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Original Research Article

Investigating the influence of taurine on thiol antioxidant status in Wistar rats with a multi-analytical approach☆

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ABSTRACT

Taurine (2-aminoethanesulfonic acid) is an organic acid widely distributed in animal tissues. It is involved in many physiological processes. Thus, it is widely discussed especially due to its antioxidant properties. In this study, we focused on the effect of taurine supplementation on the concentration of antioxidants in blood plasma and erythrocytes of Wistar rats. Taurine was applied in feed mixture in the dosage of 0, 1, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500 and 4000 mg/kg. We monitored both enzymatic and non-enzymatic antioxidants – glutathione peroxidase, glutathione reductase, and superoxide dismutase and reduced/oxidized glutathione and metallothionein. Using three different methods 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Ferric Reducing Antioxidant Power (FRAP) and free radicals, we determined antioxidant capacity. In addition, we monitored levels of uric acid and glucose. Our results revealed significant changes in both enzymatic and non-enzymatic parameters with the increasing taurine supplementation.

Introduction

Taurine is present in the majority of the human tissues in low concentrations (Awapara, 1950; Roe and Weston, 1965; Davison and Kaczmarek, 1971; Baskin and Dagirman, 1973; Fukuda et al., 1982; Sturman, 1993; Bouckenooghe et al., 2006). In addition, it occurs in foodstuffs, especially in seafood (Silva et al., 2011) and meat (Lau et al., 1990; Schmid, 2009). Moreover, taurine has significant anti-inflammatory

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properties (Marcinkiewicz, 2009) and participates in different physiological processes as it stabilizes cell membranes (Condron et al., 2010), regulates fatty tissues metabolism (Ueki and Stipanuk, 2009) and levels of calcium ions in blood (Ribeiro et al., 2010). As it follows from these facts, taurine is important in the prevention of cardiovascular diseases (Huxtable and Bressler, 1974; Hayes et al., 1975; Huxtable and Chubb, 1977; Thurston et al., 1981; Pion et al., 1987), obesity, type II diabetes and cancer (Zulli 2011; Caletti et al., 2012; Das and Sil, 2012; Feng et al., 2012; Giles et al., 2012; Ribeiro et al., 2012; Taranukhin et al., 2012; Zinellu et al., 2012). Due to biological functions of taurine, this compound is, moreover, intensively studied due to its possibility to regulate oxidation stress (Aruoma et al., 1988; Gurer et al., 2001; Hanna et al., 2004; Ghosh et al., 2009; Marcinkiewicz, 2010; Srivastava et al., 2010; Wu and Prentice, 2010). Protective role of taurine against oxidative stress in murine erythrocytes has been demonstrated (Sinha et al., 2008; Zitka et al., 2010; Hynek et al., 2012a). Treatment with taurine before cadmium intoxication prevented the toxin-induced oxidative impairments in the erythrocytes of the experimental animals (Sinha et al., 2008). Development and function of skeletal muscle (Ishikura et al., 2011; Ito and Azuma, 2011; Silva et al., 2011), the retina (Zeng et al., 2009) and the central nervous system (Wu and Prentice, 2010) may be regulated by taurine. Beside metabolic regulation, taurine also plays an important role in innate immunity (Nagl et al., 2000) and is directly related to antioxidant properties in clinical (Zulli, 2011; Shivananjappa and Muralidhara, 2012), toxicological (Turna et al., 2011; Yildirim and Kilic, 2011; Shao et al., 2012b) and oncological studies (Henderson et al., 2001; Gottardi and Nagl, 2010; Shalby et al., 2011; Das et al., 2012).

Based on the aforementioned papers investigating the impact of taurine on an organism, complex experiments allowing the understanding of the regulation of homeostasis during oxidative stress are still missing. For this reason, we aimed our experiment at the investigation of both enzymatic and non-enzymatic antioxidant systems, such as glutathione peroxidase, glutathione reductase, and superoxide dismutase and reduced/oxidized glutathione and metallothionein. Our work brings new knowledge about the role of taurine in homeostasis during oxidative stress. In addition, its supplementation is discussed with the oxidative stress itself. A wide scale of taurine doses was chosen to cover expected amounts taken by humans from food sources.

Materials and methods

Chemicals

Taurine and other chemicals were purchased from Sigma Aldrich Chemical Corp. (St. Louis, Missouri, USA), unless noted otherwise. The stock solution of 800 mM taurine was prepared by dilution with Milli Q distilled water. The deionised water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

Laboratory animals and their keeping

In total, 60 males of Wistar rats (Biotest, Konárovice, Czech Republic) were used in the experiment. At the beginning of experiment, the animals were 28 days old and differences in body weight were in a range ±2.5 g. The animals were kept in an air conditioned room with stable temperature of 23 ± 1 °C, humidity 60%, light period 12 h with light intensity up to 200 lx, content of CO_2 up to 0.25%, NH_3 up to 0.0025%. Food and water were provided ad libitum during the whole experiment. All animals were in good condition and no aberrations in behavior and development were observed. The experiment was approved and supervised by ethical committee, Mendel University in Brno, Brno, Czech Republic.

Experimental design

Animals were divided into 12 experimental groups (5 males in each group). They were supplemented with taurine through feed mixture, which was enriched by taurine in the dosage of 0, 1, 250, 500, 750, 1000, 1500, 2000, 2500, 3000, 3500 and 4000 mg/kg per four weeks. Each animal consumed 35 g of feed mixture per day in average. Animals were put to death after 28 days. Blood sample was obtained by the puncture to heart into heparin-coated micro test tubes (Dispolab, Germany). Subsequently the centrifugation step followed (700 × *g*, 10 min) and components (erythrocytes, blood plasma) were analyzed.

Taurine determination

Preparation of erythrocytes sample

A sample (0.1 g of erythrocytes, fresh weight) was deeply frozen by liquid nitrogen. After it, 1 ml of 0.2 M phosphate buffer (pH 7.0) was added. Sample was vortexed for 15 min and centrifuged at 24,000 × *g* (20 min at 4 °C). A volume of 380 μl of supernatant was taken and mixed with 20 μl of 100% trifluoroacetic acid (TFA). Sample prepared like this was further centrifuged (24,000 × *g*, 20 min, 4 °C). After it, a volume of 237 μl was taken and it was mixed with 12.5 μl of 100% TFA. After centrifugation (24,000 × *g*, 20 min, 4 °C), supernatant (200 μl) was mixed with 200 μl of diluting buffer (thiodiglycol 5 ml/l, citric acid 14 g/l, sodium chloride 11.5 g/l, sodium azide 0.10 g/l). Finally, this sample was prepared for the analysis using the AAA 400 apparatus.

