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Scavenging and antioxidant potential of physiological taurine concentrations against different reactive oxygen/nitrogen species

Max W. S. Oliveira, Juliane B. Minotto, Marcos R. de Oliveira, Alfeu Zanotto-Filho, Guilherme A. Behr, Ricardo F. Rocha, José C. F. Moreira, Fábio Klamt

Center of Oxidative Stress Research, Department of Biochemistry – Basic Health Sciences Institute (ICBS)/Federal University of Rio Grande do Sul (UFRGS), 2600 Ramiro Barcelos, Porto Alegre (RS) 90035-003, Brazil

Correspondence: Fábio Klamt, e-mail address: 00025267@ufrgs.br

Abstract:
While several studies have been conducted on the antioxidant properties of the β-amino acid taurine, these studies all used concentrations lower than what is found physiologically. This study investigates the scavenging and antioxidant properties of physiological taurine concentrations against different reactive species. No reactivity between taurine and hydrogen peroxide was found; however, taurine exhibited significant scavenging potential against peroxyl radical, nitric oxide, and superoxide donors. This study also evaluated if taurine was able to minimize the *in vitro* CuZn-superoxide dismutase damage (SOD) induced by peroxynitrite. Taurine prevented both the formation of nitrotyrosine adducts and the decrease in SOD activity caused by peroxynitrite. In addition, taurine prevented the *ex vivo* damage caused by *tert*-butyl hydroperoxide in rat liver slices. These experimental data show that taurine, at different physiological concentrations efficiently scavenges many reactive oxygen and nitrogen species. This finding supports the hypothesis that the antioxidant properties of taurine may be critical for the maintenance of cellular functions, and it suggests a more important function of taurine that requires further investigation.

Key words:
taurine, scavenger, antioxidant, physiological concentrations

Abbreviations: AAPH – 2,2'-azobis(2-methylpropionamidine)-dihydrochloride, DTNB – 5,5'-dithiobis-(2-nitrobenzoic acid), MDA – malondialdehyde, MPO – myeloperoxidase, NBT – nitro blue tetrazolium, NOS – nitric oxide synthase, RNS – reactive nitrogen species, $ROO^{\bullet}$ – peroxyl radical, ROS – reactive oxygen species, SNP – sodium nitroprusside, SOD – superoxide dismutase, t-BHP – *tert*-butyl hydroperoxide, TEMED – N,N,N',N'-tetramethyl-ethylene-diamine, TnCl – taurine chloramine, TBARS – tiobarbituric acid reactive species

Introduction

Taurine (2-aminoethanesulfonic acid), a sulfur-containing β-amino acid, is found in all animal cells at millimolar concentrations. Concentrations of taurine in the plasma and extracellular fluids are much lower, typically

ranging from 10 to 100 μM [46]. Although this β-amino acid is not incorporated into proteins, intracellular concentrations of taurine can reach up to 80 mM, depending on the tissue type. Taurine levels in heart (25–30 mM), brain (30–40 mM), and lung (11–17 mM) tissue are higher than in liver tissue (around 10 mM). However, the highest levels of taurine are found in neutrophils, where the cytosolic concentration is 50 mM, and in the retina, where the concentration is 50–70 mM [14, 34, 48].

Several biological functions have been attributed to taurine, such as bile acid conjugation, maintenance of calcium homeostasis, osmoregulation, and membrane stabilization [18, 32]. Previous studies have shown that especially high taurine concentrations are found in tissues with high oxidative activity (retina, nerves, kidney and heart); while lower concentrations are found in tissues with primary glycolytic activity [17]. Despite the fact that taurine is a very stable molecule and difficult to oxidize, these data suggest that the high concentrations of taurine in these cell types could exert a physiological scavenging potential against reactive species generated by oxidative metabolism.

