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ORIGINAL ARTICLE

Mechanism underlying the antioxidant activity of taurine: prevention of mitochondrial oxidant production

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Abstract An important function of the β-amino acid, taurine, is the regulation of oxidative stress. However, taurine is neither a classical scavenger nor a regulator of the antioxidative defenses, leaving uncertain the mechanism underlying the antioxidant activity of taurine. In the present study, the taurine antagonist and taurine transport inhibitor, β-alanine, was used to examine the mechanism underlying the antioxidant activity of taurine. Exposure of isolated cardiomyocytes to medium containing β-alanine for a period of 48 h led to a 45% decrease in taurine content and an increase in mitochondrial oxidative stress, as evidenced by enhanced superoxide generation, the inactivation of the oxidant sensitive enzyme, aconitase, and the oxidation of glutathione. Associated with the increase in oxidative stress was a decline in electron transport activity, with the activities of respiratory chain complexes I and III declining 50–65% and oxygen consumption falling 30%. A reduction in respiratory chain activity coupled with an increase in oxidative stress is commonly caused by the development of a bottleneck in electron transport that leads to the diversion of electrons from the respiratory chain to the acceptor oxygen forming in the process superoxide. Because β-alanine exposure significantly reduces the levels of respiratory chain complex subunits, ND5 and ND6, the bottleneck in electron transport appears to be caused by impaired synthesis of key subunits of the electron transport chain complexes. Co-administration of taurine with β-alanine largely prevents the mitochondrial effects of β-alanine, but treatment of the cells with 5 mM taurine in the absence of β-alanine has no effect on the mitochondria, likely because taurine treatment has little effect on cellular taurine levels. Thus, taurine serves as a regulator of mitochondrial protein synthesis, thereby enhancing electron transport chain activity and protecting the mitochondria against excessive superoxide generation.

Keywords Taurine · β-Alanine · Mitochondrial protein synthesis · Oxidative stress · Electron transport

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Introduction

Taurine is a β-amino acid found in very high concentration in excitable tissues (Huxtable 1992). Although the β-amino acid is not involved in peptide bond formation, it is considered an essential or semi-essential nutrient (Knopf et al. 1978; Gaull 1986; Bouckenooghe et al. 2006). A strict nutritional requirement for taurine occurs in species with limited ability to synthesize taurine in the liver and with a stringent dependence on taurine for bile acid conjugation. These species develop several pathological conditions, including a retinopathy, cardiomyopathy and developmental defects, in response to taurine depletion (Knopf et al. 1978; Pion et al. 1987; Sturman 1993). Many of these conditions also develop in animals treated with a taurine transport inhibitor or in taurine transporter null mice, as the transporter is required for the maintenance of high intracellular taurine levels (Heller-Stilb et al. 2002; Ito et al. 2008). Although the pathological properties of the taurine deficient condition have been characterized, the mechanism underlying the development of the pathology remains unclear.

It has recently been proposed that the actions most likely linked to the development of the taurine deficient cardiomyopathy are the maintenance of contractile function through improvements in calcium transport, the modulation of protein phosphorylation and the regulation of oxidative stress (Schaffer et al. 2010). According to Eley et al. (1994) and Schaffer et al. (2000) changes in Ca^{2+} availability and Ca^{2+} sensitivity of the myofibrils may contribute to the contractile defects observed in β-alanine-mediated taurine deficient cardiomyopathy. However, taurine deficiency is also associated with severe damage to the cell, which has been attributed in part to oxidative stress. In the case of the heart, taurine deficiency potentiates oxidative damage mediated by toxic doses of doxorubicin (Harada et al. 1980) or angiotensin II (Schaffer et al. 2003a, b). Nonetheless, the mechanism underlying the antioxidant activity of taurine has not been established. Aruoma et al. (1988) have clearly demonstrated that taurine is incapable of scavenging the standard oxidants. Moreover, there is no conclusive evidence that taurine regulates the levels of the antioxidant defense system. The present study introduces the novel idea that taurine's antioxidant activity is linked to improved mitochondrial function, which diminishes mitochondrial superoxide generation. To test this idea, isolated cardiomyocytes were incubated with medium containing β-alanine, a taurine analogue that reduces taurine levels (an effect related to the taurine transporter) and interferes with the actions of taurine (an effect related to the structural similarities of taurine and β-alanine; like a partial agonist β-alanine inhibits reactions involving taurine, such as conjugation). Also examined is the effect of taurine treatment alone or co-administration of taurine and β-alanine, the latter combination which prevents the mitochondrial changes mediated by β-alanine alone.

Methods

Cardiomyocytes preparation, taurine content, and oxygen consumption

Neonatal cardiomyocytes were prepared from 2 to 3-day-old Wistar rats as described previously by Grishko et al. (2003). Briefly, cells were isolated after homogenization and exposure to a mixture of trypsin, chymotrypsin, and elastase. After a pre-plating step to remove nonmyocytes, the unattached cells were suspended in minimum essential medium (MEM) containing 10% newborn calf serum and 0.1 mM 5-bromo-2-deoxyuridine. The cells were plated and incubated overnight to allow for attachment of viable cardiomyocytes. The serum-containing medium was replaced the next day with serum-substitute. The neonatal cardiomyocyte culture was maintained for 2 days at 37°C prior to being exposed for 48 h to medium containing no addition for 48 h (control), 5 mM taurine for 48 h, 5 mM taurine + 5 mM β-alanine for 48 h or 5 mM β-alanine alone for 4, 16, 24 or 48 h. Taurine content was measured using the procedure described by Schaffer et al. (2002).

Oxygen consumption was measured employing the methods described by Ricci et al. (2008). Cells were suspended in respiration buffer consisting of 0.3 mol/L mannitol, 10 mmol/L KCl, 5 mmol/L $MgCl_2$, and 10 mmol/L K_2PO_4, pH 7.4. The β-alanine treated and control cells were transferred to a sealed water-jacketed chamber fitted with a calibrated Clark oxygen electrode. The cells were allowed to stabilize for $\sim$10–15 min, at which point the linear change in oxygen content of the cellular medium was measured as a function of time. After the measurement, the protein content of the cells used to measure oxygen consumption was determined. The rate of oxygen consumption was normalized relative to the amount of cellular protein of the cells used in the measurement of oxygen consumption. In some experiments the basal rate of oxygen consumption of β-alanine treated and control cells was determined. After determination of the basal rate of oxygen consumption, either the uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 5 μM) or 1 μM oligomycin, which inhibits respiration by blocking ATP synthase activity and reduces respiration, were added to the medium. The rate of oxygen consumption of the oligomycin, β-alanine treated cells and the CCCP, β-alanine treated cells was $\sim$30% less than their respective control cells.