Preparation of blood plasma sample

A sample of blood plasma (380 μl) was mixed with 20 μl of 100% TFA.Sample prepared like this was further centrifuged (24,000 × *g*, 20 min, 4 °C), after it a volume of 237 μl was taken and it was mixed with 12.5 μl of 100% TFA. After centrifugation (24,000 × *g*, 20 min, 4 °C), supernatant (200 μl) was mixed with 200 μl of diluting buffer (thiodiglycol 5 ml/l, citric acid 14 g/l, sodium chloride 11.5 g/l, sodium azide 0.10 g/l). Finally, this sample was prepared for the analysis using the AAA 400 apparatus.

Taurine analysis

For determination of taurine, an ion-exchange liquid chromatography (Model AAA 400, Ingos, Czech Republic) with

post-column derivatization with ninhydrin and VIS detector was used. A glass column with inner diameter 3.7 mm, and 350 mm in length was filled manually with a strong cation exchanger in sodium cycle LG ANB (Ingos) with approximately 12 μm particles and 8% porosity. The glass column was tempered within the range from 35 to 95 °C. A double-channel VIS detector with the volume of flow cuvette of 5 μl was set to two wavelengths – λ = 440 and 570 nm. A solution of ninhydrin (Ingos) was prepared in the mixture of 75% (v/v) metylcelosolve (Ingos) and 25% (v/v) 4 M acetate buffer (pH 5.5). Stannous chloride ($SnCl_2$, Lachema, Czech Republic) was used as a reducing agent. The prepared solution of ninhydrin was stored under an inert atmosphere (N_2) and cooled at 4 °C. Elution of amino acids was performed according to program using a discontinuous gradient of elution buffers of different ionic strength and pH, and also using a temperature gradient. During the analysis, the flow rate was 0.3 ml/min under the pressure of 4.5–6 MPa. Temperature was set to 120 °C in the heat generator. Temperature was set to 60 °C in the column.

Determination of reduced and oxidized glutathione

Reduced (GSH) and oxidized (GSSG) glutathione were assayed using high performance liquid chromatography with electrochemical detection (HPLC-ED). Two solvent delivery pumps (Model 582 ESA Inc., Chelmsford, MA, USA) operating in a range of 0.001–9.999 ml/min, column Zorbax eclipse AAA C18 (150 μm × 4.6 μm; 3.5 μm particle size; Agilent Technologies, Santa Clara, CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA) with three cells including carbon porous working and auxiliary electrodes and Pd/H_2 reference electrode were used for assay purposes. The assayed solution (20 μl) was injected using autosampler (Model 542 HPLC, ESA) with carousel adjusted at 8 °C. The column was heated at 32 °C. Mobile phase consisted of 80 mM TFA (A) and methanol (B). The compounds of interest were separated by the following linear gradient: 0 → 1 min (3% B), 1 → 2 min (10% B), 2 → 5 min (30% B), 5 → 6 min (98% B). Mobile phase flow rate of 1 ml min^{-1} and working electrode potential 900 mV were used. Time of analysis was 20 min.

Determination of total proteins, glutathione peroxidase, glutathione reductase, superoxide dismutase, antioxidant capacity, glucose and uric acid

Spectrophotometric measurements were carried out using an automated chemical analyser BS-400 (Mindray, China). It is composed of a cuvette space tempered to 37 ± 1 °C, a reagent space with a carousel for reagents (tempered to 4 ± 1 °C), a sample space with a carousel for preparation of samples and an optical detector. Transfer of samples and reagents is provided by a robotic arm equipped with a dosing needle (error of dosage up to 5% of volume). Cuvette contents are mixed by an automatic mixer including a stirrer immediately after addition of reagents or samples. Contamination is reduced due to its rinsing system, including rinsing of the dosing needle as well as the stirrer by MilliQ water. For detection itself, the following range of wavelengths can be used – 340, 380, 412, 450, 505, 546, 570, 605, 660, 700, 740 and 800 nm.

Determination of total protein content

Determination of total protein content using Bradford method is described in the following paper (Bradford, 1976). Briefly, reagent Coomassie Brilliant blue G-250 (0.01% Coomassie Brilliant Blue G-250, 4.7% CH_3CH_2OH, 8.5% H_3PO_4, v/v) in volume of 190 μl was pipetted into cuvette. Further, sample (10 μl) was added. Mixture was incubated at 37 °C for 10 min. Absorbance was measured at 595 nm, reagent itself was used as a blank.

Determination of glutathione reductase (GR) activity

Determination of GR activity was performed according to Sochor et al. (2012b). A Glutathione Reductase Assay Kit (Sigma Aldrich) was used for glutathione reductase (GR) activity determination. GR assay was based on the reduction of glutathione (GSSG) by NADPH in the presence of glutathione reductase. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GR activity. Reagents R1 and R2 were prepared by dissolving in an assay buffer (100 mM potassium phosphate buffer, pH 7.5, with 1 mM EDTA). A 260 μl volume of the reagent R1 (1.15 mM oxidized glutathione in the assay buffer) was poured with 10 μl of sample and 30 μl volume of reagent R2 (1 mM NADPH in GR assay buffer) into a plastic cuvette. The decrease in absorbance was measured at 340 nm for 126 s using kinetic program. The same device and conditions were used for GR as in the case of total proteins assay.

Determination of glutathione peroxidase (GPX) activity

Glutathione Peroxidase Cellular Activity Assay Kit (CGP1, Sigma Aldrich) was used for GPX assay. The kit uses an indirect determination method. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPX, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH. The decrease in NADPH absorbance measured at 340 nm during the oxidation of NADPH to $NADP^+$ is indicative of GPX activity, since GPX is the rate limiting factor of the coupled reactions. A 260 μl volume of reagent R1 (0.3 mM NADPH in an assay reagent) was pipetted into a plastic cuvette with subsequent addition of 10 μl of sample and after mixing, a 30 μl volume of reagent R2 (3 mM tert-butyl hydroperoxide), which starts the reaction, was added to the cuvette. The decrease in absorbance was measured at 340 nm using kinetic program for 126 s. The spectrophotometer calculates GPX activity according to a calibration curve.