Various *in vitro* and *in vivo* studies have demonstrated that taurine has cytoprotective effects [44]. While the current mechanism(s) underlying the several protective effects of taurine are not well known, these effects are often attributed to an antioxidant activity [13, 14, 27, 36, 39–42, 45]. Taurine is known to react and detoxify hypochlorous acid (HOCl) generated by activated neutrophils from myeloperoxidase (MPO), hydrogen peroxide and chloride during the oxidative burst [7]. This protective function involves the formation of stable taurine chloramine (TnCl), and it is believed to be the reason for high taurine levels (50 mM) found in neutrophils. Moreover, taurine can inhibit the formation of the apaf-1/caspase-9 apoptosome complex, inhibiting mitochondria mediated apoptosis [50]. Despite these findings, we previously demonstrated that TnCl is a potent inducer of programmed cell death in cancer cells [22].

Previous studies, which sought to determine the free radical scavenging potential of taurine, observed minimal direct chemical scavenging actions against many oxygen-derived radicals [2, 13, 45]. One factor that is not often adequately addressed in the assessment of the antioxidant role of taurine is its high intracellular concentrations in certain types of cells. For instance, the scavenging potential of taurine against a ROS-generating system was previously investigated and was found to be ineffective. However, the highest concentration of taurine used in these studies was 10 mM [49]. Thus, it is unclear whether the relatively low reactivity of taurine at these concentrations is applicable to cells that contain high levels of taurine. The objective of the present study was to examine the *in vitro* antioxidant properties of physiological taurine concentrations against different reactive species. We also wanted to determine whether taurine could protect cells from t-BHP toxicity in rat liver slices to clarify possible *in vivo* antioxidant properties of this β-amino acid.

Materials and Methods

Animals

Adult male Wistar rats were obtained from the Central Animal House of Federal University of Rio Grande do Sul. The animals were caged in groups of five with free access to food and water, and they were maintained on a 12-h light-dark cycle (lights on 7:00 am) at a room temperature of 22 C ± 1 C. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and with the approval of the Ethics Committee of the Federal University of Rio Grande do Sul.

Chemicals

Taurine was purchased from FLUKA (USA), and CuZnSOD from bovine erythrocytes was acquired from Roche (USA). 2,2'-Azobis(2-methylpropionamidine)dihydrochloride (AAPH) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were purchased from Aldrich Chemical (Milwaukee, WI, USA). Folin-Ciocalteu reagent was obtained from Merck (Germany). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Taurine solutions were prepared fresh daily in ultrapure Milli-Q water or in the specified buffer. All experiments were carried out using 1, 15, 30 and 60 mM taurine or β-alanine (used as a β-amino acid control). Trolox®, a synthetic analog of vitamin E, was used as a standard antioxidant at 1 mM. The final pH of each solution and incubation system was monitored during all experiments to determine the effects of pH variation on the results obtained here. Protein content was

measured by the Lowry method using bovine serum albumin as the standard [33].

Biochemical analysis

To assess the *in vitro* reactivity of taurine against hydrogen peroxide, different taurine concentrations were incubated with 1 mM hydrogen peroxide for one hour. The remaining concentration of H_2O_2 was then determined using an extinction coefficient value ($\varepsilon_{240\ nm}$) of 43.6 $M^{-1}cm^{-1}$[6].

The *in vitro* scavenging activity of taurine against peroxyl radicals was estimated by the total reactive antioxidant potential (TRAP) as previously described [30, 31]. Briefly, the reaction mixture (3.7 ml) containing AAPH (10 mM) and luminol (4 mM) in 0.1 M glycine buffer (pH 8.6) was incubated at room temperature for 2 h. The thermal decomposition of water-soluble azobis (2-amidinopropane hydrochloride) produces peroxyl radicals ($ROO^{\bullet}$) at a known steady rate. Peroxyl radicals react with luminol, resulting in chemiluminescence (CL). The addition of 300 µl of taurine at different concentrations decreases the CL proportionally to its antioxidant potential. The TRAP profile was obtained by measuring the CL emission in a liquid scintillation counter (Wallac 1409) as counts per minute (CPM). The CL intensity was monitored for 50 min after the addition of taurine. The areas under the curve of the chemiluminescence traces were used to statistically compare the scavenging potential of taurine against ROO as compared to the control traces. The "induction time" is reflective to the time required to consume the active antioxidants present in the sample.

