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ORIGINAL ARTICLE

Taurine plays an important role in the protection of spermatogonia from oxidative stress

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Abstract It has been demonstrated that taurine has various physiological functions in the body. We demonstrated that taurine is abundant in the serum, liver, muscle and testis of the Japanese eel (*Anguilla japonica*). In the eel testis, taurine is found mainly in spermatogonia and is weakly expressed also in the Sertoli cells. We have further found in the eel testis that taurine is actively accumulated via the sodium/chloride-dependent taurine transporter (TauT; SLC6A6), which is expressed in germ cells. In our current study, the effects of taurine on the anti-oxidant response were examined. Taurine was found to promote the total superoxide dismutase (SOD) activity in the testis. Moreover, our results indicate that taurine does not affect the mRNA levels of copper–zinc (Cu/Zn) SOD or manganese SOD, but promotes the translation of Cu/Zn SOD. Overall, our present data suggest that taurine may modulate Cu/Zn SOD at the translational level and thereby may play an important role in the protection of germ cells from oxidative stress.

Keywords Taurine · Cu/Zn SOD · Spermatogonia · Oxidative stress · Japanese eel

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Introduction

Taurine (2-aminoethanesulfonic acid) is a free β-amino acid that is present at high concentrations in several tissues. Taurine has been detected in the central nervous system, in the optic nerve and retina, in liver, cardiac, smooth and skeletal muscles, and in the testis and ovary (Campistron et al. 1986; Magnusson et al. 1989; Lee et al. 1992; Quesada et al. 1993; Lobo et al. 2000, 2001). Taurine has been shown to have various functions such as bile formation in the liver (Vessey 1978), osmoregulation (Lasserre and Gilles 1971), the stabilization of membranes (Huxtable and Bressler 1973), and calcium binding and transport regulation (Lazarewicz et al. 1985; Lombardini 1985). Taurine is transported into cells via the sodium/chloride-dependent taurine transporter (TauT; SLC6A6).

TauT has been previously characterized in a range of tissues in different organisms Ó Flaherty et al. 1997; Liu et al. 1992; Jhiang et al. 1993; Takeuchi et al. 2000; Zarate and Bradley 2007; Kozlowski et al. 2008; Pint et al. 2011), but not to our knowledge in the testis. However, it has been reported that taurine plays important roles in testosterone secretion in the rat testis (Yang et al. 2010) and that it promotes golden hamster sperm motility (Boatman et al. 1990). We have also reported that 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), the fish progestin, promotes taurine synthesis in the Japanese eel (*Anguilla japonica*) testis via the up-regulation of cysteine dioxygenase (CDO) mRNA and that taurine enhances spermatogonial proliferation (Higuchi et al. 2011). These data suggest that taurine may play important roles in the male reproductive system. Hence, it is of some importance to further elucidate the functional characteristics of taurine and TauT in the testis.

It has been shown in a number of studies that taurine supplementation is effective in the growth and in the

maintenance of normal physiological conditions in several fish species such as the Japanese flounder (Park et al. 2002; Kim et al. 2003, 2005), yellowtail (Matsunari et al. 2005) and red sea bream (Takagi et al. 2010). Taurine also acts as an antioxidant and protects against the toxicity of various substances in vivo (Green et al. 1991; Gürer et al. 2001; Balkan et al. 2002). Recently, also it was reported that taurine protects superoxide dismutase (SOD) pathway from cadmium-induced or arsenic oxidative stress (Sinha et al. 2008; Manna et al. 2009; Das et al. 2009). However, the mechanism underlying this property of taurine has not been fully determined.

The SODs are enzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide, which oxygen, in turn, is broken down to water by catalase in the peroxisomes, and by glutathione peroxidase in the cytosol and mitochondria. Three distinct types of SODs have been identified in mammals. Two isoforms have copper (Cu) and zinc (Zn) atoms in their catalytic center and are located either in the cytoplasm (Cu/Zn SOD) or in the extracellular elements (EC SOD). The third isoform has manganese (Mn SOD) as a cofactor and is located in the mitochondria (Zelko et al. 2002). Cells are normally protected against oxidative damage by multiple enzymatic mechanisms and by antioxidant molecules. The SODs are the first and most important line of antioxidant enzyme defense systems against oxidative stress such as that caused by superoxide anion radicals.

Germ cells are highly specialized cells that are responsible for the propagation of DNA, which directs the development of next generations. It is essential for organisms to maintain the integrity of germ cell DNA. In this regard, it has been demonstrated that developing rat germ cells and spermatozoa contain higher levels of SOD activity than the SOD activity levels of many somatic cells (Bauché et al. 1994) and that human spermatogonia showed immunoreactivity to Cu/Zn SOD, whereas only weak expression of this enzyme is observed in spermatocytes, and in other more differentiated germ cells and Sertoli cells (Nonogaki et al. 1992).

The aim of our present study was to further investigate the functional characteristics of taurine and TauT in the Japanese eel testis. We demonstrate that the accumulation of taurine in germ cells via the activities of TauT plays an important protective role against oxidative stress through the induction of Cu/Zn SOD.