Determination of superoxide dismutase (SOD) activity

Kit 19160 SOD (Sigma Aldrich) was used for assay of superoxide dismutase (SOD). Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion ($O_2^{\bullet -}$) into hydrogen peroxide and molecular oxygen. The kit uses an indirect determination method. SOD Assay Kit utilizes Dojindo's tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a

superoxide anion. Since the absorbance at 450 nm is proportional to the amount of superoxide anion, the SOD activity as an inhibition activity can be quantified by measuring the decrease in the color development at 450 nm. A 200 μl volume of reagent R1 (WTS solution 20 times diluted with buffer) was pipetted into a plastic cuvette and agent was incubated at 37 °C for 108 s. Afterwards, a 20 μl volume of sample was pipetted and in 378 s the reaction was started by adding a 20 μl volume of reagent R2 (enzyme solution 167 times diluted with buffer). It was incubated for 72 s and then absorbance was measured at λ = 450 nm. Kinetic reaction was measured for 180 s and absorbance was read every 9 s.

Determination of antioxidant activity by the ABTS test

The ABTS radical method is one of the most used assays for the determination of the concentration of free radicals. It is based on the neutralization of a radical-cation arising from the one-electron oxidation of the synthetic chromophore 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS): $ABTS^{\bullet}$ – e-$ABTS^{\bullet}+$. This reaction is monitored spectrophotometrically by the change of the absorption value. Procedure for the determination was taken from publication of Sochor et al. (2010). A 150 μl volume of reagent (7 mM $ABTS^{\bullet}$ (2,2′-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) and 4.95 mM potassium peroxodisulphate) was poured with 3 μl of sample. Absorbance was measured at 660 nm. For calculating of the antioxidant activity, difference between absorbance at the last 10th minute and second minute of the assay procedure was used.

Determination of antioxidant activity by the FRAP method

The FRAP method (Ferric Reducing Antioxidant Power) is based on the reduction of complexes of 2,4,6-tripyridyl-s-triazine (TPTZ) with ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), which are almost colorless, and eventually slightly brownish. This chemical forms blue ferrous complexes after its reduction. Procedure for the determination was taken from publication of Sochor et al. (2010). Reagent preparation: Solution 1: 10 mmol l^{-1} solution of TPTZ in 40 mmol l^{-1} of hydrochloric acid. Solution 2: 20 mmol l^{-1} solution of ferric chloride hexahydrate in ACS water. Solution 3: 20 mmol l^{-1} acetate buffer, pH 3.6. These three solutions (TPTZ, $FeCl_3$, acetate buffer) were mixed in a 1:1:10 ratio. A 150 μl volume of reagent was injected into a plastic cuvette with subsequent addition of a 3 μl of sample. Absorbance was measured at 605 nm for 10 min. Difference between absorbance at the last 10th minute and second minute of the assay procedure was used for calculating of the antioxidant activity.

Determination of antioxidant activity by the Free Radicals method

The Free Radicals method is based on ability of chlorophyllin (the sodium-copper salt of chlorophyl) to accept and donate electrons with a stable with a stable change of maximum absorption. This effect is conditioned by an alkaline environment and the addition of catalyst. Procedure for the determination was taken from publication of Pohanka et al. (2012c). A 150 μl volume of reagent (chlorophyllin extract, bicarbonate buffer and catalyst) was injected into a plastic cuvette with subsequent addition of a 6 μl sample. Absorbance was measured at 450 nm in the second minute of assay and the 10th minute. Difference of the two values of absorbance was considered as an outputting value.

Assay of metallothionein

In total, 200 μl of sample was analyzed using differential pulse voltammetry (DPV) based on Brdicka reaction. The assay was performed in compliance with the previously described protocol (Adam et al., 2008, 2010b; Sochor et al., 2010; Hynek et al., 2012b). 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Zofingen, Switzerland), standard reaction cell with three electrodes and cooled sample holder (4 °C) were used for assay purposes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 served as a working electrode. A silver/silver chloride electrode was the reference and platinum electrode was auxiliary electrode. The sample was injected in the reaction cell containing 1800 μl of Brdicka supporting electrolyte [1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer consisting of $NH_3(aq) + NH_4Cl$, pH = 9.6]. The DPV assay parameters were following: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude −250 mV. All experiments were carried out at 4 °C (Julabo F12 cooler).

Determination of uric acid

A volume of 200 μl of R1 reagent (Greiner, Germany, 1 mM 2,4,6-tribromo-3-hydroxybenzoic acid in 100 mM phosphate buffer, pH 7.0) was mixed with 4 μl of sample and 50 μl of R2 reagent (Greiner, Německo, 0.3 mM 4-aminoantipyrine, 10 μM $K_4[Fe(CN)_6]$, peroxidase 2 kU/l, uricase 30 U/l in 100 mM phosphate buffer, pH 7.0). Absorbance was measured for 6 min at λ = 546 nm. Absorbance of R1 reagent mixed with sample and absorbance of mixture after 6 min of incubation were used for the calculation of uric acid concentration.

Determination of glucose

A volume of 200 μl of reagent (0.1 M phosphate buffer pH 7.5, 0.75 mM phenol, 0.25 mM 4-aminoantipyrine (4-AAP), glucose oxidase ≥ 15 kU/l, peroxidase ≥ 1.5 U/l) was pipetted into plastic cuvette. Subsequently, a volume of 20 μl of sample was added. Absorbance was measured for 10 min at λ = 505 nm. Values of absorbance of reagent and mixture after 10 min incubation were used for the calculation the glucose concentration.