In vitro generation of nitric oxide ($NO^{\bullet}$) was achieved by the decomposition of 20 mM sodium nitroprusside (SNP) in PBS buffer (pH 7.4) as described [26, 37], with modifications for use in a 96-well microplate. Different concentrations of taurine were incubated with SNP for one hour at 37°C. Afterwards, 20 µl of Griess reagent was added, and the remaining concentration of nitrite was determined at 540 nm. As nitrite is the only stable final product of the autoxidation of NO in aqueous solution [3, 15, 37], only nitrite was measured by the Griess reaction [20]. The results were expressed as a percentage of the generated nitrite, using sodium nitrite as a standard.

The scavenging activity of taurine against superoxide anions was assessed in two different assays. First, this activity was quantified by the inhibition of superoxide-dependent adrenaline auto-oxidation to adrenochrome by different concentrations of taurine using spectrophotometric measurements at 480 nm, as previously described [38]. The results are expressed as absorbance/time (seconds). The area under the curve of the graph was used for statistical analysis and compared against the control values. Five units of CuZnSOD (E.C.: 1. 15. 1. 1) were used to determine assay specificity. Superoxide scavenger activity was also measured spectrophotometrically by monitoring superoxide-dependent reduction of NBT to the blue chromogen, formazan at 560 nm in the presence of different taurine concentrations [47]. Next, 0.1 mM NBT and 0.02 units/ml Xanthine Oxidase (XO) (E.C.: 1. 1. 3. 22) were added to 50 mM Tris-HCl at pH 7.4, containing 0.012 mM Tween 20 and 1 mM EDTA. This mixture was warmed to 37°C, and a 10-fold concentrated taurine solution was added to reach a final volume of 200 µl. NBT reduction was monitored for 1 h with 2-minute intervals between readings in a 96-well microplate reader (Molecular Devices). The rate of superoxide formation was expressed as a percentage of superoxide formation.

Peroxynitrite solutions were prepared from acidified hydrogen peroxide and sodium nitrite as described previously [43], and the concentration was determined using an extinction coefficient value at 302 nm ($\varepsilon_{302\ nm}$) of 1670 $M^{-1}cm^{-1}$. Samples consisted of CuZnSOD (2 units) mixed with different taurine concentrations. Nitration was carried out by incubating samples with 100 µM peroxynitrite for 1 h, and the extent of nitration was determined by Dot-Blot analysis using anti-nitrotyrosine antibody. For Dot-Blot analysis, samples were applied to a nitrocellulose membrane. After blocking the membrane with 5% albumin, it was incubated overnight with a rabbit anti-nitrotyrosine antibody (1:2000) (BD Biosciences, CA, USA), followed by a horseradish peroxidase-conjugated secondary antibody (1:10000) (DakoCytomation, USA). Dots were visualized by chemiluminescence using an ECL kit from NEM (Boston, MA, USA). Quantification of dot intensity was performed using ImageJ 1.36b software (National Institutes of Health, USA). SOD activity was assessed in a 10% native polyacrylamide gel stained with 4-nitroblue tetrazolium (NBT). The bands were revealed through reduction of NBT (0.2 mg/ml) by the superoxide produced through photochemical reduction of riboflavin (2.8 µM) with N,N,N',N'-Tetramethyl-ethylene-diamine (TEMED, 28 mM) as modified from previous studies [4]. A cali-

bration curve was obtained by measuring *in gel* activities of different concentrations of CuZnSOD (Fig. 5B).

The antioxidant activity of taurine was also evaluated *ex vivo,* using *tert*-butyl hydroperoxide (t-BHP) as an oxidant. Rat liver slices were preincubated with different concentrations of taurine for 30 min at 37°C. Preincubations were done in a medium of oxygen-balanced Krebs-Ringer phosphate buffer (pH 7.4) with 10 mM glucose in a shaking water bath under a carbogenic mixture (95% O_2/5% CO_2). After preincubation, 1 mM t-BHP was added. Rat liver slices were then removed, homogenized with PBS (pH 7.4), and stored at –80 C until further analysis.