Western blot analysis

Western blot analysis was performed according to the protocol described by Grishko et al. (2003). Cells were lysed in ice cold isolation buffer. The homogenate was centrifuged at 800 g for 6 min at 4°C and the supernatant was retrieved and centrifuged again at 16,000*g* for 20 min at 4°C. The 16,000 g pellet was defined as the mitochondrial fraction and used to determine ND5, ND6, COI, and succinate dehydrogenase content. The pellet was suspended in RIPA lysis buffer. Protein concentration was determined by the bicinchoninic acid (BCA) method using bovine serum albumin (BSA) as a standard. The mitochondrial sample was mixed with an equal volume of 5× electrophoresis sample buffer and the samples were then boiled for 3 min before being analyzed. During Western blot analysis, mitochondrial samples were subjected to one-dimensional electrophoresis using 12% SDS-PAGE. The proteins were then transferred to nitrocellulose membranes and the membranes were blocked. After incubation with the appropriate antibody, the membranes were washed and then incubated with a secondary antibody. The Western

blots were analyzed by the enhanced chemiluminescence reaction.

Complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), and complex III (cytochrome *c* oxidoreductase) activities

Extraction of mitochondrial proteins was conducted according to the procedures described by Grishko et al. (2003). Complex I activity was determined from the decrease in absorbance of NADH at 340 nm using a Cary 15 ultraviolet-visible spectrophotometer (Ricci et al. 2008). Mitochondria were first incubated at 37°C for 5 min with the assay medium containing 10 mM Tris–HCl, pH 8.0, 80 μM decylubiquinone, 1 mg/mL BSA, 0.25 mM potassium cyanide and 0.4 μM antimycin A. The absorbance change was monitored for 3 min before the addition of 5 μM rotenone, after which the reaction was measured for an additional 2 min. Complex I activity, reported as mmol/min/mg protein, was calculated from the difference between NADH oxidation activity in the presence and absence of rotenone.

Extraction of mitochondrial proteins was conducted according to the procedures described by Grishko et al. (2003). The 16,000 g pellet obtained was defined as the mitochondrial fraction and used to determine complex II [succinate dehydrogenase (SDH)] activity. The pellet was suspended in 50 mM potassium phosphate buffer, pH 7.4. Succinate dehydrogenase activity was measured from the reduction of 2,6-dichlorophenolindophenol at 600 nm using a Cary 15 ultraviolet-visible spectrophotometer (Ricci et al. 2008). Mitochondria were first incubated with 50 mM potassium phosphate buffer and 20 mM succinate at 37°C for 3 min. Next, the assay medium containing 500 μM 2,6-dichlorophenolindophenol, 20 mM potassium cyanide, 20 μg/mL rotenone, 20 μg/mL antimycin A, and 50 mM potassium phosphate buffer was added to the sample. Finally, 25 μM decylubiquinone was added to start the reaction. The absorbance change was monitored for 3 min. Succinate dehydrogenase activity was reported as μmol/min/mg protein.

After extraction of mitochondrial proteins (Grishko et al. 2003), the samples were centrifuged first at 800*g* for 6 min and the supernatant subsequently centrifuged at 16,000*g* for 20 min. After a further centrifugation, the 16,000 g pellet was defined as the mitochondrial fraction and used to determine complex III activity. The pellet was suspended in 50 mM Tris–HCl, pH 7.4. Complex III activity was determined from the increase in absorbance due to the reduction of oxidized cytochrome *c* at 550 nm using a Cary 15 ultraviolet-visible spectrophotometer (Chen et al. 2003). Mitochondria were first incubated at 37°C for 10 min with assay medium containing 250 mM sucrose, 1 mM EDTA, 50 μM oxidized cytochrome *c*, 2 mM potassium cyanide, 10 μg/mL rotenone, and 50 mM Tris–HCl, pH 7.4. Next, 10 mM decylubiquinol was added to the sample and the absorbance change was monitored for 3 min before addition of 40 μM antimycin A, after which the activity was measured for an additional 2 min. Complex III activity, reported as mmol/min/mg protein, was calculated from the difference between cytochrome *c* reduction activity with and without antimycin A.

Enzyme activity and oxidative stress

Aconitase assay was conducted using the Bioxytech Aconitase-340 kit (Foster City, CA, USA). Mitochondrial samples were incubated at 37°C in the dark for 15 min with assay buffer (10 mM Tris–HCl, pH 7.4), substrate (trisodium citrate in Tris–HCl, pH 7.4), enzyme (isocitrate dehydrogenase with Tris–HCl) and NADH reagent. An increase in absorbance at 340 nm was monitored for 5 min. Aconitase activity was reported as mU/mg protein. Succinate dehydrogenase (SDH) activity (see complex II activity) is unaffected by oxidative stress and was used to normalize aconitase activity, which is sensitive to oxidative stress.

Oxidized and reduced glutathione levels were measured using the glutathione assay kit (Cayman Chemical, Ann Arbor, MI, USA). The data normalized for protein content are expressed as the % change in reduced glutathione and oxidized glutathione content, as well as the glutathione redox ratio (reduced glutathione/oxidized glutathione).

Neonatal cardiomyocytes were incubated with MitoSox (5 μM), a mitochondria specific superoxide dye. After 30 min, the dye-containing media was removed and replaced with fresh media. Cells were then incubated for an additional 30 min prior to analysis. Some of the cells were incubated for 4 h in untreated fresh media (control) and MitoSox fluorescence determined. Other cells were transferred to media containing 5 mM *β*-alanine and MitoSox fluorescence was determined 4 h later. The control data normalized for protein content was assigned a value of 1.0 and the data normalized for protein content for the *β*-alanine group was expressed as relative levels of MitoSox fluorescence. The asterisk denotes a significant difference between the control and *β*-alanine groups.

Statistical analysis

All results were reported as mean ± SEM. The statistical significance of the data was determined using the Student's *t* test for comparison within groups and analysis of variance (ANOVA) combined with Tukey's post hoc test for comparison between groups. Values of $P < 0.05$ were considered statistically significant.