Descriptive statistics

Data were processed using MICROSOFT EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± standard deviation (SD) unless noted otherwise (EXCEL®). The detection limits (3 signal/noise, S/N) were calculated according to Long and Winefordner (1983), whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

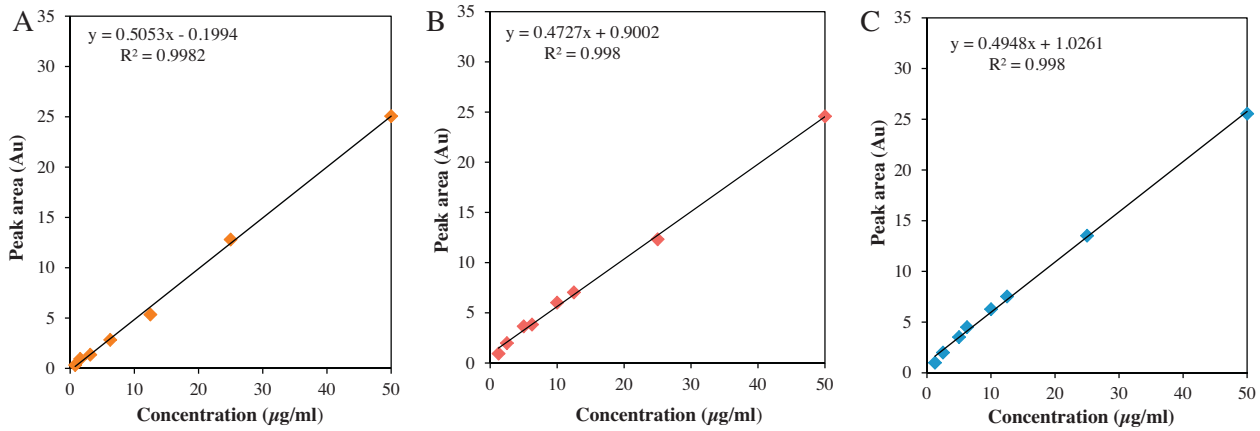

Fig. 1 – Calibration curve of taurine in (A) sodium citrate diluting buffer, (B) in rat plasma and (C) in erythrocytes.

Results and discussion

Application of taurine did not cause any observable pathology process or significant alteration in the animals' behavior. Weight of the animals was stable for the whole experiment with small alterations, which were insignificant. When the animals were anatomized, no difference in organs from the examined animals and animals treated with taurine was found. Calibration curves of taurine were prepared in different matrixes such as diluting sodium citrate buffer, rat plasma and erythrocytes. They were linear up to the concentration of taurine 50 $\mu g\,ml^{-1}$. They all had similar equations and coefficient of determination 0.998 as shown in Fig. 1.

Taurine levels were significantly increased in both erythrocytes and blood plasma. In dependence on the taurine supplementation, content of taurine increased with the increasing taurine dosage, and the increase was revealed in both plasma Fig. 2A and erythrocytes Fig. 2B.

GSH and GSSG levels

In the aforementioned effects of taurine on the body, we mentioned disparate biological effects. The fact that taurine acts as an antioxidant is important for understanding of taurine effects. Extensive intake of taurine can improve total antioxidant capacity in the body. We did the experiment on Wistar rats with different dose of applied taurine. The antioxidant properties were well responding to the taurine dose and the alterations in the assessed parameters were observed in a dose-dependent manner. Among the antioxidants, thiol group(s) containing compounds has an important role in the body. Moreover, they can participate in the detoxification of heavy metals (Kleckerova et al., 2011; Gumulec et al., 2013).

Contents of GSH and GSSG were determined by HPLC. The method is suitable to precise determination of the markers without sensitivity to interference. We revealed that both GSH and GSSG increased with dose of taurine in a dose-dependent manner (Fig. 3A and B, respectively). In the plasma, the increase in both GSH and GSSG levels were quite extensive as GSH increased to about 223% (compare to controls) in animals exposed to taurine in the dosage of 1000 mg/kg and GSSG decreased to about 9% at the same dose of taurine. Increase of taurine dose above 1000 mg/kg had only moderate effect on GSH and GSSG. GSH increased from 323% to 382% when taurine dose was up to 4000 mg/kg, and GSSG was decreased only moderately compared to GSH (Fig. 3). It decreased from 9% to 15%. Compared to the plasma, taurine has only moderate effect on blood cells. GSH levels in blood cells had (similarly to blood plasma) increasing tendency with the increasing content of taurine in feed. The increase was distinct up to

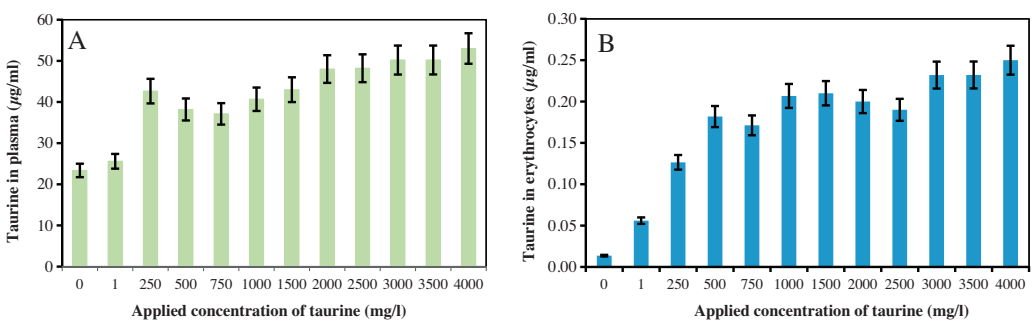

Fig. 2 – Content of taurine in (A) rat plasma (10 μl) and (B) erythrocytes (0.1 g). Ionex HPLC was used for determination of taurine. The injection was 100 μl with the volume of flow cuvette of 5 μl and at wavelength λ = 440 nm; *n* = 3.

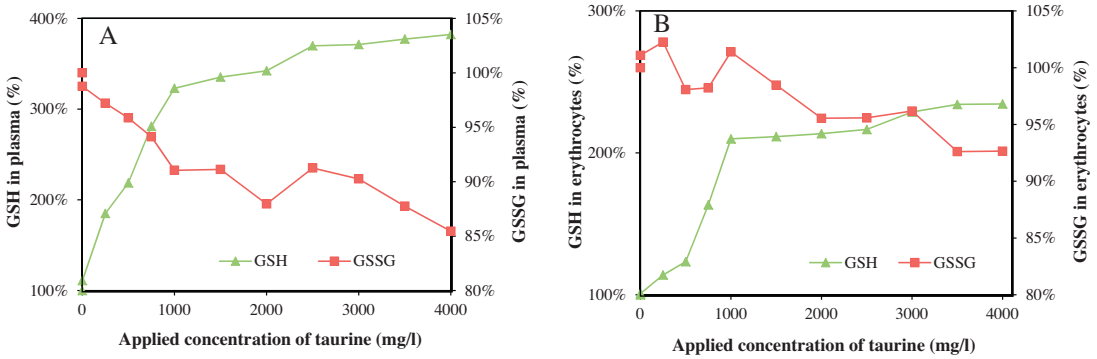

Fig. 3 – Content of GSH and GSSG in (A) plasma (10 μl) and (B) erythrocytes (0.1 g). HPLC ED was used for determination of GSH and GSSG. The injection was 10 μl at the applied potential of 900 mV; *n* = 3.