To analyze oxidative alterations in proteins induced by t-BHP, the remaining levels of reduced protein thiol (-SH) in the samples were measured. A sample aliquot was diluted in 0.1% SDS and 10 mM 5,5-dithiobis 2-nitrobenzoic acid (DTNB). Ethanol was added to produce the intense yellowish color of the product of the reaction between the sulfhydryl (-SH) groups and DTNB. After 20 min, -SH levels were spectrophotometrically determined at 412 nm [10]. Results are expressed as mol (-SH)/mg protein.

As an index of lipid peroxidation, tiobarbituric acid reactive species (TBARS) formation was measured using a hot acid reaction. This is a widely adopted method for measuring lipid oxidation [9]. The homogenates of rat liver slices were mixed with 0.6 ml of 10% trichloroacetic acid (TCA) and 0.5 ml of 0.67% thiobarbituric acid, and heated in boiling water for 25 min. The levels of TBARS were spectrophotometrically determined at 532 nm. Results are expressed as nmol MDA equivalents/mg protein.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM); p values were considered significant when $p < 0.05$. The differences between the experimental groups were analyzed by one-way ANOVA analysis followed by Tukey's *post-hoc* test.

Results

To address the possible antioxidant role of taurine at physiological concentrations, we investigated the *in vitro* scavenging potential of taurine against different reactive species. In all of the experiments, Trolox® (1 mM), a standard antioxidant, and β-alanine (at the same concentrations of taurine), a β-amino acid control, were used. Figure 1 shows that there is no significant reactivity between taurine and 1 mM hydrogen peroxide at any concentration tested. β-Alanine also showed no reactivity with H_2O_2 (data not shown). However, taurine (15, 30 and 60 mM) was able to quench the peroxyl radical generated by AAPH decomposition, as shown by a decrease in the peroxyl-mediated luminol chemiluminescence (Fig. 2). β-Alanine showed no detectable reactivity towards peroxyl radicals (data not shown). Trolox® was used as a negative control. Taurine (60 mM) was also able to decrease nitrite formation from the decomposition of nitric oxide, and was as effective as Trolox® (1 mM) for scavenging $NO^{\bullet}$ (Fig. 3). Moreover, using two different assays, the scavenging activity of taurine against $O_2^{\bullet-}$ donors was observed at concentrations of 30 and 60 mM (Fig. 4). CuZnSOD was used to determine assay specificity (see materials and methods section).

Because $O_2^{\bullet-}$ and NO• are precursors for the endogenous synthesis of $ONOO^-$ in the cells, and CuZnSOD is a well-known target of peroxynitrite, we evaluated whether taurine was able to prevent CuZnSOD damage by $ONOO^-$. We found that exposure to high taurine concentrations was able to decrease the *in vitro* formation of 3-nitrotyrosine adducts in CuZnSOD mediated by $ONOO^-$ (Fig. 5A). In addition, as shown in Figure 5B, 60 mM taurine was capable of

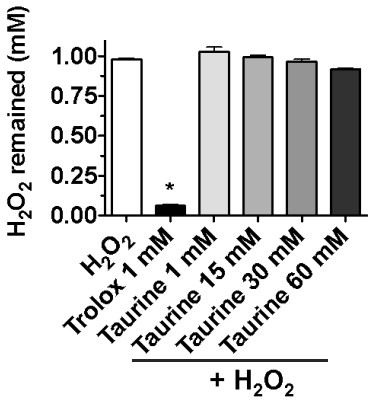

Fig. 1. Scavenging activity of taurine against hydrogen peroxide. Different taurine concentrations were incubated with 1 mM hydrogen peroxide for 1 h, and the remaining H_2O_2 was determined as described in the Materials and Methods section. Data are expressed as the mean ± SEM, and the experiments were performed in triplicate (n = 4). * Statistically different from H_2O_2 alone ($p < 0.001$)

