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Glutamyl cycle in the rat liver appears to be sex-gender specific

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ABSTRACT

Numerous studies show sexually dimorphic responses of drug metabolizing enzymes in the liver, but it is less clear whether xenobiotic detoxification mediated by glutathione is sex-gender specific. Therefore, we investigated whether sex-gender differences exist in the biosynthesis and metabolism of GSH in the rat liver.

Livers were obtained from Sprague–Dawley rats of both sexes for measurement of glutathione, its precursors and metabolites by capillary electrophoresis, whereas H_2S and malondialdehyde were measured by colorimetric assays. The expression of glutamylcysteine ligase (GCL), the key enzyme in glutathione synthesis was detected by Western blotting and immunohistochemistry.

It was observed that L-methionine, glutathione, taurine and malondialdehyde (a marker of lipid peroxidation) were similar in livers from both sexes, while L-cysteine levels were significantly higher and H_2S was lower in female rat livers. Furthermore, L-methionine and L-cysteine, L-cysteine and glutathione, L-cysteine and taurine were positively associated only in male livers. Finally, the female liver expressed less GCL than the male liver.

These data suggest that the glutamyl cycle in the liver is sexually dimorphic. This difference could be linked to the increased sensitivity of females to drugs and xenobiotics.

1. Introduction

It is well known that there are sex-gender differences with regard to the prevention, epidemiology, evolution, prognosis, therapy and outcome of diseases, although the intrinsic mechanisms of these differences are still unclear (Legato, 2009). Importantly, studies have shown sexually dimorphic responses of the liver, one of the best known involves the expression and activity of hepatic drug metabolizing enzymes (Waxman and Holloway, 2009). Women have been found to be more susceptible than men to drug-induced (including herbal remedies) acute liver failure (Boelsterli and Goldlin, 1991; Miller, 2001; Ostapowicz et al., 2002; Hemieda, 2007; McConnachie et al., 2007). In the case of paracetamol, this sex-gender difference in hepatotoxicity seems to depend on glutamylcysteine ligase (GCL) (Botta et al., 2006; McConnachie et al., 2007) the rate limiting step in glutathione (GSH) synthesis (Griffith, 1999), a tripeptide that is crucial for counteracting oxidative stress, maintaining redox state and xenobiotic detoxification (Meister and Anderson, 1983; Zappacosta et al., 2002). The GCL catalyzes the reaction between L-glutamate and L-cysteine (Wu et al., 2004). This sulfur amino acid, once inside the cell, is largely incorporated into GSH and proteins while a smaller part is degraded into sulfate, H_2S and taurine (Finkelstein and Martin, 1984; Finkelstein et al., 1988; Chen et al., 2004; Lu, 2009).

The inability of most cells to import GSH highlights the importance of *de novo* GSH biosynthesis in maintaining GSH homeostasis. GSH biosynthesis depends on several factors including GCL activity (Lu, 2009), which is physiologically regulated by (a) the availability of L-cysteine (Lu, 2009); (b) non-allosteric feedback competitive inhibition by GSH; (c) aging; (d) hormones such as corticosteroids, insulin and exogenous estrogens that increase GCL expression and GSH levels in the livers of both male and female mice; (e) lipid peroxidation products that may contribute to the rapid activation of GCL (Backos et al., 2010). For most cells, the glutamyl cycle allows GSH to be used as a continuous source of L-cysteine, an important function because L-cysteine can oxidize itself to cystine, thus reducing potentially toxic oxygen free radicals (Lu, 2009).

Because the underlying biological mechanisms of the greater sensitivity of females to chemical-induced liver injury are still not clear, we decided to investigate sex-gender differences related to

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the biosynthesis and metabolism of GSH, which is a crucial detoxification mechanism (Di Simplicio et al., 1984; Lee et al., 2008).

2. Materials and methods

2.1. Animals

Eighteen male and 18 female Sprague–Dawley rats (7 weeks old) were purchased from Harlan, Italy. Rats were housed 2–3 per cage, maintained on a 12 h light/dark cycle and were allowed food and water *ad libitum*. The experimental protocols were carried out in accordance with Italian law (DL 116, 1992) and the NIH principles of laboratory animal care (NIH 80-33, revised 1996). All experimental procedures were approved by the Department of Veterinary Public Health, Food Security and Collegial Organs for the Protection of Health (Ministry of Health) as requested by Italian law.

Animals were euthanized by decapitation, the abdominal cavity was opened to expose the liver, which was rapidly removed, washed, weighed, homogenized in cold PBS, stored at −80 °C and used within 1 month. Part of the liver was used for immunohistochemical staining, being fixed in 4% paraformaldehyde and embedded in paraffin. Total liver protein content was measured by the Lowry method (Lowry et al., 1951).

2.2. Thiol measurement

For thiol analysis, 100 μl of standard or samples were mixed with 10 μl of TBP (10%), vortexed for 30 s and subsequently incubated at 4 °C for 10 min. At the end of incubation 100 μl of 10% trichloroacetic acid (TCA) were added and the mixture vortexed for 10 s and then centrifuged for 10 min at 3000 × *g*. 100 μl of supernatant were mixed with 100 μl of 300 mmol/l Na_3PO_4 at pH 12.5 and with 25 μL of 5-IAF (4.1 mmol/l), and subsequently incubated at room temperature (RT) for 10 min. The mix was diluted 1/100 before being injected for capillary electrophoresis with a laser-induced fluorescence detector (Zinellu et al., 2003).