the taurine concentration of 1000 mg/kg (for 210% compared to control), from the concentration of 1000 mg/kg only moderate (to 234% in the concentration of 4000 mg/kg). GSSG in blood cells was not extensively influenced as well. GSSG levels had decreasing tendency with the increasing taurine concentration. In the highest applied concentration (4000 mg/kg), the decrease was only for 7%. The moderate effect on blood cells compared to blood plasma can be explained by limited distribution of taurine into the cells and its low intracellular concentration. There are some papers focused on the connection between cysteine, glutathione and metallothionein. Ueki et al., who investigated the compensation of hepatic taurine in the mice with liver-specific knockout of cysteine deoxygenase, showed that taurine and hypotaurine are important in the control of body cysteine levels (Ueki et al., 2012). Oxidative degradation of cysteine that predominates when cysteine is in an excess leads to the production of the taurine and sulphate (Stipanuk and Ueki, 2011). On the other hand, cysteine (together with glutamic acid and glycine) serves as a building stone of glutathione that has significant protective and antioxidant properties. In this light, the increased plasmatic level of GSH is not surprising. Taurine was reported to be able to induce production of GSH in disparate organs (Anand et al., 2011). This induction is connected with the protective effect of taurine on glutathione and glutathione-dependent enzymes (Pushpakiran et al., 2004). On the other hand, glutathione and glutathione derivatives are modulated by taurine, which means that all these compounds have protective and modulatory effects in some tissues (Janaky et al., 2008). We determined accumulation of GSH in blood plasma under taurine treatment. The presence in blood can be a consequence of accumulation of GSH in organs and followed leaking into the plasma. In addition, the pool of glutathione may be preserved due to the antioxidant properties of taurine and its ability to conjugate xenobiotics (Emudianughe et al., 1983).

Assay of MT

MT is an intracellular protein rich for cysteine residua (Eckschlager et al., 2009; Krizkova et al., 2009; Adam et al., 2010a; Babula et al., 2010, 2012; Ryvolova et al., 2011). It is both antioxidant being able to undergo to oxidized form and a chelator of heavy metals (Masarik et al., 2011, 2012a; Sochor et al., 2012a). Content of MT was determined using an electrochemical device and Brdicka reaction. The results were recalculated to milligram of protein and they are introduced in Fig. 4. Content of MT increased with the increasing taurine

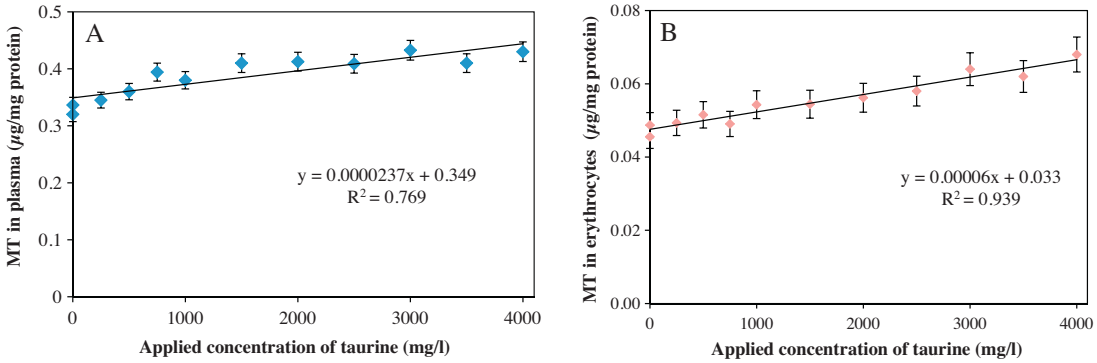

Fig. 4 – Content of MT in (A) plasma and (B) erythrocyte samples. DPV Brdicka reaction was used for determination of MT. DPV parameters were as follows: initial potential of −0.7 V, end potential of −1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude 250 mV; *n* = 3.

dosage, and the increase was revealed in both plasma and blood cells (Fig. 4A and B, respectively). The increase of MT content was in a linear manner with the increasing dose of taurine. In the plasma samples, the increase was described by an equation $y = 0.0000237x + 0.349$ and coefficient of determination was relatively high ($R^2 = 0.769$), which indicates strong correlation. Erythrocytes had linear correlation for MT to taurine as well. The correlation in the blood cells samples is described by an equation of $y = 0.00006x + 0.033$. Coefficient of determination was higher in erythrocytes than in the plasma samples ($R^2 = 0.939$). The findings about correlation of MT to taurine are surprising and not widely investigated in the current literature. This fact indicates possible connection between taurine and metallothionein. However, knowledge is almost missing and this connection must be further investigated. On the other hand, the fact that MT can be induced by high doses of taurine can be important in therapy of heavy metals as MT is involved in detoxification of the metals. Significant changes in erythrocyte metallothionein in response to dietary zinc uptake were observed by Thomas et al. (1992) or Sullivan et al. (1998).

On the other hand, zinc is heavy metal of which excess may cause oxidative stress. Reduced zinc toxicity by dietary taurine supplementation in male Wistar rats was observed by Yeh et al. (2011). In the light of above-mentioned fact, we can conclude that the protective effect of metallothionein that is involved in the heavy metals detoxification may be supplemented and supported by taurine and thus the levels of MT remain unaltered, or higher. The role of taurine in connection with zinc is discussed in some tissues, especially in retina (Lima et al., 2004; Nusetti et al., 2005), so, these tissues may provide the great model in the further investigation of connection between taurine and metallothionein in future. Though data about possible connection between MT and taurine are scared, some conclusions can be done from papers where the both markers were assayed. This is in connection with Erikson and Aschner, whose revealed toxicity mechanisms of manganese (Erikson and Aschner, 2002). They proved different effect of manganese on *TauT* expression, the gene for taurine transporter, and MT. While *TauT* was expressed in rat astrocytes, mRNA for MT was significantly reduced. In another experiment, MT was found to be able to recover taurine level after burden of astrocytes by methylmercury (Vitarella et al., 1996). Sinha et al. (2009) observed protective effect of orally administered taurine on cadmium-induced oxidative stress in murine liver. Taurine protective effect was connected also with the alterations in metallothionein levels. Owing to results reported in our work, we demonstrate positive regulatory effect of exogenous taurine on MT level. The application of taurine can improve resistance of the body on disparate toxicological insults. On the other hand, more specialized experiment should be done to reveal possible close connection between taurine and metallothionein.