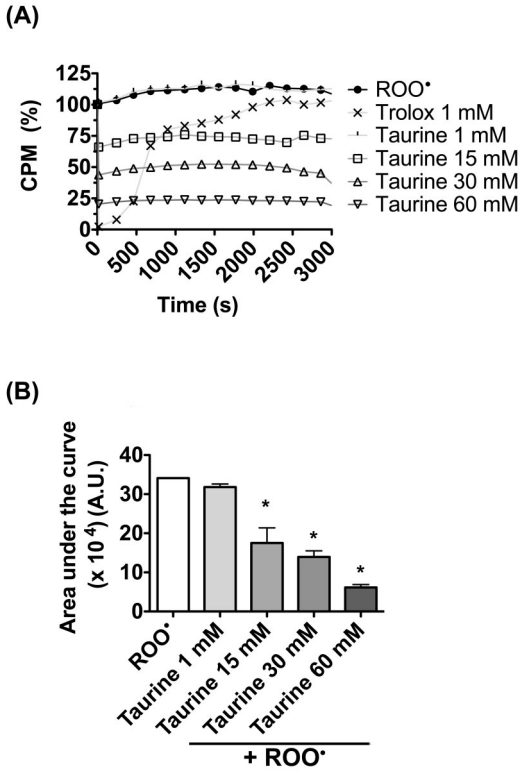

Fig. 2. Scavenging activity of taurine against a peroxyl radical (ROO•) donor. Different taurine concentrations were incubated with AAPH and luminol and the remaining ROO• concentration was determined as described in the Materials and Methods section. Results are presented as (**A**) the kinetics of the thermal decomposition of AAPH plus luminol and with co-incubation of taurine in counts per minute (CPM), and (**B**) the area under the curve from the data shown in graph A. Data are expressed as the mean ± SEM, and the experiments were performed in triplicate (n = 6). * Statistically different from ROO• alone ($p < 0.001$)

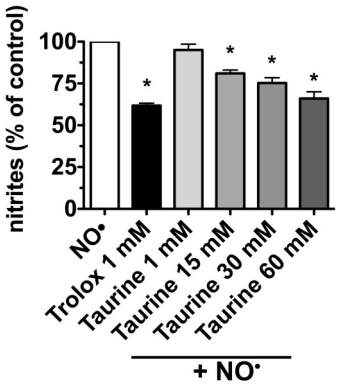

Fig. 3. Scavenging activity of taurine against a nitric oxide (NO•) donor. Different taurine concentrations were incubated for 1 h with 20 mM sodium nitroprusside (SNP), and the remaining nitrite was determined by the Griess reaction, as described in the Materials and Methods section. Data are expressed as the mean ± SEM, and experiments were performed in quintuplicate (n = 4). * Statistically different from NO donor alone ($p < 0.001$)

reversing CuZnSOD inactivation mediated by peroxynitrite as well. Taurine alone had no direct effect on CuZnSOD activity.

To test whether physiological concentrations of taurine could prevent oxidative damage in a mammalian tissue system, rat liver slices were preincubated with taurine and subsequently exposed to t-BHP. Two oxidative parameters were analyzed: t-BHP-induced lipid peroxidation and protein sulfhydryl oxidation (Fig. 6). All taurine doses inhibited t-BHP-induced lipid peroxidation (Fig. 6A). Likewise, all taurine concentrations prevented the decrease in protein sulfhydryl levels induced by t-BHP (Fig. 6B).