Materials and methods

Animals

Cultivated male Japanese eels (*A. japonica*) were purchased from a commercial eel supplier and were maintained in a 500 l circulating freshwater tank at 23 °C. The fish were cultured for 1 week, and then killed by administering a 0.1 % dose of ethyl aminobenzoate anesthetic. The brains, gills, hearts, livers, spleens, muscles, blood and the testes were then sampled from each animal. Blood samples were held for 16 h at 4 °C. Afterwards, then the blood sample was centrifuged at 1,300×*g* for 10 min at 4 °C. Subsequently, the serum was collected and kept at −30 °C until experiment. The eels were not fed during the culture period. These experiments were conducted in accordance with the institutional animal ethics guidelines of Ehime University (Protocol Number 128, Permit Number H19-001).

Full cloning of taurine transporter and manganese SOD (Mn SOD)

cDNAs were reversely transcribed from RNA preparations of Japanese eel testes using SuperScript II (Invitrogen, Carlsbad, CA). The reactions contained 0.5 μg of oligo(dT)18 and 5 μg of total RNA in a 20 μl final volume. PCR was then carried out using the degenerate primers listed in Supplementary Table 1. Various cycling parameters were used until an amplicon of the expected size was obtained. To obtain the 5′- and 3′-ends of Japanese eel TauT and Mn SOD, SMART cDNAs were generated from testis RNA using a SMARTerTM RACE cDNA Amplification Kit (Clontech, Mountain View, CA). 5′- and 3′-RACE was then performed in accordance with the manufacturer's instructions using the primers listed in Supplementary Table S1. The obtained clones were then validated by repeat sequencing using a Genetic Analyzer 3130 (Applied Biosystems, Tokyo, Japan).

RT-PCR

Tissues were homogenized by a bead beating method using glass beads (1 mm) for 20–30 s with Mini-BeadBeater-1 (BioSpec Products, Inc., Bartlesville, OK) in Sepasol RNA I super (Nacarai Tesque, Kyoto, Japan). Total RNA was extracted from the homogenized lysates, and then cDNAs were then reversely transcribed using SuperScript II (Invitrogen). Gene-specific primers are listed in Supplementary Table S1. PCR was then performed in a thermal cycler (Takara, Kyoto, Japan) under the reaction condition listed in Supplementary Table S1. Each cycle consisted of a denaturation step at 94 °C for 1 min, annealing at 58.5 °C for 1 min, and extension at 72 °C for 1 min.

Testicular organ culture

Testicular organ cultures were established as described previously (Miura et al. 1991) with minor modifications. Briefly, freshly removed eel testes were cut into 1 × 1 × 0.5 mm^3 pieces and placed on floats of 1.5 %

Table 1 Free amino acid concentrations in the eel serum (μM), liver, muscle and testis (nano-mole/100 mg tissue) of the Japanese eel

Amino acid	Serum (μM)	Liver (nmole/100 mg tissue)	Muscle (nmole/100 mg tissue)	Testis (nmole/100 mg tissue)
Taurine	90.6 ± 4.97	220.0 ± 4.12	92.4 ± 5.62	360.0 ± 2.61
Alanine	74.7 ± 2.33	148.3 ± 3.90	25.0 ± 1.94	38.5 ± 3.30
Arginine	32.2 ± 2.25	0.8 ± 0.12	29.2 ± 4.67	6.8 ± 1.01
Aspartic acid	4.8 ± 0.84	20.1 ± 6.65	4.9 ± 0.80	23.4 ± 0.59
Cysteine	3.6 ± 0.90	0.6 ± 0.12	ND	23.0 ± 2.03
Glutamic acid	20.2 ± 1.26	118.5 ± 1.44	8.4 ± 0.84	123.5 ± 6.55
Glycine	37.6 ± 1.78	46.9 ± 1.72	32.0 ± 2.83	23.0 ± 2.03
Histidine	13.8 ± 0.82	7.4 ± 0.18	13.7 ± 2.40	1.7 ± 0.34
Isoleucine	19.0 ± 1.23	8.3 ± 1.37	6.6 ± 1.08	7.4 ± 2.99
Leucine	32.7 ± 2.53	14.4 ± 2.91	9.2 ± 1.29	9.0 ± 1.55
Lysine	85.1 ± 1.56	24.8 ± 2.76	77.2 ± 5.42	17.9 ± 3.32
Methionine	16.5 ± 0.05	5.5 ± 0.90	4.7 ± 0.22	4.6 ± 0.46
Phenylalanine	17.6 ± 1.56	5.9 ± 1.22	4.6 ± 0.10	2.1 ± 0.71
Proline	6.2 ± 0.04	ND	ND	ND
Serine	33.0 ± 2.68	12.2 ± 1.76	5.4 ± 0.24	13.3 ± 0.83
Threonine	78.1 ± 2.10	13.2 ± 1.81	6.9 ± 0.28	18.8 ± 1.32
Tryptophan	3.9 ± 0.44	0.4 ± 0.17	0.2 ± 0.02	0.05 ± 0.01
Tyrosine	15.4 ± 0.91	4.2 ± 1.99	3.9 ± 0.18	3.4 ± 0.42
Valine	40.4 ± 2.30	15.2 ± 1.16	9.7 ± 1.58	12.3 ± 1.18
β-Alanine	3.6 ± 1.09	10.6 ± 1.26	11.9 ± 2.6	1.1 ± 0.27