Assay of enzymes acting as high molecular antioxidants

Antioxidant enzymes or high molecular weight antioxidants are the group of compounds involved in the protection against free radicals. They are necessary in maintenance of oxidative homeostasis within cells, tissues and the whole body. The antioxidant enzymes are expressed whenever an oxidative insult appears as they are readily to suppress it. SOD, CAT, GR and GPX are probably the most important antioxidant enzymes found in the body. SOD catalyses dismutation of superoxide into less toxic products. When superoxide presented in an excess level, SOD become expressed. Beside the presence of direct radicals, release of GSH, cysteine or dithiothreitol leads to the production of SOD as well (Choi et al., 2008).

The levels of GR, GPX and SOD determined in our experiments are shown in Fig. 5. The activities were related to one milligram of protein. Taurine caused increase in SOD activity in a dose-dependent manner (Fig. 5A). On the other hand, this increase was from 90 μmol/min/mg in the controls and up to approximately 120 μmol/min/mg in the highest dose of taurine. The effect of taurine supplementation on SOD levels were investigated especially by Nonaka et al. (1999, 2000, 2001). Taurine prevents down-regulation of extracellular expression of SOD mediated by homocysteine (Nonaka et al., 1999, 2000). We can suppose that taurine supplementation, which led to the increase of taurine in blood plasma and erythrocytes, caused up-regulation in SOD expression and thus increase in SOD levels in monitored blood plasma/erythrocytes. GR seems to be insensitive to taurine and its activity was not significantly altered due to the taurine supplementation (Fig. 5B). In the contrary to GR, taurine caused significant increase in GPX activity (Fig. 5B). The

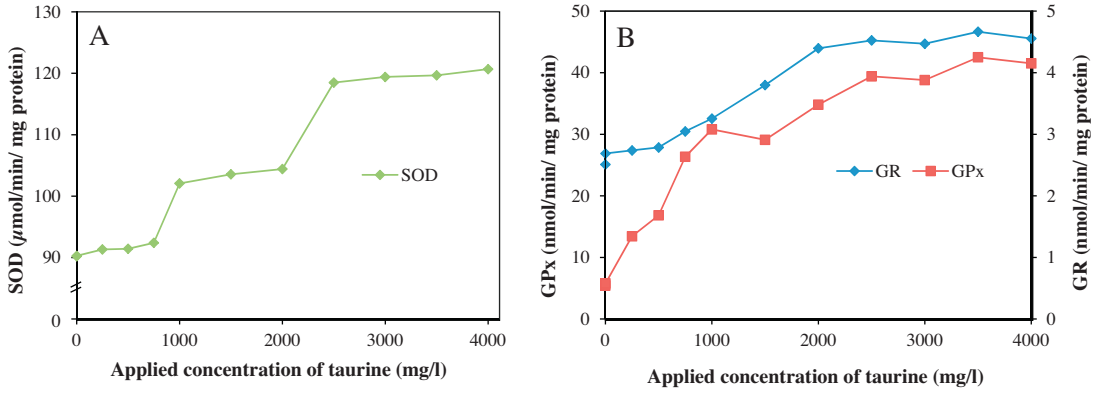

Fig. 5 – Activities of (A) SOD (absorbance at 450 nm, 180 s) in blood cells; *n* = 3 and (B) GR (absorbance at 340 nm, 126 s) and GPX (absorbance at 340 nm, 126 s).

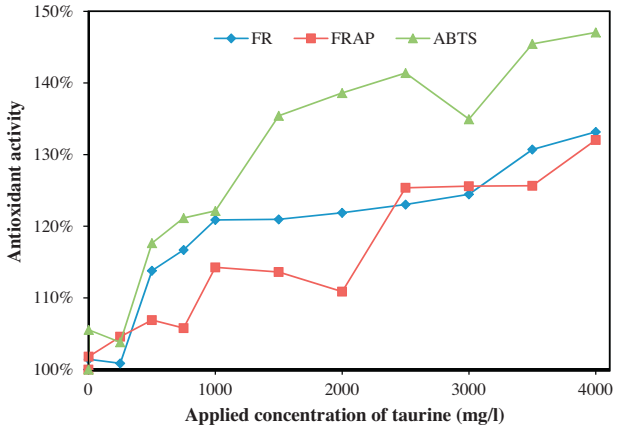

Fig. 6 – Levels of low molecular weight antioxidants in blood cells assayed by FR (6 μl sample, absorbance at 450 nm, 10 min), FRAP (3 μl sample, absorbance at 605 nm, 10 min), and ABTS (3 μl sample, absorbance at 660 nm, 10 min) tests. The results are expressed as percent of the control value (control variant presented 100%); *n* = 3.

increase was in a dose-dependent manner. The highest dose of taurine caused almost ten times increase in GPX activity compared to the control animals. The findings about SOD, GR and GPX confirms the earlier conclusions made by scientists based on the taurine analysis in organs of laboratory animals. They proved up-regulatory effect of taurine on SOD and GPX, whereas GR was not extensively affected (Sahin et al., 2011; Abbasoglu et al., 2012).

Assay of antioxidant activity

Antioxidant activity represents the basic representation of low molecular weight antioxidants total level. Disparate analytical tools are suitable for the assay purposes as obvious from cited papers (Masarik et al., 2012b; Pohanka et al., 2012a,b; Sochor et al., 2013). For the experimental purposes, we chose FR, FRAP and ABTS as the most reliable photometric methods for assay of low molecular weight antioxidants. The results were recalculated to the antioxidant activity of a standard compound trolox. The experimental data are introduced in Fig. 6. It is obvious that the low molecular weight antioxidants were increased in a dose-dependent manner. In addition, this finding is confirmed by all of the used analytical methods. When compared the value received by assay of samples from animals exposed to the higher doses of taurine and the control untreated animals, we can see significant enhancement of the antioxidants activity. FR value was increased for about 34%, FRAP for about 33%, and ABTS for about 47%. Regulation of antioxidants content by taurine cannot be explained by a simple phenomenon. It is probably a complicated multifactorial process, in which regulation of basal metabolism and expression of genes for certain enzymes cause enhancement of antioxidants levels. The findings well corresponds to the cited papers in which antioxidant effect of taurine was revealed (Agackiran et al., 2012; Ji et al., 2012; Shao et al., 2012a; Tsounapi et al., 2012).

