinemia on oxidative and metabolic status in liver and serum of rats. Wistar rats received daily subcutaneous injections of proline from their 6th to 28th day of life. Twelve hours after the last injection the rats were sacrificed and liver and serum were collected. Results showed that hyperprolinemia induced a significant reduction in total antioxidant potential and thiobarbituric acid-reactive substances. The activities of the antioxidant enzymes catalase and superoxide dismutase were significantly increased after chronic proline administration, while glutathione (GSH) peroxidase activity, dichlorofluorescin oxidation, GSH, sulfhydryl, and carbonyl content remained unaltered. Histological analyses of the liver revealed that proline treatment induced changes of the hepatic microarchitecture and increased the number of inflammatory cells and the glycogen content. Biochemical determination also demonstrated an increase in glycogen concentration, as well as a higher synthesis of glycogen in liver of hyperprolinemic rats. Regarding to hepatic metabolism, it was observed an increase on glucose oxidation and a decrease on lipid synthesis from glucose. However, hepatic lipid content and serum glucose levels were not changed. Proline administration did not alter the aminotransferases activities and serum markers of hepatic injury. Our findings suggest that hyperprolinemia alters the liver homeostasis possibly by induction of a mild degree of oxidative stress and metabolic changes. The hepatic alterations caused by proline probably do not implicate in substantial hepatic tissue damage, but rather demonstrate a process of adaptation of this tissue to oxidative stress. However, the biological significance of these findings requires additional investigation. J. Cell. Biochem. 113: 174–183, 2012.

KEY WORDS: HYPERPROLINEMIA; HISTOLOGICAL ANALYSIS; OXIDATIVE STRESS; LIVER; SERUM; GLYCOGEN CONTENT

L-Proline is a proteinogenic amino acid, which has been described by playing regulatory roles in physiologic or pathophysiologic situations [Phang et al., 2010]. Such attribution is primarily based on the particularity of proline has an own metabolic system, that can serves to special functions as redox-regulation, energy source, stress signaling, and apoptosis [Phang et al., 2001, 2008].

Hyperprolinemia is a biochemical hallmark present in two inherited metabolic disorders caused by distinct defects in the L-proline catabolic pathway. Hyperprolinemia type I (HPI) and type II (HPII) are caused by deficiencies in the activities of proline oxidase and Δ^1-pyrroline-5-carboxylic acid dehydrogenase, respectively. Clinically, patients may exhibit diverse phenotypes; whereas, some patients have neurological, renal, and/or auditory defects, others

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are asymptomatic [Mitsubuchi et al., 2008]. Beyond that, mild hyperprolinemia is also present in some hepatic disorders, such as alcoholic liver disease [Vargas-Tank et al., 1988] and cirrhosis [Shaw et al., 1984]. This correlation is pertinent, as far as proline is primarily catabolized in the liver, but also in brain and kidney [Phang et al., 2001].

Studies addressing the proline metabolism in plants, fungi, and mammals suggest that this amino acid may have opposing effects on the intracellular redox environment [Krishnan et al., 2008; Phang et al., 2010; Wyse and Netto, 2011]. Because the catabolism of this amino acid involves the transfer of electrons from substrate proline to flavine adenine dinucleotide (FAD), proline may be a direct substrate for the generation of ATP [Hagedorn and Phang, 1983]. Nevertheless, it has been shown that proline-derived electrons can directly reduce oxygen to produce superoxide autogenously [Liu et al., 2005; White et al., 2007]. In this setting, proline acts as a prooxidant in the microenvironment of diseases as cancer, which may be an important mechanism for reducing carcinogenesis [Rivera and Maxwell, 2005; Phang et al., 2008]. Besides, previous works showed that proline can mediate oxidative stress in brain of rats [Delwing et al., 2003a,b]. On the other hand, it has been described that proline is able to perform chemical reaction, producing adducts with hydroxyl radical [Rustgi et al., 1977; Floyd and Nagy, 1984; Alia et al., 2001; Halliwell and Gutteridge, 2007]. In this context, studies have shown that proline can modulate the intracellular redox environment and protect mammalian cells against oxidative stress [Krishnan et al., 2008]. Therefore, proline may have multiple functions in stress adaptation, exhibiting dual functions as a pro-oxidant, and as a reactive oxygen species (ROS) scavenger [Krishnan et al., 2008; Phang et al., 2010].

It is known that high concentration of free radicals can elicit oxidative stress and promote cellular injury. Following liver injury, several cell types can secrete inflammatory cytokines that stimulate the recruitment of inflammatory cells which in turn, induce the generation of more ROS [Decker, 1990; Hernandez-Gea and Friedman, 2011]. In this context, in vitro and in vivo observations suggest that oxidative stress and associated damage may represent a common link between different forms of chronic liver injury [Ha et al., 2010; Nair et al., 2010].

Given the putative role of proline in mechanisms redox and considering that little is known about the effects of hyperprolinemia on liver, either due to inherited or hepatic disorders, in the present study we evaluated the hepatic oxidative and metabolic status in rats subjected to chronic experimental hyperprolinemia. Firstly, we investigated the effect of proline on several oxidative stress parameters, namely total radical-antioxidant potential (TRAP), glutathione (GSH) levels, thiobarbituric acid-reactive substances (TBARS), reactive species production (DCFH oxidation), sulfhydryl and carbonyl content, as well as on antioxidant enzymes activities, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in liver of rats. Besides, histological analyses were performed in order to verify the occurrence of morphological changes and evaluate the glycogen/glycoprotein content in hepatic tissue sections of hyperprolinemic rats. Hepatic metabolic status was assessed by measuring synthesis and concentration of glycogen and lipid, as well as on glucose oxidation to CO_2. Glucose levels and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were also determined in serum of rats.