Results

The taurine analogue, β-alanine, is considered an inhibitor of taurine's actions, as it both interferes with the actions of taurine and facilitates cellular taurine depletion. Therefore, to provide information on the mechanism of taurine's actions, β-alanine was used to reduce taurine levels and suppress taurine action. As seen in Fig. 1, incubation of isolated neonatal cardiomyocytes with medium containing 5 mM β-alanine led to a time-dependent decrease in cellular taurine content, with levels reaching 55% of normal content after a 48 h incubation. By contrast, co-administration of 5 mM β-alanine and 5 mM taurine led to no significant change in taurine levels while cells incubated with medium containing 5 mM taurine only led to a small 10% increase in intracellular taurine content (Fig. 1). Cells incubated with medium lacking β-alanine and taurine (control) retained normal levels of taurine throughout the 48 h incubation period.

One of the important actions of taurine is the suppression of oxidative stress. The antioxidant activity of taurine has been generally noted in cells or tissue exposed to high, extracellular levels of taurine that are considered pharmacological. To test whether a reduction in intracellular taurine levels and interference with the intracellular actions of taurine cause a rise in oxidative stress, two markers of mitochondrial oxidative stress were determined in β-alanine treated, taurine + β-alanine co-treated, taurine treated and untreated cells. One of the markers, aconitase activity, is commonly used to detect mitochondrial oxidative stress because oxidants destroy the iron–sulfur centers of the citric acid cycle enzyme, leading to a loss of enzyme activity. By contrast, the citric acid cycle enzyme, succinate dehydrogenase, is largely insensitive to oxidative stress (<10%) and serves as a control in this study. As expected, β-alanine treatment had no effect on succinate dehydrogenase activity, but it reduced aconitase activity by 45% following a 16–48 h treatment period (Fig. 2a). By contrast, aconitase activity in taurine treated and taurine + β-alanine co-treated cells was indistinguishable from that of the control cells. Another marker of oxidative stress, the glutathione redox state (reduced glutathione/oxidized glutathione ratio), fell 43% following 48 h of β-alanine treatment but remained unaffected by a 48 h incubation with medium lacking β-alanine, containing both taurine and β-alanine or containing only taurine (Fig. 2b). The final measure of oxidative stress is MitoSox fluorescence, which rose 19% after a 4 h incubation with medium containing 5 mM β-alanine; longer periods of incubation were not performed because the confocal microscope method used to evaluate MitoSox fluorescence was not stable over a prolonged period of cell incubation. Co-administration of taurine and β-alanine or incubation of the cells with only taurine had no effect on MitoSox fluorescence (Fig. 2c). Together, these data reveal that the mitochondria of the β-alanine treated cardiomyocyte are subjected to significant oxidative stress.

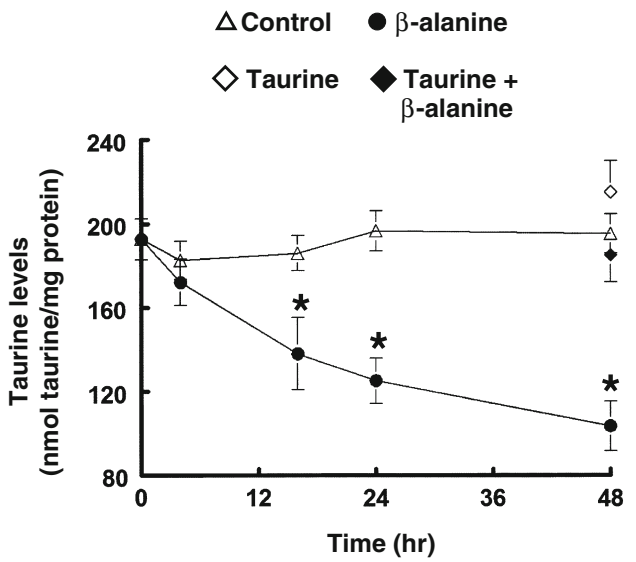
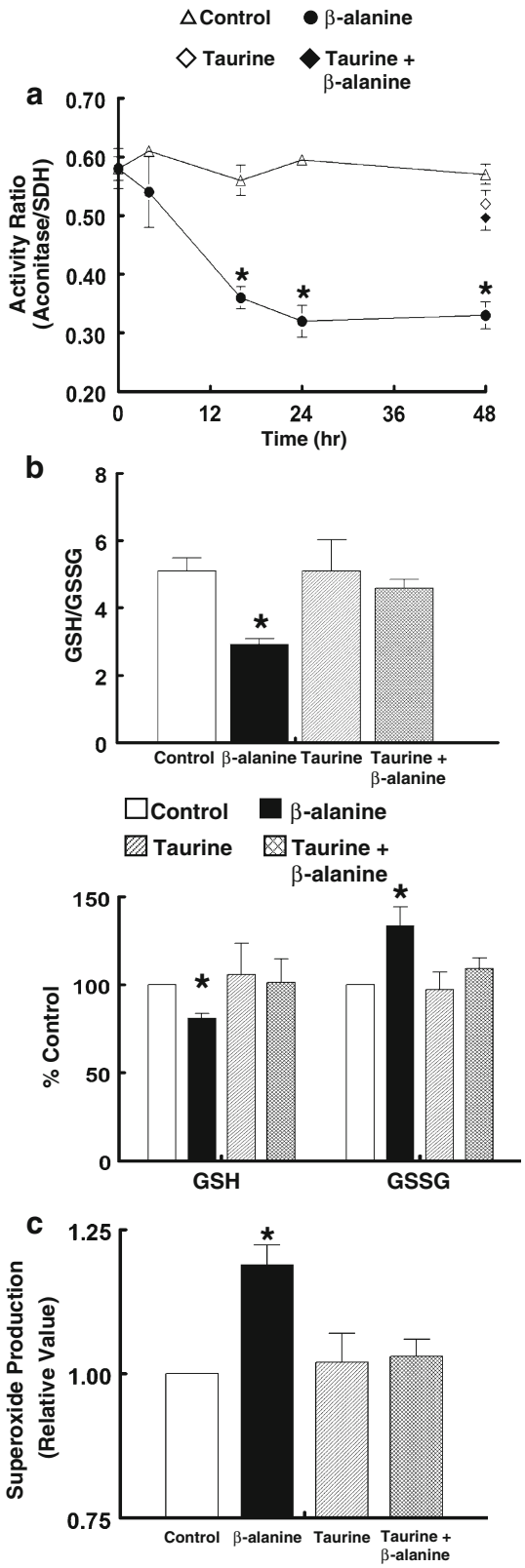