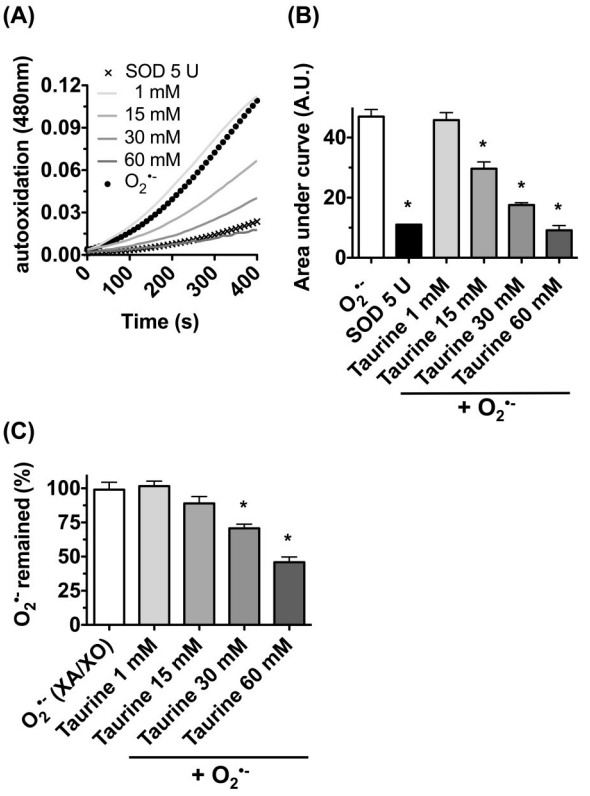

Fig. 4. Scavenging activity of taurine against anion superoxide ($O_2^{\bullet-}$) donors. (**A**) Kinetics of superoxide-dependent adrenaline auto-oxidation to adrenochrome, and the effect of different taurine concentrations. (**B**) Area under curve from data shown in graph A. Data are expressed as the mean ± SEM, and the experiments were performed in quintuplicate (n = 5). * Statistically different from adrenaline auto-oxidation alone ($p < 0.001$). (**C**) Effects of different taurine concentrations on superoxide-mediated NBT reduction in a xanthine/xanthine oxidase (XA/XO) system. Data are expressed as the mean ± SEM, and the experiments were performed in quadruplicate (n = 4). * Statistically different from X/XO system alone ($p < 0.01$)

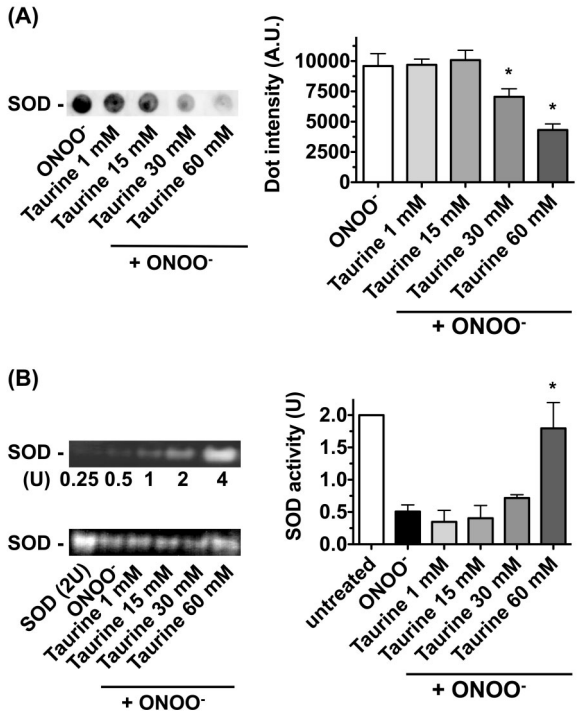

Fig. 5. Scavenging activity of taurine against peroxynitrite ($ONOO^{\bullet-}$). (**A**) Representative dot-blot showing the preventive effect of taurine in the formation of 3-nitrotyrosine groups in CuZnSOD by peroxynitrite. On the right side, densitometric analysis of the data is shown. Data are expressed as the mean ± SEM, and the experiments were performed in triplicate (n = 4) *Means statistically different from induced control ($p < 0.01$). (**B**) Calibration curve of SOD activity (upper figure). Preventive effects of taurine on the decrease in SOD activity induced by peroxynitrite (lower figure). On the right side, densitometric analysis of the data is shown. Data are expressed as the mean ± SEM (n = 5). * Statistically different from induced control ($p < 0.001$)