MATERIALS AND METHODS

ANIMALS

Male or female Wistar rats were obtained from the Central Animal House of the Department of Biochemistry of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were maintained on a 12 h light/12 h dark cycle at a constant temperature ($22 \pm 1^{\circ}C$), with free access to water and commercial protein chow. Animal care followed the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 80–23, revised 1996) and was approved by the University Ethics Committee.

PROLINE ADMINISTRATION

Chronic hyperprolinemia was chemically induced by daily subcutaneous administration of proline from the 6th to the 28th day of life as described by Pontes et al. [1999] and Moreira et al. [1989]. Proline (Sigma Chemical Co.) was dissolved in 0.9% NaCl and administered twice a day. During the first 8 days of treatment (6th–13th day of life) rats received 12.8 μmol Pro/g body weight, from the 14th to 17th day they received 14.6 μmol Pro/g body weight, from the 18th to 21st day they received 16.4 μmol Pro/g body weight, and from the 22nd to 28th day of life they received 18.2 μmol Pro/g body weight. Control animals received saline injections in the same volumes as those applied to proline-treated rats. The animals were killed 12 h after the last injection by decapitation without anesthesia, when the blood levels of proline had returned to normal [Moreira et al., 1989]. Rats subjected to this treatment achieved plasma proline levels between 1.0 and 2.0 mM, which are similar to those found in hyperprolinemic patients [Moreira et al., 1989; Phang et al., 2001].

TISSUE AND HOMOGENATE PREPARATION

The rats were sacrificed by decapitation without anesthesia 12 h after the last injection of proline. Blood was collected and the serum was separated by centrifugation, at $800 \times g$ for 5 min at $25^{\circ}C$. The liver was quickly removed and processed as follow.

For oxidative stress parameters determination, the liver was homogenized in 10 volumes (1:10 w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $800 \times g$ for 10 min at $4^{\circ}C$, to discard nuclei and cell debris. The pellet was discarded and the supernatant was taken to biochemical assays.

For measurement of hepatic lipids, frozen liver samples were thawed on ice and was homogenized in 20 volumes (1:20 w/v) of deionized water and centrifuged at $800 \times g$ for 10 min at $4^{\circ}C$. The pellet was discarded and the supernatant was taken to triglycerides measure and extraction/isolation of lipids to dried lipid extracts according to Folch et al. [1957] to cholesterol determination.

THIOBARBITURIC ACID REACTIVE SPECIES (TBARS)

TBARS levels were determined according to the method described by Ohkawa et al. [1979]. Briefly, 50 μl of 8.1% sodium dodecyl sulfate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5, 1.5 ml of 0.8%

thiobarbituric acid, and 700 μl of distilled water were added to 500 μl of tissue homogenate in a Pyrex tube. The mixture was vortexed and the reaction was carried out in a boiling water bath for 1 h. After cooling with tap water, the mixture was allowed to cool on water for 5 min, centrifuged at 750 × *g* for 10 min, and the resulting pink color was determined in a spectrophotometer at 535 nm. A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. The results were reported as nmol of TBARS per mg protein.

2′,7′-DICHLOROFLUORESCIN OXIDATION ASSAY (DCFH)

Reactive species production was measured following a method based on 2′,7′-dichlorofluorescin (DCFH) oxidation [LeBel et al., 1992]. Samples (60 μl) were incubated for 30 min at 37 °C in the dark with 240 μl of 100 μM DCFH diacetate (DCF-DA) solution in a 96-well plates. DCF-DA is deacetylated by intracellular esterases to form non-fluorescent DCFH, which is rapidly oxidized by some reactive oxygen and/or nitrogen species (ROS and/or RNS) present in samples, producing the highly fluorescent compound de-esterified dichlorofluorescein (DCF), which can be measured at $\lambda_{ex} = 488$ nm and $\lambda_{em} = 525$ nm. A calibration curve was performed with standard DCF (0.25–10 mM) and the levels of reactive species were calculated as nmol DCF/mg protein.

TOTAL RADICAL-TRAPPING ANTIOXIDANT POTENTIAL (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) thermolysis in Perkin-Elmer Microbeta Microplate Scintillation Analyzer (PerkinElmer Life and Analytical Sciences, Waltham, MA) [Lissi et al., 1992; Evelson et al., 2001]. Two hundred and forty microliter of a system containing ABAP (10 mM) dissolved in 50 mM sodium phosphate buffer pH 8.6 plus luminol (5.6 mM), was added to a microplate and the initial chemiluminescence was measured. Ten microliter of 300 μM Trolox (water-soluble α-tocopherol analogue, used as a standard) or 10 μl of liver supernatant was then added to each plate well, producing a decrease in the initial chemiluminescence value. This value is kept low, until the antioxidants present in the sample are depleted, when chemiluminescence returns to its initial value. The time spent by the sample to keep chemiluminescence return to its low initial value is directly proportional to the antioxidant capacity of the tissue. The results were represented as nmol Trolox/mg protein.

SUPEROXIDE DISMUTASE (SOD)

This method for the assay of SOD activity is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide (O_2^-) which is a substrate for SOD [Marklund, 1985]. Briefly, to 15 μl of each sample were added 215 μl of a mixture containing 50 mM Tris buffer pH 8.2 with 1 mM EDTA and 30 mM CAT. After that, 20 μl of pyrogallol were added and the absorbance was immediately recorded each 30 s for 3 min at 420 nm in SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, California). The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. One SOD unit is defined as the amount of SOD necessary to inhibit 50% of pyrogallol autoxidation and the specific activity is reported as SOD units/mg protein.