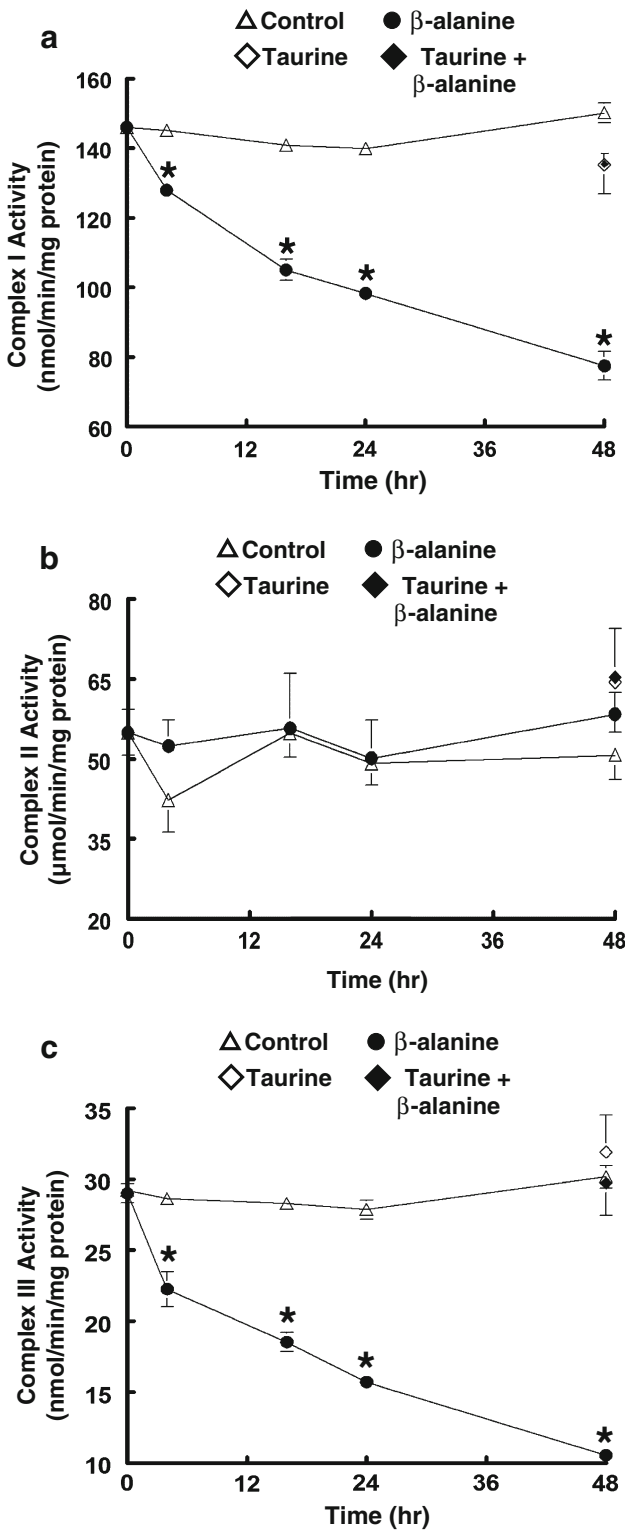
Fig. 1 Decline in taurine content in β-alanine-treated cardiomyocytes. Isolated neonatal cardiomyocytes were exposed to medium containing 5 mM β-alanine for 4, 16, 24 or 48 h, at which point the β-alanine-treated cells (*filled circle*) were harvested and taurine levels measured. Control cells (*open triangle*) were exposed to medium lacking 5 mM β-alanine for 4, 16, 24 or 48 h before being harvested for taurine analysis. Some cells were incubated in medium containing either 5 mM taurine (*open diamond*) or 5 mM taurine + 5 mM β-alanine (*filled diamond*) for 48 h before being harvested for taurine determination. Taurine values shown represent mean ± SEM of 4–6 different cell preparations. *Asterisks* denote a significant difference between β-alanine-treated and both untreated and taurine + β-alanine co-treated cells ($P < 0.05$)

Ricci et al. (2008) have shown that the formation of a bottleneck in the electron transport chain can lead to the production of superoxide, as electrons are diverted from the respiratory chain to the acceptor oxygen. To determine whether β-alanine treatment facilitates the formation of a bottleneck in the electron transport chain, respiratory chain activity was assessed by measuring both the activities of complexes I–III and the rate of oxygen consumption of β-alanine treated and untreated cells. Figure 3a shows that after 4 and 48 h of β-alanine treatment the activity of complex I fell 15 and 45%, respectively. Neither taurine treatment nor taurine + β-alanine co-treatment appreciably altered complex I activity. Similarly, complex III activity of the β-alanine treated cell fell, reaching levels 80 and 35% of control after 4 and 48 h of β-alanine treatment (Fig. 3b). By contrast, cells incubated with medium lacking β-alanine, containing taurine or containing both taurine and β-alanine over the same period of time showed no decline in complex III activity. Complex II activity was unaffected