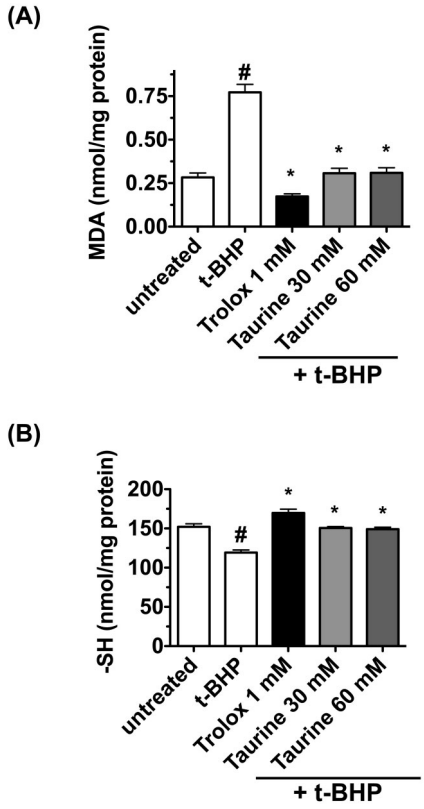

Fig. 6. Taurine is able to prevent oxidative stress mediated by tert-Butylhyperoxide (t-BHP) in liver slices. (**A**) Quantification of lipid peroxidation assessed by TBARS. (**B**) Total reduced sulfhydryl content. Data are expressed as the mean ± SEM, and experiments were performed in quintuplicate (n = 3). * Statistically different from t-BHP group ($p < 0.05$). # Statistically different from control ($p < 0.001$)

Discussion

Several reviews discussing the activity of taurine in physiological processes have been published. However, no consensus on the antioxidant role of taurine in mammalian systems has been reached, and the overall function of this β-amino acid is still being debated. Moreover, none of these studies evaluated the antioxidant activity of taurine at the effective physiological concentrations achieved in cells with high oxidative metabolism.

The objective of this study was to determine whether taurine, at physiological concentrations, is able to act as an antioxidant or scavenger molecule. We found that taurine, at physiologic concentrations, primarily above 15 mM, acts as a good *in vitro* scavenger of reactive oxygen (peroxyl radical and anion superoxide) and nitrogen (nitric oxide and peroxynitrite) species. The original data presented here support an antioxidant/scavenger function for taurine, and shows that taurine acts as an important intracellular ROS/RNS scavenger and antioxidant.

Hydrogen peroxide is a reactive oxygen species and a signaling molecule that can be produced in almost all tissues. It can cross cell membranes and react with iron to form more harmful species such as $^{\bullet}OH$ [16]. Taurine did not show any reactivity towards H_2O_2, suggesting minimal interaction between these molecules, as seen previously [2]. However, these incubations were done at lower concentrations of taurine (up to 1 mM). The undetectable reactivity against H_2O_2 and the high concentrations of taurine in pro-inflammatory cells (e.g., neutrophils) should allow enough H_2O_2 flux for hypochlorous acid (HOCl)

synthesis by myeloperoxidase (MPO) during the oxidative burst.

Taurine showed efficient reactivity against nitric oxide, being effective from 15 to 60 mM. $NO^{\bullet}$ exerts several effects in biological systems (e.g., regulation of vascular smooth muscle tone, and platelet aggregation) [19], and can modulate the activity of the mitochondrial electron transport chain by the inhibition of cytochrome c oxidase [1]. It also reacts with superoxide anions to generate peroxynitrite or other radicals that can damage the mitochondria. The reactivity of taurine with this radical may be important *in vivo* to avoid S-nitrosylation modification of enzymes such as glyceraldehyde-3-phosphate-dehydrogenase (G3PDH), and to avoid damage of iron-sulfur proteins [16]. In addition, the mitochondrial electron transport chain is the main site of ROS production. In this pathway, electrons leak from the chain and partially reduce molecular O_2, yielding superoxide radicals. This radical can cause cellular damage by itself or react with other compounds to yield radicals that are more reactive. Taurine concentrations above 15 mM may prevent the superoxide generation by this system. Other systems that generate a superoxide flux (XA/XO) were also used here to demonstrate the scavenging ability of taurine.