CATALASE (CAT)

CAT activity was assayed using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies). The method used is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer pH 7.0, and 0.1–0.3 mg protein/mL [Aebi, 1984]. One CAT unit is defined as one μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as CAT units/mg protein.

GLUTATHIONE PEROXIDASE (GPx)

GPx activity was measured using tert-butyl-hydroperoxide as substrate [Wendel, 1981]. NADPH disappearance was monitored at 340 nm using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies). The medium contained 2 mM GSH, 0.15 U/mL GSH reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide, and 0.1 mM NADPH. One GPx unit is defined as one μmol of NADPH consumed per minute and the specific activity is represented as GPx units/mg protein.

REDUCED GLUTATHIONE CONTENT (GSH)

This method is based on the reaction of GSH with the fluorophore *o*-phtalaldeyde (OPT) after deproteinizing the samples and was measured according to Browne and Armstrong [1998]. Initially, metaphosphoric acid was used to deproteinize the samples, which were then centrifuged at 1,000 × *g* for 10 min. Briefly, to 15 μl of each sample were taken 200 μl of a mixture containing 15 μl of OPT 1 mg/mL (prepared in methanol) plus 185 μl of 100 mM sodium phosphate buffer pH 8.0 with 5 mM EDTA in a 96-well plates. The assay was allowed to stand in the dark for exactly 15 min. After that, the fluorescence was measured at $\lambda_{ex} = 350$ nm and $\lambda_{em} = 420$ nm. A calibration curve was also performed with a commercial GSH solution, and the results were calculated as nmol GSH/mg protein.

TOTAL SULFHYDRYL CONTENT

This assay was performed as described by Aksenov and Markesbery [2001], which is based on the reduction of 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, which in turn become oxidized (disulfide), generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. Briefly, 50 μl of homogenate were added to 1 ml of PBS buffer pH 7.4 containing 1 mM EDTA. The reaction was started by the addition of 30 μl of 10 mM DTNB and incubated for 30 min at room temperature in a dark room. Results were reported as nmol TNB/mg protein.

PROTEIN CARBONYL CONTENT

Oxidatively modified proteins present an enhancement of carbonyl content [Stadtman and Levine, 2003]. In this study, protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm [Reznick and Packer, 1994]. Briefly, 100 μl of homogenate

were added to plastic tubes containing 400 μl of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This was kept in the dark for 1 h and vortexed each 15 min. After that, 500 μl of 20% trichloroacetic acid were added to each tube. The mixture was vortexed and centrifuged at 20,000 × *g* for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 ml ethanol/ethyl acetate (1:1 v/v), vortexed, and centrifuged at 20,000 × *g* for 3 min. The supernatant was discarded and the pellet re-suspended in 600 μl of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60°C for 15 min. After that, it was centrifuged at 20,000 × *g* for 3 min and the absorbance was measured at 370 nm (UV) in a quartz cuvette with a Hitachi U-2001 double-beam spectrophotometer with temperature control (Hitachi High Technologies America, Inc., Life Sciences Division, Pleasanton, CA). Results were represented as protein carbonyl content (nmol/mg protein).

HISTOLOGICAL ANALYSIS

For each group, six rats were anesthetized with a mixture of ketamine and xylazine (75 and 10 mg/kg, respectively), and submitted to transcardiac perfusion with 0.9% saline followed by fresh 4% formaldehyde solution, pH 7.4. The liver was removed and post-fixed in 4% formaldehyde solution for 6 h at 4°C. Fragments from the central portion, left, and right lobes were processed and embedded in Paraplast®. Semiserial 5 μm-thick sections were placed in glass slides and stained with hematoxylin and eosin (HE) for morphological analysis or submitted to periodic acid-Schiff (PAS) staining for the analysis of glycoproteins/glycogen [Bancroft and Stevens, 1990]. Pictures were taken using a CCD camera coupled to an Olympus CX-40 microscope. The software Adobe Photoshop was used to construct the panels.

HEPATIC GLYCOGEN SYNTHESIS

For the measurement of hepatic glycogen synthesis, the liver was dissected and cut into 300 μm slices using a McIlwain tissue chopper (100–120 mg). It was incubated in a beaker with a medium containing Krebs Ringer bicarbonate buffer (pH 7.4), 5 mM D-glucose, and 0.2 μCi D[U-^{14}C]glucose for glycogen synthesis from glucose or 0.2 mM L-alanine and 0.2 μCi L[U-^{14}C]alanine for glycogen synthesis from alanine. Incubations were carried out in ambient content that was gassed with a 95% O_2:5% CO_2 mixture for 1 h. Liver slices were incubated at 37°C for 1 h in a metabolic shaker (60 cycles/min), according to the method of Dolnikoff et al. [2001]. Incubation was stopped by placing the bottles in ice. Afterward, 1 ml of 60% KOH was added to each beaker. After 15 min in a boiling water bath, 3 ml of 96% ethanol was added to the tubes to precipitate glycogen. After precipitation, glycogen was suspended in 0.2 ml of water, the scintillation liquid (OptiPhase HiSafe3 from PerkinElmer-USA) was added and the samples were assessed in a scintillation counter. Results were reported as pmol of glucose or alanine incorporated into glycogen.