◀ **Fig. 2** Taurine deficiency mediates oxidative stress. **a** Isolated cardiomyocytes were treated with 5 mM *β*-alanine (*filled circle*) for 4, 16, 24, and 48 h, at which point aconitase and succinate dehydrogenase activities were measured. Control cells (*open triangle*) were incubated with medium lacking all additions while taurine treated (*open diamond*) and taurine + *β*-alanine (*filled diamond*) treated cells were incubated for 48 h with medium containing 5 mM taurine and 5 mM taurine + 5 mM *β*-alanine, respectively. Mitochondria were isolated from all cell groups and after extraction were assayed for aconitase and succinate dehydrogenase activity. Unlike aconitase activity, succinate dehydrogenase (*SDH*) activity is unaffected by oxidative stress and therefore serves as a control. Values are expressed as activity ratio (aconitase/SDH) and represent mean ± SEM of 4–6 preparations. *Asterisks* denote a significant difference between *β*-alanine treated cells and both control and taurine + *β*-alanine treated cells. **b** Some cells (*β*-alanine) were treated for 48 h with 5 mM *β*-alanine before being harvested while other cells were exposed to medium lacking all additions (control) or containing either 5 mM taurine or 5 mM taurine + 5 mM *β*-alanine for 48 h. After harvesting the cells, levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) were determined. Data in the *upper panel* show the effects of *β*-alanine, taurine, and taurine + *β*-alanine on the glutathione redox state (GSH/GSSG) while the *lower panel* show the % change in GSH and GSSG content mediated by *β*-alanine treatment, taurine treatment or co-treatment with *β*-alanine and taurine; control cells were incubated with medium lacking taurine and *β*-alanine and control values were fixed at 100%. *Asterisks* denote a significant difference between the *β*-alanine treated group and the other three groups (control, taurine, and taurine + *β*-alanine). **c** MitoSox-loaded cardiomyocytes were incubated for 4 h with medium containing 5 mM *β*-alanine, 5 mM taurine, 5 mM taurine plus 5 mM *β*-alanine or lacking all additions (control). MitoSox fluorescence, a measure of mitochondrial superoxide content, was measured with a confocal microscope before and following the 4 h incubation. Control values were fixed at 1.0. The values for the *β*-alanine, taurine, and taurine plus *β*-alanine groups were normalized relative to the control values and represent mean ± SEM of 3–5 preparations. *Asterisks* denote a significant difference between the *β*-alanine-treated and the other groups ($P < 0.05$)

in any of the experimental groups (Fig. 3b). As expected, the impairment in electron transport chain activity was associated with a decrease in mitochondrial respiration (Ricci et al. 2008). As shown in Fig. 4, cellular oxygen consumption was diminished 25–30% by *β*-alanine treatment, but not by exposure of isolated cardiomyocytes to medium lacking *β*-alanine. Addition of the ATP synthase inhibitor, oligomycin, to the medium also suppressed cellular oxygen consumption by ~25%. When the cells were treated with both *β*-alanine and oligomycin, oxygen consumption was reduced ~50%, suggesting that the effects of oligomycin and *β*-alanine treatment are additive Fig. 4b). Similarly, the effects of *β*-alanine treatment and the uncoupler, CCCP, are additive (Fig. 4b). While CCCP stimulated respiration by 93% in the untreated control cells, the treatment of cells with both *β*-alanine and CCCP increased oxygen consumption only ~65% (Fig. 4). Because *β*-alanine-treated cells suppressed respiration to a similar degree (25–35%) in the presence and absence of oligomycin and CCCP, it follows that *β*-alanine causes an intrinsic change in the electron transport chain that is consistent with the formation of a bottleneck in the pathway.



◀ **Fig. 3** Effect of *β*-alanine-mediated taurine depletion on activities of complexes I–III. Some cells were exposed for designated times to medium containing (*filled circle*) or lacking (*open triangle*) *β*-alanine. Other cells were exposed to medium containing 5 mM taurine (*open diamond*) or 5 mM taurine + 5 mM *β*-alanine (*filled diamond*) for 48 h. **a** After isolation of mitochondria from each group of cells, complex I activity was determined by NADH oxidation, as monitored by a decrease in absorbance at 340 nm. **b** Complex II activity of each cell group was determined by monitoring 2,6 dichlorophenolindophenol reduction at 600 nm. **c** Complex III activity of each cell group was determined by cytochrome *c* reduction, as monitored by an increase in absorbance at 550 nm. Values shown represent mean ± SEM of 3–5 preparations. The *asterisks* denote a significant difference between the *β*-alanine-treated group (4–48 h) and both the control and the taurine + *β*-alanine group ($P < 0.05$)

One mechanism that adequately explains the effect of *β*-alanine on mitochondrial function assumes that *β*-alanine interferes with the synthesis of mitochondria encoded proteins. Because mitochondria encoded proteins are subunits of respiratory chain complexes, impaired synthesis of the mitochondrial proteins hampers the assembly of the respiratory chain complexes, reducing their activity. To test this idea, the levels of ND5 and ND6, which are subunits of complex I, and COI, which is a subunit of cytochrome *c* oxidase, were examined in *β*-alanine treated and untreated cells. A small decline in ND5 protein content was observed after 4 h of *β*-alanine treatment, which reached a new steady state level of 30% below the control by 16 h of treatment; control cells incubated with medium lacking *β*-alanine experienced no change in ND5 content (Fig. 5a). The effect of *β*-alanine treatment on ND6 was greater (40% decrease) than that noted for ND5. By comparison, COI levels were unaffected by a 48 h *β*-alanine treatment (Fig. 5b). Neither taurine treatment alone nor taurine + *β*-alanine co-treatment had a major effect on ND5, ND6 or COI levels (Fig. 5b).

Discussion

The most important observation of the present study is that *β*-alanine-mediated taurine depletion leads to impaired electron transport, which in turn increases mitochondrial superoxide generation. Blocking *β*-alanine-mediated taurine depletion through co-administration of taurine and *β*-alanine prevents mitochondrial dysfunction and the generation of reactive oxygen species by the mitochondria. This finding provides a rational mechanism for the "elusive" antioxidant activity of taurine. The major sources of superoxide generation in the mitochondria are complexes I and III, whose activities are depressed in the taurine-deficient cardiomyocyte but not in cells co-treated with taurine and *β*-alanine. It is widely accepted that slowing of electron flux through the respiratory chain can result in a diversion of electrons from complexes I and III to an alternate acceptor, such as oxygen (Turrens 2007). Taurine prevents the diversion of electrons into superoxide generation by improving the function of the electron transport chain.