2.3. Taurine detection

A volume of 50 μl of tissue homogenate was mixed with 50 μl IS homocysteic acid (200 μmol/l) and 100 μl of TCA (10%) were then added to precipitate the proteins. After centrifugation at 3000 × *g* for 5 min, 10 μl of clear supernatant were mixed with 90 μl of 100 mmol/l Na_2HPO_4, pH 9.5 and 11 μl of 15 mmol/l FITC. After 20 min incubation time at 100 °C, samples were diluted 100-fold and injected into a capillary electrophoresis apparatus. Analysis was performed in an uncoated fused silica capillary, 75 μm I.D. and 47 cm length (40 cm to the detection window), injecting 18 nl of sample. Separation was carried out in a 20 mmol/l tribasic sodium phosphate buffer, pH 11.8, 23 °C at normal polarity 22 kV (Zinellu et al., 2009).

2.4. Measurement of H_2S

H_2S was determined using the method described in Hua et al. (2009) with slight modifications. In brief, 1 ml of liver homogenate was mixed with 100 μl of zinc acetate (1%, w/v) and incubated at 37 °C for 10 min. After proteins precipitation with 20% TCA, supernatant was collected by centrifugation (14,000 rpm × 20 min, 4 °C) and diluted twice with distilled water and incubated with N,N-dimethyl-*p*-phenylenediamine sulfate (2 g/l) and ferric chloride ($FeCl_3$, 3 g/l) in HCl 6 N for 20 min at RT followed by a centrifugation at 14,000 rpm for 5 min at 4 °C. The absorbance of the resulting solution was measured at 670 nm and H_2S concentration was calculated using a calibration curve of sodium hydrosulfide in water (100–3.125 μM). H_2S blanks were obtained incubating the supernatant with $FeCl_3$ only. Results are expressed as nmol/mg protein.

2.5. Malondialdehyde (MDA) detection

MDA levels were measured according to Esterbauer and Cheeseman with slight modifications (Esterbauer and Cheeseman, 1990). 200 μl of tissue homogenate were mixed with 200 μl of dilute acetic acid (1:3 in H_2O). 150 μl of 10% sodium dodecylsulphate (SDS) to easily precipitate the proteins and 200 μl of Tris–HCl 50 mM were added to this mixture. This solution was kept in incubation for 10 min at RT and then 500 μl of a solution of thiobarbituric acid (0.75% in dilute acetic acid and NaOH 1 N (1:1) were added. The mixture was boiled for 60 min, quenched in ice (10 min), added with 400 μl of acetic acid (1:3) and then centrifuged at 4 °C at maximum speed for 20 min. The quantification was performed spectrophotometrically at 532 nm by measuring the absorbance produced by the sample. Standards of MDA at known concentration (50–5 μM) were used to obtain a reference curve.

2.6. Western blot analysis of GCL, actin, β-tubulin and GAPDH

Western blot analysis of total actin, β-tubulin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and GCL expression were performed on liver homogenates. 80 μg of protein for each sample were loaded. The reaction was carried out using anti-actin IgG fraction of antiserum developed in the rabbit (A5060, Sigma–Aldrich, Italy), a polyclonal rabbit anti-β-tubulin (2146S, Cell Signalling Technology, DBA Italia, Italy), a monoclonal rabbit anti-GAPDH (2118S, Cell Signalling Technology, DBA Italia, Italy) and a polyclonal rabbit anti-GCLc (sc-2275S, Cell Signalling Technology, DBA Italia, Italy). Specific protein was detected by chemiluminescence reaction (LumiGLO, Cell Signalling Technology, DBA Italia, Italy), followed by densitometric analysis of immunoblot by dedicated software (Labworks).

Western blot analysis showed that total actin and GAPDH expression were significantly higher in females than in males ($P = 0.048$ and $P = 0.017$, respectively; data not shown) whereas β-tubulin expression was significantly lower in female than in male tissue ($P = 0.006$; data not shown) and therefore we were unable to normalize Western blot data for these proteins. The reported differences were in line with those found by others (Perrot-Sinal et al., 2001; Ferguson et al., 2005; Verma and Shapiro, 2006).

2.7. Immunohistochemistry of GCL

Immunohistochemistry was performed using a polyclonal rabbit anti-GCLc (sc-2275S, Cell Signalling Technology, DBA Italia, Italy) and the LSAB+ System-HRP Kit (Dako, Italy).

For antigen retrieval, 3-μm thick sections were heated at intermediate power in a conventional microwave oven in citrate buffer (10 mM, pH 6) for 10 min. The slides were blocked with 3% hydrogen peroxide for 20 min and incubated with primary antibody (1:50) in PBS/BSA 1% overnight at 4 °C, washed, incubated with biotinylated goat anti-rabbit IgG in PBS for 30 min, washed, incubated with streptavidin-HRP reagent for 30 min, washed, and incubated with diaminobenzidene substrate for 2 min. Finally, the slides were counterstained with hematoxylin, dehydrated in graded ethanol washes and washed in xylene. Liver sections were examined in a blinded manner under light microscopy (DM4000B, Leica).

2.8. Statistical analysis

Each sample was determined in duplicate and statistical analysis of data was done by comparing parameters obtained from male and female rat livers. To assess the distribution of samples