HEPATIC GLYCOGEN CONTENT

The hepatic glycogen concentration was measured using the Krisman method [Krisman, 1962]. Briefly, the liver was dissected and approximately 100 mg of tissue was treated with 1 ml of KOH 30%. After 15 min in boiling water bath, 3 ml of 96% ethanol was added to precipitate glycogen. Liver glycogen was measured with the colorimetric method and results were reported as mg glycogen/100 mg tissue.

GLUCOSE OXIDATION TO CO_2 AND LIPID SYNTHESIS FROM D[U-^{14}C]GLUCOSE

For the measurement of CO_2 production from D[U-^{14}C]glucose and lipid synthesis, liver slices (between 100 and 120 mg) were incubated in 1.0 ml Krebs Ringer bicarbonate buffer pH 7.4, containing 5.0 mM glucose plus 0.2 μCi D[U-^{14}C]glucose. Before incubation, the reaction medium was gassed with a 95% O_2:5% CO_2 mixture for 1 min. Flasks were sealed with rubber caps and the slices were incubated at 37°C for 1 h in a metabolic shaker (60 cycles/min), according to the method of Dolnikoff et al. [2001]. Incubations were stopped by adding 0.25 ml 50% trichloroacetic acid through the rubber cap. Then 0.2 ml of 1 M sodium hydroxide was injected into the central wells. The flasks were shaken for an additional 30 min at 37°C to trap CO_2. Afterwards, the contents of the central well were transferred to vials and assayed for CO_2 radioactivity in a liquid-scintillation counter. The flask contents were homogenized and transferred to tubes. After centrifugation, the precipitate was washed three times with 10% trichloroacetic acid and lipids were extracted with chloroform/methanol (2:1). The chloroform/methanol phase was evaporated in vials and radioactivity was measured. All the results were expressed with respect to the initial specific activity of the incubation medium.

HEPATIC LIPIDS CONTENT

For measurement of hepatic lipids, frozen liver samples were thawed on ice, homogenized in deionized water, and triglycerides were assayed by commercially available diagnostic kits supplied by Labtest®. To hepatic cholesterol determination, extraction and isolation of lipids to dried lipid extracts were done according to Folch et al. [1957]. Then, cholesterol was enzymatically assayed in lipid extract by colorimetric Labtest® kit. Results were reported as mg/g tissue.

SERUM BIOCHEMICAL PARAMETERS

The activities of ALT and AST and glucose levels were determined in serum of rats using commercially available diagnostic kits supplied by Labtest® (Labtest, MG, Brazil). ALT and AST activities were expressed as units/L and glucose levels as mg/dL.

PROTEIN DETERMINATION

Protein was determined by the method of Lowry et al. [1951] using bovine serum albumin as standard.

STATISTICAL ANALYSIS

All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Data were analyzed by Mann–Whitney *U*-test for unpaired sample and values of $P < 0.05$ were considered to be significant. Descriptive data were expressed as mean ± SD.

RESULTS

Initially, we investigated the effects of chronic proline administration on several parameters of oxidative stress in the liver of rats. Table I shows that proline-treated rats presented a significant reduction in TRAP ($P < 0.05$) and TBARS levels ($P < 0.05$). In contrast, DCFH oxidation and GSH, sulfhydryl and carbonyl content were not altered ($P > 0.05$). Enzymatic antioxidant defenses were also evaluated by determination of SOD, CAT, and GPx activities. As can be seen in Table II, SOD ($P < 0.01$) and CAT ($P < 0.05$) activities were significantly increased after chronic proline administration as compared to control, while GPx activity remained unaltered ($P > 0.05$). Because SOD and CAT are oxidant-detoxifying enzymes that work in sequence converting superoxide anion to water [Halliwell and Gutteridge, 2007], a ratio between SOD and CAT enzyme activities were applied. The hepatic SOD/CAT ratio did not change in proline-treated rats, when compared to control [control $= 38.2 \pm 7.3$ and proline $= 41.2 \pm 6.7$, expressed in arbitrary units; ($P > 0.05$)].

The liver structural integrity was evaluated by morphological analysis in control and proline-treated rats. In the control animals (Fig. 1A,C), we can observe the normal appearance of the hepatic cells. In contrast, hyperprolinemic rats (Fig. 1B,D) exhibited a slight disruption of the hepatic microarchitecture and a higher presence of inflammatory cells as lymphocytes and neutrophils around the hepatic cells and at the portal space (ps). These proline-associated changes were accompanied by a markedly increase in the glycogen content revealed by the PAS staining (Fig. 2B,D), when compared to the respective control (Fig. 2A,C).

Based on histological evidences, we also performed biochemical analyses in order to measure glycogen/glycoproteins content in liver. Figure 3 shows that hyperprolinemia significantly increased glycogen synthesis from both D[U-^{14}C]glucose ($P < 0.05$) (A) and L[U-^{14}C]alanine ($P < 0.05$) (B), as well as glycogen concentration ($P < 0.05$) (C). Regarding to hepatic metabolism, Figure 4 depicts the increased rate of D[U-^{14}C]glucose oxidation to CO_2 (A) ($P < 0.05$) and decreased D[U-^{14}C]glucose conversion to lipid (B) ($P < 0.05$). In addition, hepatic lipid content such as triglycerides [control $= 30.2 \pm 3.5$ and proline $= 31.9 \pm 2.5$; $P > 0.05$)] and cholesterol [control $= 1.55 \pm 0.17$ and proline $= 1.53 \pm 0.08$; $P > 0.05$)] were not changed by proline administration as compared to control.

Serum biochemical parameters, including glucose levels, as well as ALT and AST activities were also evaluated. Table III shows that proline administration did not alter the glucose levels, ALT, and AST activities ($P > 0.05$).