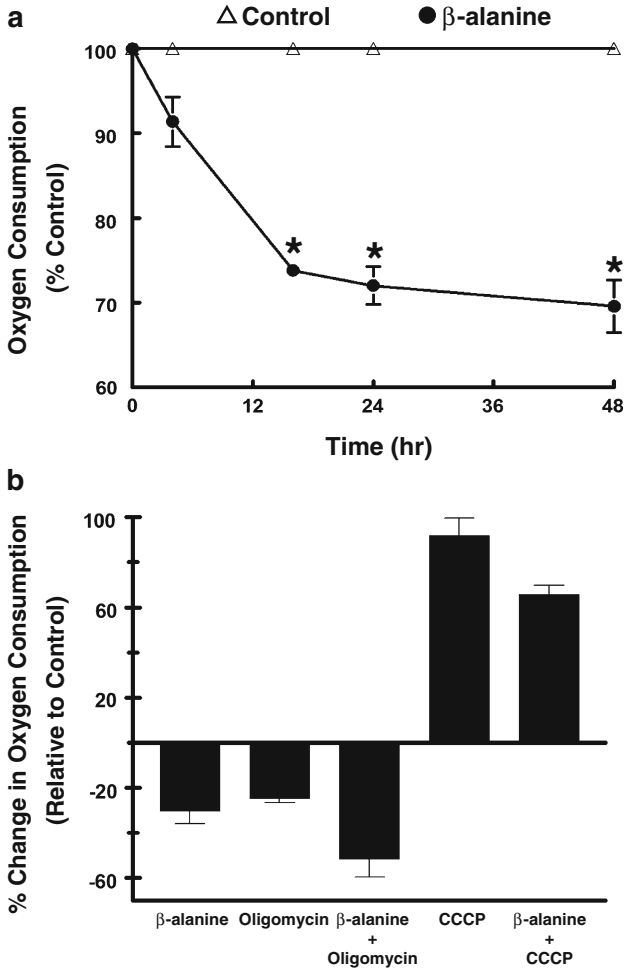


Fig. 4 Reduction in oxygen consumption of taurine-deficient cardiomyocytes. **a** Isolated cardiomyocytes were exposed to either no additions (*open triangle*) or 5 mM *β*-alanine (*filled circle*) for 4, 16, 24 or 48 h, at which point cells were transferred to a sealed, water-jacketed chamber fitted with a calibrated Clark oxygen electrode. The basal rate of oxygen consumption normalized for cellular protein content was determined. The normalized rate of oxygen consumption of the control cells was fixed at 100%. Shown is the decline in normalized oxygen consumption relative to the control mediated by *β*-alanine treatment for 4, 16, 24, and 48 h. Data represent mean ± SEM of 3–5 different preparations. *Asterisks* denote a significant difference between the *β*-alanine-treated groups and the control groups ($P < 0.05$). **b** Some untreated and *β*-alanine-treated cells were exposed to the ATP synthase inhibitor, oligomycin (1 μM), while others were exposed to the uncoupler, CCCP (5 μM). Shown are the effects of oligomycin and CCCP on oxygen consumption of control and *β*-alanine treated cells. The effects of oligomycin, CCCP and *β*-alanine treatment are additive. Data represent mean ± SEM of 3–5 different preparations

Several lines of evidence reveal that the electron transport chain is compromised in the *β*-alanine-mediated, taurine-deficient cardiomyocyte. First, activities of complexes I and III, which are considered the primary mitochondrial sources of superoxide generation in the

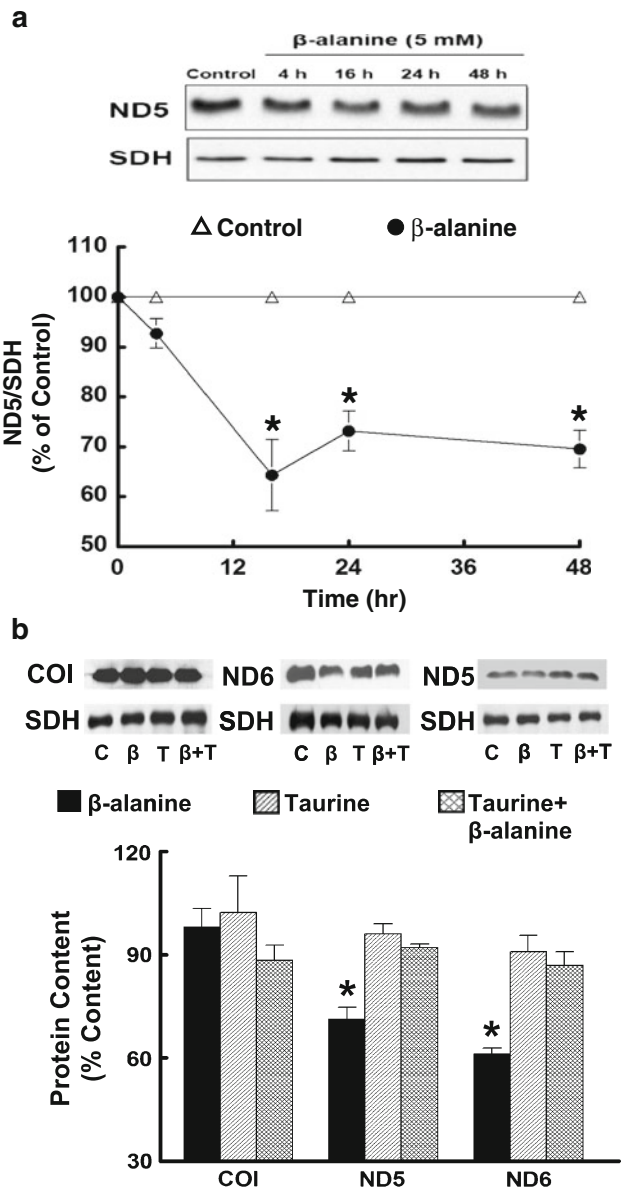


Fig. 5 Downregulation of ND5 and ND6, but not COI in taurine-depleted cardiomyocytes. Mitochondria obtained from control (*open triangle*), *β*-alanine-treated (*filled circle*), taurine treated and taurine + *β*-alanine treated cells were subjected to Western blot analysis of ND5, ND6, and COI. **a** Time course of ND5 decline in cells exposed to medium containing *β*-alanine exposure for various periods of time (4–48 h). Values shown represent mean ± SEM of the ND5/SDH ratio of 6–8 preparations. Shown are representative gels for ND5 and succinate dehydrogenase (*SDH*). *Asterisks* denote a significant difference between the *β*-alanine treated cells and control cells. **b** Changes in ND5, ND6 and COI levels mediated by 48 h exposure to no additions (C), *β*-alanine (*β*), taurine (T) and taurine + *β*-alanine (*β* + T). Shown in the *upper panel* are representative gels of ND5, ND6, COI, and SDH. Values shown in the *lower panel* represent mean ± SEM of 6–8 preparations and are expressed as % control, where control is fixed at 100%. *Asterisks* denote significant difference between the *β*-alanine-treated and the control groups ($P < 0.05$)