Assay of glucose and uric acid

Owing to glucose, we did not found significant involvement of taurine in glycaemia (Fig. 7A). Though plasmatic level of glucose was slightly decreased (for about 7% compared to controls), the alterations in glucose level are small to make a valid conclusion. However, this result is quite surprising because we expected more extensive impact of taurine on glycaemia. The effect of taurine on glycaemia was introduced in comprehensive review (de la Puerta et al., 2010). Team of Japanese scientists (Ishikura et al., 2008) monitored levels of glucose in volunteers during prolonged exercise. Supplementation with taurine led to the more stable glycaemia compared to non-supplemented group of volunteers. Moreover, the study of the taurine effect carried out on the streptozocin-induced diabetic rats revealed their prolonged survival and reduced glycaemia levels that was in direct connection with taurine supplementation (Di Leo et al., 2000). These results suggest that taurine supplementation can maintain the blood

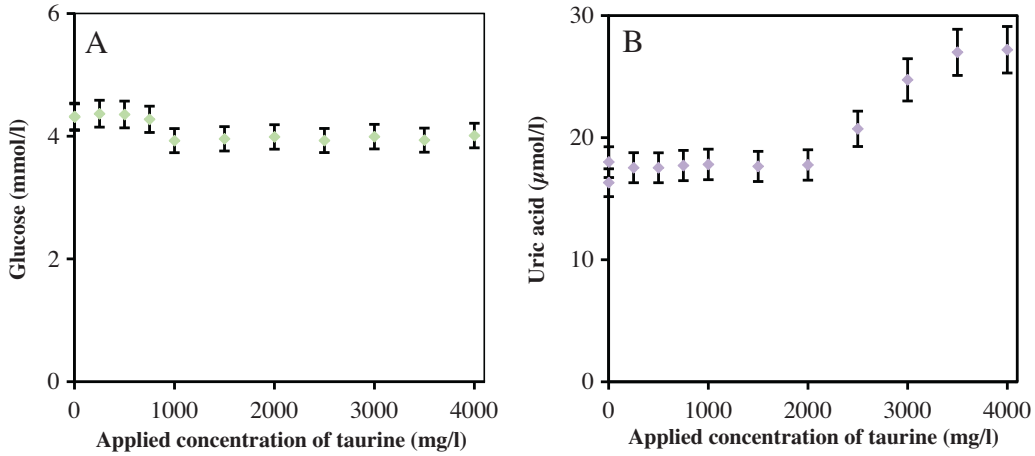

Fig. 7 – Assay of (A) glucose (20 μl sample, absorbance at 505 nm, 10 min) and (B) uric acid (4 μl sample, absorbance at 546 nm, 6 min); *n* = 3.

Table 1 – Correlation of the tested markers. Mathematical analysis carried out by program Statistica (Czech Republic).

	UA	FR	FRAP	ABTS	SOD	GR	GPx	GSSG	GSH	TAU	MT
Gl	−0.639	0.789	0.892	0.855	0.940	0.948	0.852	0.785	0.796	0.802	0.749
UA	×	−0.937	−0.747	−0.884	−0.705	−0.761	−0.912	−0.943	−0.934	−0.794	−0.658
FR		×	0.893	0.963	0.867	0.887	0.963	0.954	0.964	0.846	0.761
FRAP			×	0.886	0.977	0.901	0.904	0.853	0.870	0.854	0.794
ABTS				×	0.893	0.955	0.953	0.950	0.955	0.867	0.787
SOD					×	0.951	0.906	0.844	0.869	0.832	0.767
GR						×	0.932	0.900	0.909	0.870	0.794
GPx							×	0.983	0.977	0.908	0.782
GSSG								×	0.990	0.908	0.800
GSH									×	0.884	0.777
TAU										×	0.821

glucose concentration during prolonged exercise through enhanced catecholamine sensitivity or reduce glycaemia in experimentally induced diabetes. Interesting clinical and epidemiological studies provide evidence on the usefulness of taurine supplementation both in the prevention and treatment of diabetes and its complications (Franconi et al., 1995; Carneiro et al., 2009; Bianchi et al., 2012). Therefore, uric acid in plasma was the next parameter monitored in the examined plasma (Fig. 7B). Uric acid is one of the most presented antioxidant in plasma (Barros et al., 2012). Uricemia was increased in a dose-dependent manner. It increased from 16 μmol/l (controls) to 27 μmol/l in the animals supplemented by the highest dose of taurine. Increase in uric acid level under taurine supplementation was observed by Feuer et al. (1981), who orally supplemented chickens by glutaurine for 4 weeks.