TABLE I. Effect of Chronic Hyperprolinemia on Some Parameters of Oxidative Stress, Such as Dichlorofluorescin Oxidation Assay (DCFH), Thiobarbituric Acid-Reactive Substances (TBARS), Total Radical-Trapping Antioxidant Potential (TRAP), Glutathione (GSH), Sulfhydryl, and Carbonyl Content in the Liver of Rats

Parameters	Control	Proline
DCFH oxidation	18.01 ± 2.41	18.08 ± 2.84
TBARS	1.07 ± 0.07	$0.92 \pm 0.11^{*}$
TRAP	141.74 ± 26.58	$89.94 \pm 34.70^{*}$
GSH	5.84 ± 0.85	4.81 ± 1.23
Sulfhydryl content	61.67 ± 5.48	64.47 ± 4.55
Carbonyl content	2.38 ± 0.62	2.47 ± 0.25

Data are expressed as mean ± SD as follow: DCFH (nmol DCF/mg protein), TBARS (nmol TBARS/mg protein), TRAP (nmol Trolox/mg protein), GSH (nmol GSH/mg protein), sulfhydryl (nmol TNB/mg protein), and carbonyl content (nmol carbonyl/mg protein). $^{*}P < 0.05$, compared to control (Mann–Whitney *U*-test) for 5–6 animals in each group.

TABLE II. Effect of Chronic Hyperprolinemia on Superoxide Dismutase (SOD), Catalase (CAT), and Gluthatione Peroxidase (GPx) Activities in the Liver of Rats

Enzyme activities (units/mg protein)	Control	Proline
SOD	4.1 ± 0.44	$5.6 \pm 0.34^{**}$
CAT	158.14 ± 42.61	$234.05 \pm 28.09^{*}$
GPx	423.75 ± 55.99	421.50 ± 43.65

Data are mean ± SD for 5–6 animals in each group, reported as units/mg protein. One SOD unit is defined as 50% inhibition of pyrogallol autoxidation. One CAT unit is defined as 1 mmol of hydrogen peroxide consumed per minute. One GPx unit is defined as 1 mmol of NADPH consumed per minute. $^{*}P < 0.05$; $^{**}P < 0.01$, compared to control (Mann–Whitney *U*-test).

DISCUSSION

The present study investigated the effects of chronic administration of proline on the hepatic oxidative and metabolic status in rats, utilizing a chemical experimental model of hyperprolinemia, which mimic the tissue levels of proline found in human HP II [Pontes et al., 1999; Phang et al., 2001]. By using this animal model, previous works showed that proline provokes several neurotoxic effects in rats, such as memory impairment [Delwing et al., 2006], inhibition of enzymes activities as acetylcholinesterase, Na^{+},K^{+}-ATPase, and aminotransferases [Pontes et al., 1999; Shanti et al., 2004; Delwing et al., 2005], energy deficit [Delwing et al., 2007; Ferreira et al., 2010], and induction of oxidative stress [Delwing et al., 2003a,b].

Herein, initially was investigated the effect of hyperprolinemia on several parameters of oxidative stress in liver tissue. Considering that non-enzymatic antioxidant defenses, act as the first line in the removal of ROS, TRAP was employed to evaluate the total potential of the main antioxidants found in hepatic tissue such as GSH, uric acid, ascorbic acid, and α-tocopherol [Lissi et al., 1995; Evelson et al., 2001]. Our results showed that proline significantly decreased TRAP, suggesting that high concentrations of this amino acid cause a reduction on quantity of non-enzymatic antioxidants in liver. Based on this finding, we also measured the levels of GSH, which is the antioxidant present at higher concentrations in liver and that most contributes to TRAP values [Evelson et al., 2001]. Results showed that this parameter was not altered by chronic hyperprolinemia, indicating that decreased TRAP values by proline did not reflect alteration of the GSH content. However, it is conceivable that others tissue antioxidants could be decreased in liver of hyperprolinemic rats.

Regarding the enzymatic antioxidant defenses, we observed that chronic hyperprolinemia provoked a significant increase of SOD and CAT activity, but not of GPx activity in rat liver. Interestingly, these