mitochondria, are reduced in the taurine-deficient cell. On the other hand, complex II (succinate dehydrogenase) is unaffected by β-alanine exposure, a finding consistent with its resistance to oxidative stress and changes in mitochondrial protein synthesis. Although taurine treatment has no effect on the activities of complexes I and III, it blocks the effect of β-alanine treatment on electron transport chain function. Second, oxygen consumption is diminished ~30% in the taurine-deficient cell. Interestingly, the β-alanine mediated decrease in oxygen consumption is additive with factors that affect the capacity of the mitochondria to synthesize ATP, such as CCCP (elevates respiration, reduces ATP) and oligomycin (decreases respiration, reduces ATP). These data suggest that taurine deficiency is associated with a decrease in the integrity of the electron transport chain. According to a study by Ricci et al. (2008), one of the factors that can contribute to a reduction in electron transport chain integrity is a decline in the synthesis of mitochondria encoded proteins, as they are essential for the assembly of active respiratory chain complexes. Thus, the observation that the levels of ND5 and ND6 are significantly reduced by taurine deficiency is consistent with the finding that complex I activity is also reduced (Figs. 3, 5).

The decrease in ND5 and ND6 levels without a change in COI content supports a role of taurine in the regulation of UUG decoding and mitochondrial protein synthesis. It is generally accepted that taurine forms a conjugate (5-taurinomethyluridine) with a key uridine moiety in the wobble position of mitochondrial $tRNA^{Leu(UUR)}$ (Kirino et al. 2004). This taurine-linked, post-transcriptional modification of the wobble base enhances the binding of the anticodon to the UUG codon for leucine, resulting in the decoding of UUG. Hence, $tRNA^{Leu(UUR)}$ containing the taurinomethyluridine modification exhibits equal decoding of UUG and UUA while $tRNA^{Leu(UUR)}$ lacking the taurinomethyluridine modification only decodes UUA. According to Kirino et al. (2004), most of the mitochondria encoded proteins contain leucine residues whose translation depends upon at least one UUG codon. Therefore, it is not surprising that the rate of biosynthesis of most mitochondria encoded proteins depend upon the taurinomethyluridine content of $tRNA^{Leu(UUR)}$, which in turn appears to be dependent upon taurine levels and the activity of the taurine conjugation enzyme. Because it is likely that β-alanine interferes with the formation of taurinomethyluridine, mitochondrial proteins whose mRNA contains a large number of UUG codons are highly susceptible to β-alanine treatment. The cytochrome *c* oxidase subunit COI contains no leucine residues that are translated from a UUG codon, while the number of leucine residues that are translated from UUG codons in ND5 and ND6 are 2 and 8, respectively (Kirino et al. 2004). Consequently, β-alanine treatment mediates a 40% reduction in ND6 levels, ~30% in ND5 levels and no change in COI levels, effects largely prevented by inclusion of taurine in medium containing β-alanine (Fig. 5b). Also supporting the importance of taurine conjugation in the actions of taurine and its analogues is the finding that β-alanine-induced taurine deficiency affects ND5 levels but not the mRNA content of ND5. This suggests that the regulation of ND5 levels by β-alanine likely involves an improvement in the activity of $tRNA^{Leu(UUR)}$ rather than an increase in ND5 mRNA content (data not shown).

The mechanism by which β-alanine interferes with the conjugation of $tRNA^{Leu}$ is an area of some debate. Figure 1 shows that β-alanine dramatically reduces taurine content but β-alanine also appears to act as a competitive inhibitor of taurine-linked reactions. The present study does not address which action is responsible for the effects of β-alanine on mitochondrial integrity. Nonetheless, Jong et al. (2010) proposed that β-alanine functions primarily as an inhibitor of taurine-linked reactions, an assumption that depends upon the uptake of β-alanine by the cell and intracellular competition between β-alanine and taurine. In this regard it is relevant that taurine treatment alone has no effect on either mitochondrial protein synthesis or respiratory chain function. This may relate to the inability of extracellular taurine to appreciably alter the size of the intracellular taurine pool. By contrast, addition of taurine to medium containing β-alanine prevents the β-alanine-mediated decline in mitochondrial function. Taurine also prevents the β-alanine-mediated decrease in cellular taurine content, which could also explain the abrogation of the β-alanine-mediated mitochondrial defects. Clearly, clarification of the actions of the intracellular taurine pool and of β-alanine awaits the characterization of the taurine conjugation enzyme responsible for the formation of 5-taurinomethyluridine $tRNA^{Leu}$.

The β-alanine-mediated decrease in taurine levels is a slow process, reaching levels 70% of normal after 16 h of β-alanine exposure and 55% of normal after 48 h of exposure (Fig. 1). Other steps involved in the β-alanine initiated cascade exhibit a similar time course. The final step in the cascade is the generation of reactive oxygen species, which in turn has the potential of causing cell damage. One intriguing possibility is that taurine deficiency may trigger apoptosis through initiation of the mitochondrial permeability transition, which is dependent on both calcium overload and oxidative stress (Schaffer et al. 2003a, b; Halestrap et al. 2007). According to Wu and Prentice (2010) taurine prevents glutamate toxicity by blocking calcium overload-mediated apoptosis, an effect probably involving the mitochondrial permeability transition. The present study reveals the importance of cellular taurine levels in reducing oxidative stress, which would

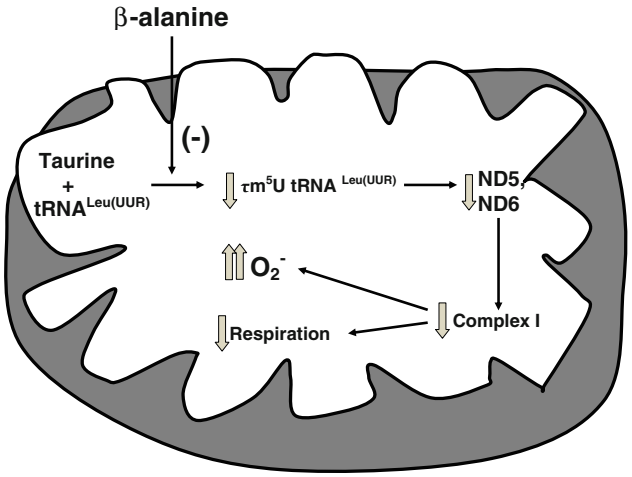


Fig. 6 Scheme for β-alanine-mediated increase in oxidative stress. β-Alanine reduces taurine actions through the downregulation of taurine levels and inhibition of taurine action. This leads to a reduction in 5-taurinomethyluridine (τm^5U) formation in the wobble position of $tRNA^{Leu(UUR)}$. As a result, UUG decoding is impaired and the biosynthesis of mitochondria encoded proteins, ND5 and ND6, decline. The assembly of respiratory chain complex I is defective, resulting in a fall in the activity of the respiratory chain complex. The formation of a bottleneck in the respiratory chain leads to the diversion of electrons from the respiratory chain to oxygen, forming in the process superoxide. The cell experiences significant oxidative stress

also be expected to play a role in taurine deficiency-linked apoptosis. Thus, taurine regulates two of the major causes of cellular toxicity, calcium overload and oxidative stress.