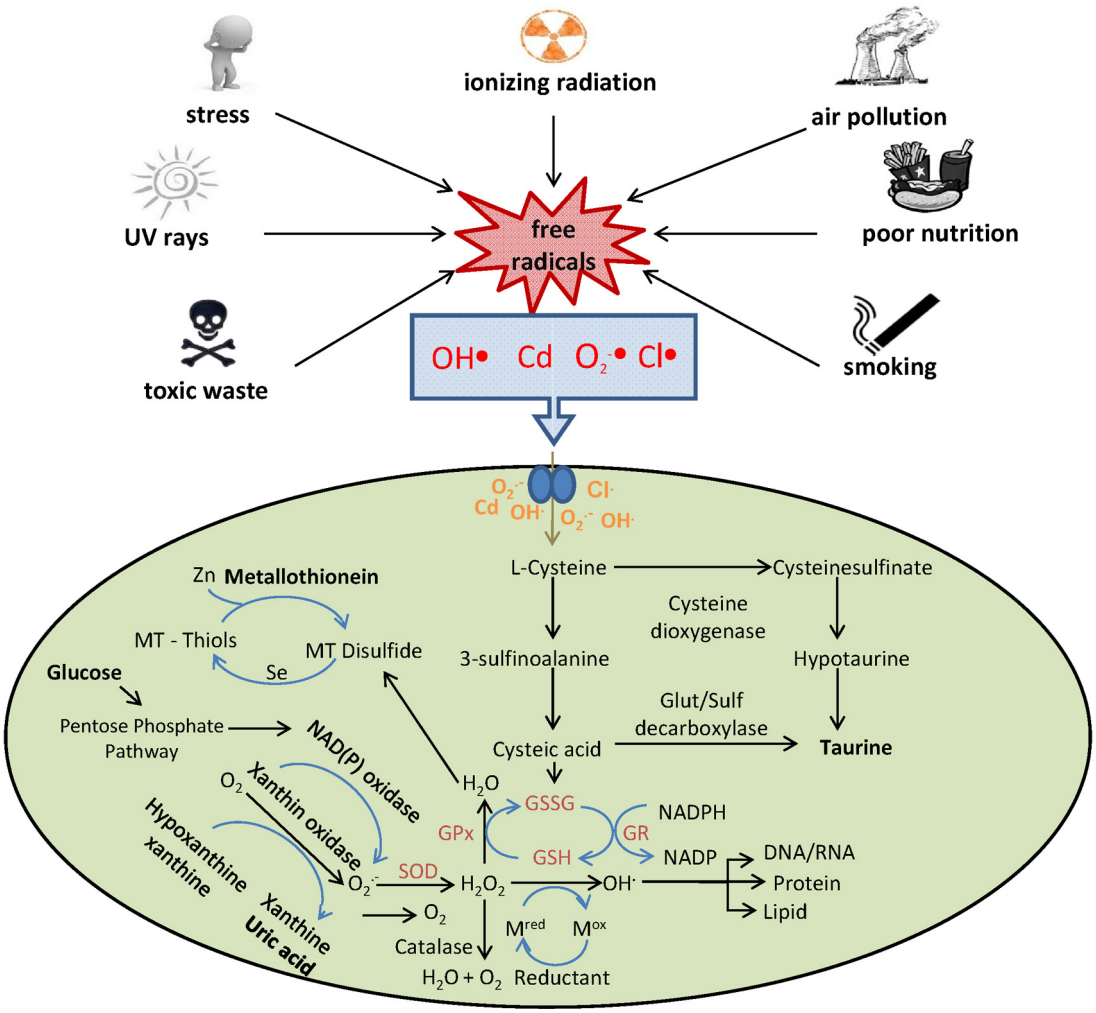

Fig. 8 – The scheme of the relation of all determined markers.
Modified according to Hayes et al. (1975), Koplove (1984), Hayes and McLellan (1999), Gropman et al. (2007) and Figueira et al. (2012).

Correlation of the results

The assayed markers were correlated one to each other. Results from the correlations are introduced in Table 1. As it is obvious in the table, only one negative correlation was determined for uric acid. The uricemia had negative correlation with all of the markers tested. The highest values were found for correlation of uric acid with FR, GPx, GSSG, and GSH. GSH participates in the other methods for assay of low molecular weight antioxidants and the antioxidant is presented in plasma in normal conditions, we can conclude that GSH probably substitutes uric acid to maintain the antioxidant activity of blood plasma. High negative correlation of GPX with uric acid can be caused by accumulation of GSH. Correlation of GSH and GPX, respectively GR can be explained by the same mechanism. Owing to the determined level of taurine, GSH is highly related to it. This fact is probably based on the close connection between GSH/GSSG metabolism and taurine. The scheme of the relation of all determined markers is shown in Fig. 8. Free radicals are generated during the both physiological and pathological processes or their origin may be connected with the environmental factors, both biotic and abiotic (UV radiation, pollution of the environment, smoking, lifestyle and diet or stress). However, there are mechanisms involved in the maintenance of homeostasis and elimination of reactive oxygen (nitrogen) species. These mechanisms are enzymatic (SOD, CAT, APX, GPX, etc.) and non-enzymatic (GSH, ascorbate, glucose etc.). GPX is an enzyme catalyzing the hydrogen peroxide elimination under simultaneous oxidation of cysteine that contains thiol (–SH) group. In this light, it is quite necessary to regenerate glutathione in reduced form (GSH). Glutathione reductase (GR) is enzyme involved in this regeneration. It utilizes NADPH in this process. The reaction results in the hydrogen peroxide detoxification and NADPH oxidation. GPX is together with CAT an important part of the enzymatic protective mechanism involved in the hydrogen peroxide detoxification. Origin of hydrogen peroxide is in the action of SOD that catalyzes the dismutation of the superoxide anion radical to hydrogen peroxide and oxygen. The excess or ineffective hydrogen peroxide detoxification leads to the formation of hydroxyl radical. Taurine represents next important non-enzymatic antioxidant in animal tissues. Taurine is a derivative of the sulphur-containing (sulfhydryl) amino acid, cysteine. For mammalian taurine synthesis occurs in the pancreas via the cysteine sulfinic acid pathway. In this pathway, the sulfhydryl group of cysteine is oxidized to cysteine sulfinic acid by the enzyme cysteine dioxygenase. Cysteine sulfinic acid, in turn, is decarboxylated by cysteinesulfinate decarboxylase to form hypotaurine. Taurine is conjugated via its amino terminal group with chenodeoxycholic acid and cholic acid to form the bile salts sodium taurochenodeoxycholate and sodium taurocholate. Glucose to stimulate NADPH oxidase, an enzyme that catalyses the production of superoxide from oxygen and NADPH. Metallothionein plays important role in the detoxification of heavy metals. It is low-molecular mass intracellular protein that role consists in the maintenance of heavy metals homeostasis. Reactive oxygen species are able to activate MT via antioxidant response element (ARE).

Conclusion

Currently, the great attention is paid to studying of the oxidative stress and methods applicable in its monitoring (Pasupathi et al., 2009; Siddique and Afzal, 2009; De et al., 2010; Pohanka et al., 2011, 2012c; De la Fuente and Hernanz, 2012; Delgado et al., 2012; Green et al., 2012; Vávrová et al., 2012). In this study, we used battery of methods for studying of taurine effect on Wistar rats. Taurine was proved to be a highly effective compound suitable for enhancing of antioxidant potency in the body. The exact mechanism of taurine action on the body is not clear. On the other hand, tight link to GSH and its metabolism was proved. Owing to the achieved results, we judge that taurine is suitable for solving pathologies related to oxidative stress. Simple application of taurine can improve not only level of low molecular weight antioxidants but also enzymatic antioxidants. Regulation of uric acid level is another interesting finding. Taurine would be implicated in pathologies in which hyperuricemia occurs.

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