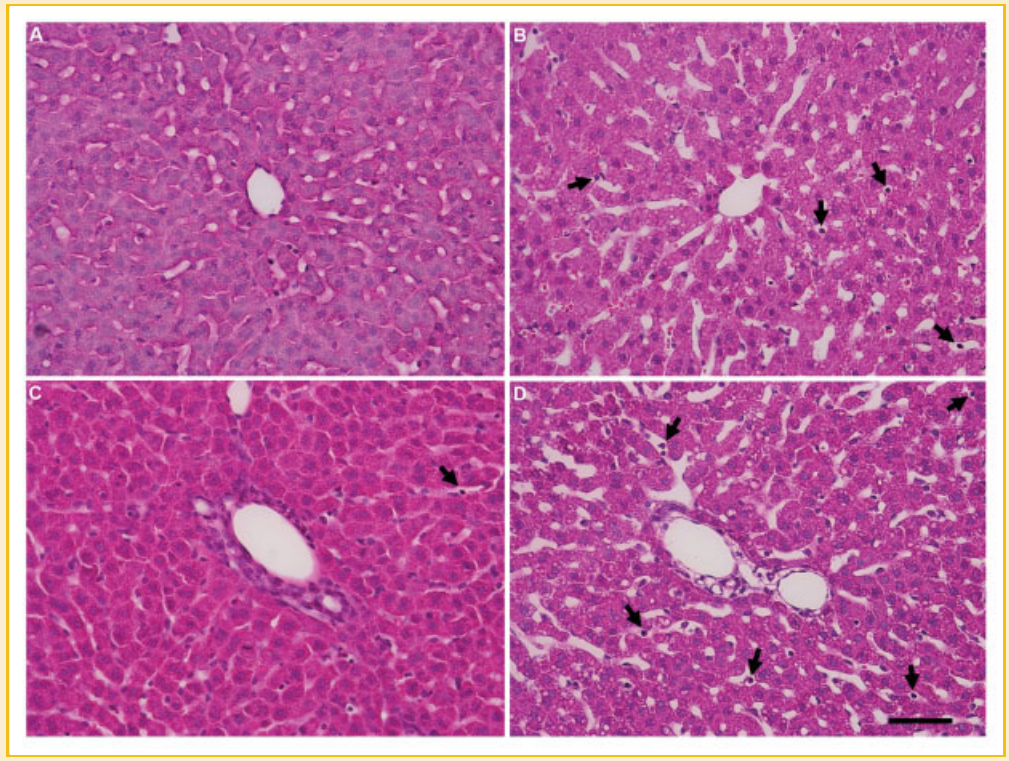

Fig. 1. Hyperprolinemia induces morphological changes. Representative photomicrographs of control rats (A,C) and hyperprolinemic rats (B,D) stained with hematoxylin and eosin (HE). The central vein (cv) region is represented in the upper line, while the portal space (ps) in the lower line. Hyperprolinemia treatment induces an increase in the frequency of inflammatory cells (arrowheads) and the presence of intracellular vacuole. Scale bar = 50 μm. n = 6. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

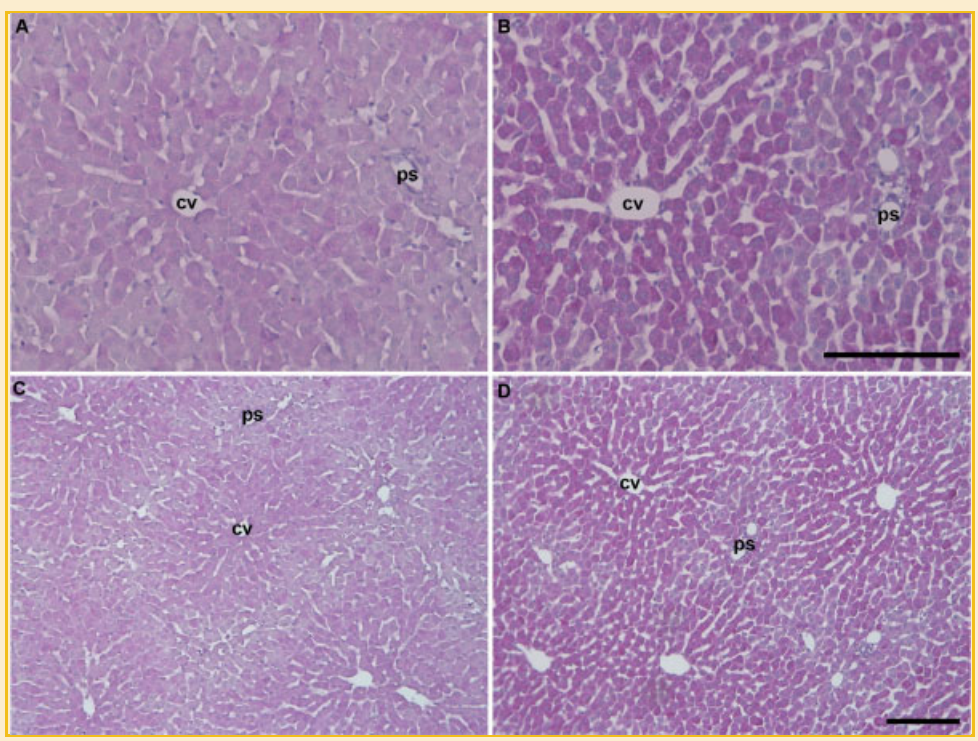

Fig. 2. Hyperprolinemia increases glycogen content in hepatocytes. Representative photomicrographs of control (A,C) and hyperprolinemic rats (B,D) stained with periodic acid–Schiff (PAS) reaction. Control rats showed a glycogen deposition (purple) in hepatocytes around to the central vein (cv), while hyperprolinemic rats showed a stronger PAS-staining around cv and spreading out to the portal space (ps), indicating an increase on glycogen content. Scale bar = 100 μm. n = 6. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