Conclusion

The present study uncovers a novel mechanism that provides an explanation for the antioxidant activity of taurine. Due to the structural similarity between taurine and β-alanine, we propose that the post-transcriptional modification of $tRNA^{Leu}$ by taurine is inhibited by β-alanine (Fig. 6). Because taurine conjugation affects the binding of the anticodon to the UUG codon, the synthesis of most of the mitochondria encoded proteins are negatively impacted. The assembly of the respiratory chain complexes depends upon an abundant supply of mitochondria encoded proteins, therefore, reduced levels of those proteins decrease the activity of the electron transport chain complexes. Because of this bottleneck in the electron transport chain, electrons are diverted from the sluggish respiratory chain to other acceptors, such as oxygen. This leads to an accumulation of damaging oxidants. By preventing this series of events, taurine functions as an indirect antioxidant.

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References

Aruoma OI, Halliwell B, Hoey BM, Butler J (1988) The antioxidant action of taurine, hypotaurine and their metabolic precursors. Biochem J 256:251–255

Bouckenooghe T, Remacle C, Reusens B (2006) Is taurine a functional nutrient? Curr Opin Coln Nutr Metab Care 9:728–733

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ (2003) Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 278:36927–36931

Eley DW, Lake N, ter Keurs HEDJ (1994) Taurine depletion and excitation-contraction coupling in rat myocardium. Circ Res 74:11210–11219

Gaull GE (1986) Taurine as a conditionally essential nutrient in man. J Am Coll Nutr 5:121–125

Grishko V, Pastukh V, Solodushko V, Gillespie M, Azuma J, Schaffer SW (2003) Apoptotic cascade initiated by angiotensin II in neonatal cardiomyocytes: role of DNA damage. Am J Physiol 285:H2364–H2372

Halestrap AP, Clarke SJ, Khalilin I (2007) The mitochondrial permeability transition pore—from molecular mechanism to reperfusion injury. In: Schaffer SW, Suleiman MS (eds) Mitochondria: the dynamic organelle. Springer Science + Business Media, New York, pp 241–269

Harada H, Cusack BJ, Olson RD, Stroo W, Azuma J, Hamaguchi T, Schaffer SW (1980) Taurine deficiency and doxorubicin: interaction with the cardiac sarcolemmal calcium pump. Biochem Pharm 39:745–751

Heller-Stilb B, van Roeyen C, Rascher K, Hartwig HG, Huth A, Seeliger MW, Warskulat U, Haeussinger D (2002) Disruption of the taurine transporter gene (taut) leads to retinal degeneration in mice. FASEB J 16:231–233

Huxtable RJ (1992) Physiological actions of taurine. Physiol Rev 72:101–163

Ito T, Kimura Y, Uozumi Y, Takai M, Muraoka S, Matsuda T, Ueki K, Yoshiyama M, Ikawa M, Okabe M, Schaffer SW, Fujio Y, Azuma J (2008) Taurine depletion caused by knocking out the taurine transporter gene leads to a cardiomyopathy with cardiac atrophy. J Mol Cell Cardiol 44:927–937

Jong CJ, Ito T, Mozaffari M, Azuma J, Schaffer S (2010) Effect of β-alanine treatment on mitochondrial taurine level and 5-taurinomethyluridine content. J Biomed Sci 17:S25

Kirino Y, Yasukawa T, Ohta S, Akira S, Ishihara K, Watanabe K, Suzuki T (2004) Codon-specific translational defect caused by wobble modification deficiency in mutant tRNA from a human mitochondrial disease. Proc Natl Acad Sci USA 101:15070–15075

Knopf K, Sturman JA, Armstrong M, Hayes KC (1978) Taurine: an essential nutrient for the cat. J Nutr 108:773–778

Pion PD, Kittleson MD, Rogers QR, Morris JG (1987) Myocardial failure in cats associated with low plasma taurine: a reversible cardiomyopathy. Science 237:764–768

Ricci C, Pastukh V, Leonard J, Turrens J, Wilson G, Schaffer D, Schaffer SW (2008) Mitochondrial DNA damage triggers mitochondrial-superoxide generation and apoptosis. Am J Physiol 294:C413–C422

Schaffer S, Solodushko V, Azuma J (2000) Taurine-deficient cardiomyopathy: role of phospholipids, calcium and osmotic stress. Adv Exp Med Biol 483:57–69

Schaffer SW, Solodushko V, Kakhniashvili D (2002) Beneficial effect of taurine depletion on osmotic sodium and calcium loading during chemical hypoxia. Am J Physiol 282:C1113–C1120

Schaffer S, Azuma J, Takahashi K, Mozaffari M (2003a) Why is taurine cytoprotective? In: Lombardini JB, Schaffer SW, Azuma J (eds) Taurine 5. Kluwer Academic/Plenum Publishers, New York, pp 307–321

Schaffer S, Solodushko V, Pastukh V, Ricci C, Azuma J (2003b) Possible cause of taurine deficient cardiomyopathy: potentiation of angiotensin II action. J Cardiovasc Pharmacol 41:751–759

Schaffer SW, Jong CJ, Ramila KC, Azuma J (2010) Physiological roles of taurine in heart and muscle. J Biomed Sci (in press)

Sturman JA (1993) Taurine in development. Physiol Rev 73:119–147

Turrens JF (2007) Formation of reactive oxygen species in mitochondria. In: Schaffer SW, Suleiman M-S (eds) Mitochondria: the dynamic organelle. Springer Science + Business Media, New York, pp 185–196

Wu JY, Prentice H (2010) Role of taurine in the central nervous system. J Biomed Sci 17(Suppl 1):S1