Although the intra-mitochondrial concentration of taurine is not known, our data suggest that it could act as a scavenger of superoxide radicals, especially in sites of high production (such as the mitochondria). β-Alanine did not exhibit the same pattern as taurine, demonstrating effectiveness only at 60 mM (data not shown). However, this concentration is unattainable *in vivo* [24]. Evidence of taurine reactivity against NO and $O_2^{\bullet-}$ led us to the next question of whether taurine could react with peroxynitrite, the reactive nitrogen species generated by these two radicals *in vivo*. Peroxynitrite is a potent damaging agent towards -SH groups, lipids and DNA, causing inactivation of proteins through nitration of tyrosine residues [51]. In this experimental model, purified CuZnSOD was incubated with peroxynitrite. We found that taurine concentrations higher than 30 mM prevented $ONOO^-$-mediated 3-nitrotyrosine adduct formation, and prevented the decrease in SOD enzymatic activity. Both experiments suggest that in tissues with high taurine concentrations, taurine can act as a peroxynitrite scavenger, preventing the nitration and inactivation of enzymes. Another study has also demonstrated a protective effect of taurine against peroxynitrite-induced Na^+/K^+ATPase inactivation [23]. Lastly, taurine reactivity with the $NO^{\bullet}$ donor (sodium nitroprusside) has been shown previously [35].

Our investigation also evaluated the total reactive antioxidant potential of taurine against peroxyl radicals. The free radical generator, AAPH, is an azo compound that undergoes thermal decomposition to yield molecular nitrogen and two carbon radicals (R), which rapidly react with oxygen to give peroxyl radicals (ROO) [25, 30, 31]. The analysis of the effect of taurine on the kinetics of peroxyl radical production by AAPH shows that 60 mM taurine is able to block this process completely (Fig. 2B). β-Alanine was again ineffective at the same taurine concentrations tested (data not shown). It was interesting to observe that this *in vitro* system is considered a good assay to replicate the *in vivo* chain reaction caused by this radical, since the major chain propagator of lipid peroxidation is the peroxyl radical [12, 29].

Traditionally, lipid peroxidation is quantified by measuring malondialdehyde (MDA), which is formed by the degradation products of polyunsaturated fatty acid hydroperoxides [11]. The main source of MDA in biological samples is the peroxidation of polyunsaturated fatty acids with two or more methylene-interrupted double bonds. MDA is able to impair several physiological mechanisms of the human body through its ability to react with molecules such as DNA and proteins [8, 28]. Because we observed a strong ability of taurine to scavenge peroxyl radicals *in vitro*, we wanted to assess whether it was capable of protecting rat liver slices preincubated with it at different concentrations against lipid peroxidation induced by t-BHP (Fig. 2). This radical is formed *in vivo* by lipoperoxidation of membranes. The $L^{\bullet}$ (alkyl) radical resulting from lipid oxidation caused mainly by $ONOO^-$ or $^{\bullet}OH$, reacts with molecular O_2 to yield $LOO^{\bullet}$. The present data show that taurine was able to inhibit *t*-BHP-induced damage to lipids in liver slices in a non-concentration-dependent manner. We also evaluated the concentration of -SH groups, which is a good indicator of redox balance in cell [5, 51]. Unaltered -SH groups are crucial for the catalytic and structural functions of many proteins [21]. Taurine is efficient in preserving -SH groups, as well as in protecting the total -SH pool from oxidation. Trolox®, a vitamin E analog, reversed the induced oxidative damage in both experiments as well.

Although the antioxidant role of taurine remains a controversial topic among researchers, the present *in vitro* and *ex vivo* data suggest that taurine may partici-

pate in cellular protection against oxidative stress by protecting the cell from lipid peroxidation and preventing the oxidation of -SH groups. From these *in vitro* studies, it seems plausible that taurine, at physiological concentrations, could act as an efficient antioxidant/scavenger against many cellular insults, including oxidative damage. Ongoing studies in our research group are investigating the antioxidant role of taurine in the mitochondria, where it may have the most important function.

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