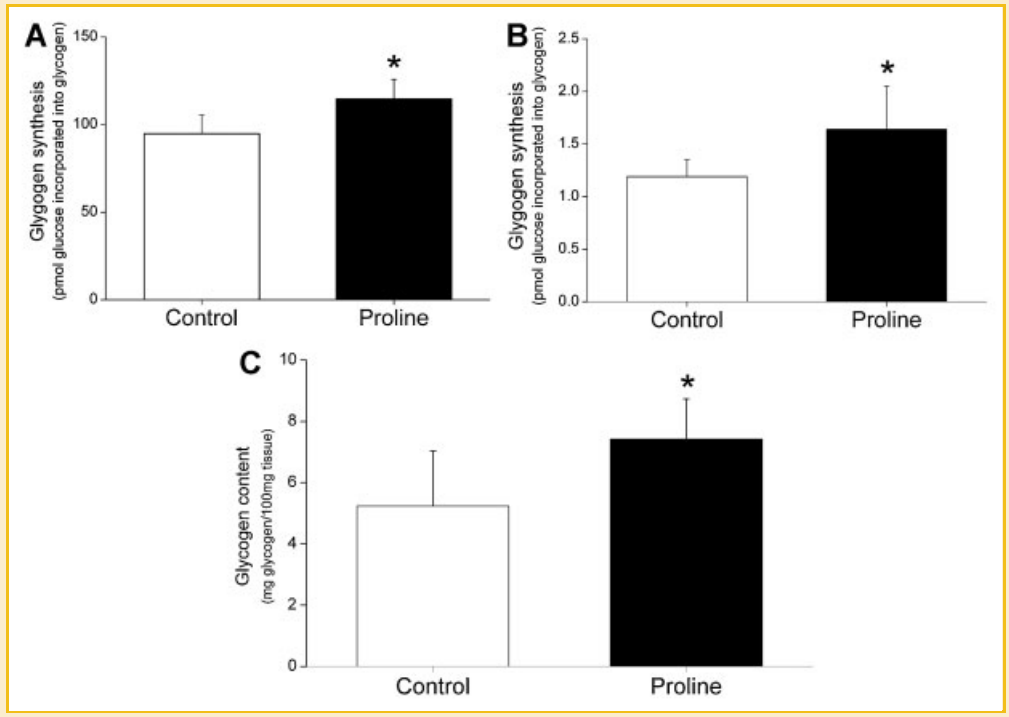

Fig. 3. Effect of chronic hyperprolinemia on glycogen synthesis from glucose (A), alanine (B), and glycogen concentration (C) in the liver of rats. Data are expressed as mean ± SD for 5–6 animals in each group. $^{*}P < 0.05$, compared to control (Mann–Whitney *U*-test).

enzymes activities increased in the same proportion (around 30% each) and there was not an imbalance in the SOD/CAT ratio. There are evidences that the continued presence of low concentrations of ROS is able to induce upregulation of antioxidant enzymes, as a cellular strategy of adaptation to oxidative stress [Halliwell and Whiteman, 2004; Halliwell and Gutteridge, 2007]. Therefore, based on data from our and other studies [Liu et al., 2005; White et al., 2007], it is conceivable to suggest that high proline levels could lead to generation of superoxide radical in hepatic tissue. Superoxide is dismutated by SOD with consequent generation of H_2O_2, which is in turn reduced by CAT. Then, the increase in activities these antioxidant enzymes in liver of rats observed in present work may be a consequence from tissue adaptation to sustained production of ROS, mainly superoxide and hydrogen peroxide, which are substrates scavenged by SOD and CAT, respectively.

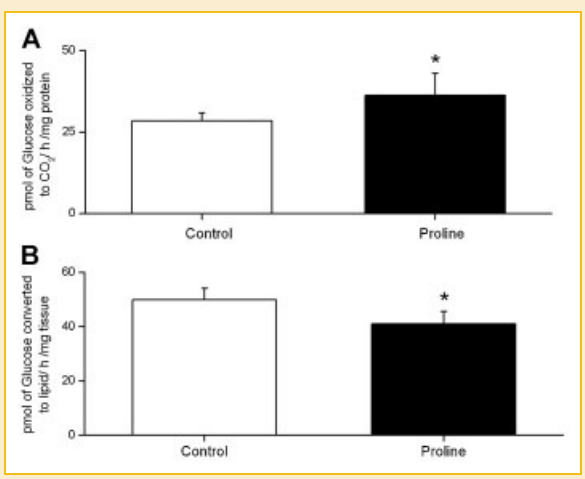

Fig. 4. Effect of chronic hyperprolinemia on glucose oxidation (A) and conversion of glucose to lipid (B) by liver tissue in rats. Data are expressed as pmol of glucose oxidized per hour per milligram of tissue and represented as mean ± SD for 5–7 animals in each group. $^{*}P < 0.05$, compared to control (Mann–Whitney *U*-test).

We also investigated the effect of hyperprolinemia on two markers of protein oxidation in liver of rats: sulfhydryl content, which is employed to verify protein damage to sulphydryl groups [Aksenov and Markesbery, 2001] and carbonyl content, formed mainly by oxidation of side chains of some amino acid residues [Dalle-Donne et al., 2003]. In this study, it was observed that liver of rats subjected to chronic hyperprolinemia did not present oxidative damage to proteins.

RNS and ROS production was assessed by DCFH oxidation assay, which is a probe used to detect peroxyl, alkoxyl, nitrogen dioxide,

TABLE III. Effect of Chronic Hyperprolinemia on Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) Activities and on Glucose Levels in Serum of Rats

Parameters	Control	Proline
ALT (U/L)	20.14 ± 2.89	18.80 ± 1.74
AST (U/L)	80.41 ± 6.24	79.60 ± 7.77
Glucose (mg/dL)	86.57 ± 17.07	90.00 ± 2.22

Data are expressed as mean ± SD for seven animals in each group. There is no difference between groups (Mann–Whitney *U*-test).

carbonate, hydroxyl, and peroxynitrite [Halliwell and Whiteman, 2004]. We showed that proline did not alter the levels of reactive species detected by DCFH assay. Additionally, lipid peroxidation was also evaluated in liver tissue by TBARS assay, which identify mainly malondialdehyde, a final product of fatty acid peroxidation [Ohkawa et al., 1979]. Interestingly, TBARS levels were significantly decreased after chronic exposure to proline. We cannot explain the exact mechanism by which proline reduces TBARS levels, but this result is in agreement with works showing that this amino acid can scavenge some free radicals, for example, by physical quenching of oxygen singlet and chemical reaction with hydroxyl radicals [Rustgi et al., 1977; Floyd and Nagy, 1984; Alia et al., 2001; Halliwell and Gutteridge, 2007]. In this context, proline accumulation has been observed in many microorganisms and plants exposed to environmental stresses, as a mechanism of protection against ROS produced during stress conditions [Alia et al., 2001; Siripornadulsil et al., 2002; Chen and Dickman, 2005; Chen et al., 2006]. Therefore, the unchanged levels of reactive species (assessed by DCFH assay) and reduced incidence on lipid peroxidation (detected by TBARS) in rat liver submitted to proline administration, could be attributed to an effective detoxification of ROS/RNS by non-enzymatic and enzymatic antioxidant defense as demonstrated by decreased TRAP and increased SOD and CAT activities, respectively. Besides, this putative role of proline in decreasing lipid peroxidation in rat liver, as well as the upregulation of antioxidant enzymes could explain at least in part, the hyperprolinemia found in patients affected by hepatic disorders, as a mechanism to protect liver from oxidative injury.

Recently, we have reported that proline elicits oxidative stress in brain of rats [Delwing et al., 2003a,b]. Nevertheless, in rat liver proline did not produced similar effects. Owing to high regenerative capacity of liver, including high antioxidant defenses and adaptability to metabolic alterations regulating body homeostasis, this organ is thought to be more resistant to damage by free radical attack [Genet et al., 2002]. In addition, it is known the increased susceptibility of central nervous system to oxidative insult, since there is a high content of polyunsaturated fatty acids (PUFAs) in brain, which are sensitive to oxidative modifications [Halliwell and Gutteridge, 2007]. Furthermore, brain has a decreased of antioxidant enzymes activities (especially CAT) when compared to liver, that may favor the onset and maintenance of a pro-oxidative state [Halliwell and Gutteridge, 2007].

The effect of chronic proline exposure on the liver structural integrity was evaluated by histological analysis. Hematoxylin/eosin staining showed that proline promotes disruption of hepatocytes arrangement and increases the number of inflammatory cells around the hepatic cells and at the ps, when compared to control rats. These histological findings could be explained on the basis that proline administration induced a mild degree of oxidative stress in the liver of rats. Since free radicals are also released in large amounts from inflammatory cells activated [Decker, 1990], their increased number reinforces the evidence of generation of ROS/RNS in liver of rats. Staining for the analysis of glycoproteins/glycogen revealed that changes in the hepatic microarchitecture are similar (although less severe) to histological phenotype to that observed in liver of patients with glycogen storage disease [Salganik et al., 2009], presenting a marked increase in the glycogen content in liver of proline-treated rats. Biochemical determination reaffirmed this finding, which demonstrate that proline increases glycogen concentration and the synthesis of glycogen from both direct (from D[U-^{14}C]glucose) and indirect (from L[U-^{14}C]alanine) pathways in the liver of rats. In this context, studies show that proline increase hepatic glucose-6-P concentration, suggesting that a proline metabolite inhibits the glucose-6-phosphatase activity and thus directs glucose-6-P away from glucose production and toward glycogen synthesis [Bode et al., 1992]. This finding could explain the glycogenic effect of proline found in present work.

Since we observed an increased deposition of glycogen in hepatocytes caused by proline (an amino acid gluconeogenic) administration [Phang et al., 2001], we also verified other possible fates of hepatic glucose after chronic treatment with proline. Results showed that hyperprolinemic rats presented an decreased conversion of D[U-^{14}C]glucose to lipid in liver. Nevertheless, hepatic lipid content such as triglycerides and cholesterol were not changed. Besides, an increased glucose oxidation capability by hepatic tissue was demonstrated when compared to controls, suggesting a stimulation of glycolytic pathway and TCA cycle. However, the glucose levels in serum of rats were not altered by proline administration. These findings might be related to the fact that pyrroline-5-carboxylate, the immediate precursor and degradation product of proline, is the sole intermediate directly connecting the tricarboxylic acid and urea cycles with amino acid metabolism [Phang, 1985].

Finally, we evaluated the activities of aminotransferases that are serum markers of hepatocyte injury. We observed that ALT and AST activities remained unchanged after chronic proline administration, suggesting that proline did not induce substantial liver damage.

Taken together, these results suggest that hyperprolinemia alters the liver homeostasis through the induction of a mild degree of oxidative stress, which appears be tolerated by liver cells because their sufficient antioxidant defense capacity to remove the reactive species formed. Additionally, proline also induces metabolic changes in liver, observed here by the increase on glycogen content and higher glucose oxidation. Then, it may be reasonable to conceive that the alterations in parameters of oxidative stress detected in present work, probably do not implicate in liver tissue damage, but demonstrate a process of adaptation this tissue to oxidative stress. Based on these findings and other studies from our laboratory, we hypothesized that some tissues are more affected by hyperprolinemia than others: while hyperprolinemia induces oxidative stress and metabolic alterations in brain, the liver appears to be more resistance to damage by ROS caused by proline. To our knowledge, there are no studies showing the effects of hyperprolinemia on oxidative and metabolic status in liver. Our data reinforce the hypothesis that proline and its interconversions function as a unique mechanism for redox balance [Araujo et al., 2001; Krishnan et al., 2008; Phang et al., 2010; Wyse and Netto, 2011], which may be related to increased levels of this amino acid in hepatic diseases. Also, we cannot rule out that liver, as a central organ for metabolism, fulfill the role to counterbalance the oxidative and metabolic effects of systemic hyperprolinemia.

However, the mechanisms and biological significance of these findings need further studies to be fully understood.

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