Values are the means ± SEM ($n = 3$)

ND not detected

agarose covered with a nitrocellulose membrane in 24-well plastic tissue culture dishes. The basal medium consisted of Leibovitz L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 1.7 mM proline, 0.1 mM aspartic acid, 0.1 mM glutamic acid, 0.5 % BSA, 1 mg/l bovine insulin, and 10 mM HEPES, adjusted to pH 7.4. To confirm the active taurine uptake in the testis, testicular fragments were cultured with taurine (0, 0.8, 80 or 8,000 μM) or with a combination of taurine (0, 0.8, 80 or 8,000 μM) and β-alanine (80 μM), a known competitive inhibitor of taurine uptake (WAKO, Osaka, Japan) for 6 days. To analyze the effects of taurine on SOD activity, testicular fragments were cultured with taurine (0, 0.8, 8, 80 or 800 μM) or with a combination of taurine (0, 0.8, 8, 80 or 800 μM) and β-alanine (80 μM) for 6 days. Testicular organ cultures were established at ratio of two testicular fragments per 1.75 ml of L-15 medium.

Western blot analysis

Tissue samples were homogenized in 20 mM Tris–HCl (pH 8.0) containing 1 mM EDTA, 1 mM PMSF and centrifuged at 10,000×*g* for 10 min at 4 °C. The supernatant was then mixed with an equal volume of sample buffer [0.125 M Tris–HCl at 4 % (wt/vol), SDS at 20 % (vol/vol), glycerol at 0.05 % (wt/vol), and bromophenol blue] containing 10 % 2-mercaptoethanol. All samples were boiled for 10 min after mixing with sample buffer. The protein concentrations were determined using a protein assay rapid kit (Wako), and 10 μg of protein was electrophoresed in 12.5 % polyacrylamide gels. The gels were then blotted onto PVDF membranes (Millipore, Billerica, MA). The immunostained product in Western blotting was identified using an anti-SLC6A6/TauT antibody (MBL, Nagoya, Japan) at a dilution of 1:3,000. After washing, the membrane was incubated with alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Vector Laboratories, Marion, IA) diluted 1:3,000 in Can Get Signal solution 2 (Toyobo) for 1 h. After further washing, AP activity was visualized using CDP-Star Detection Reagent (GE Healthcare, Tokyo, Japan) and analyzed using a LAS-4000 device (GE Healthcare). The Western blot was stripped and re-probed with the anti-α-tubulin mouse monoclonal antibody (1:2,000; Sigma–Aldrich, St Louis, MO), which was used for equal loading control. Western blot analysis was also performed to investigate the effects of taurine on the expression of Cu/Zn SOD or Mn SOD. Cultured testicular fragments that had been exposed to taurine (0, 0.8, 8, 80 or 800 μM) were homogenized in the same buffer described above. Ten micrograms of protein was resolved by SDS-PAGE and transferred to a PVDF membrane. The membrane was then immersed overnight at 4 °C in a solution containing anti-Cu/Zn SOD antibody (Stressgen, Victoria, Canada) at a dilution of 1:2,000 in Can Get Signal solution 1 or with anti-Mn SOD antibody (Stressgen) at a dilution of 1:2,000, and further processed and analyzed as described above.

Immunohistochemical analysis of SLC6AC/TauT

Eel testes were sampled and fixed in Davidson's fixative, embedded in paraffin wax, and cut into 5 μm serial sections. The sections were deparaffinized and dehydrated through Xylene/Ethanol/PBS gradients. Slides were subjected to antigen retrieval by autoclaving at 121 °C for 5 min in Tris–EDTA buffer [10 mM Tris–HCl, 1 mM EDTA, containing 0.05 % Tween-20 (pH 8.0)]. Five percentage skimmed milk in PBS (pH 7.4) was used for blocking. Immunohistochemical staining using an anti-SLC6A6/TauT antibody (MBL) was then performed using a Histofine SAB-AP (R) kit (Nichirei Biosciences, Tokyo, Japan). Negative controls were prepared by omitting the primary antibody which yielded no reaction product (data not shown).

Measurement of free amino acids

Eel liver, muscle, testis, and cultured testis tissues were homogenized in 20 volumes of distilled H_2O with an ultrasonic homogenizer. The homogenates were then centrifuged at $1{,}000\times g$, and the supernatants were collected into a clean test tube. The samples or serum was mixed with an equivalent volume of 10 % trichloroacetic acid, and centrifuged at $10{,}000\times g$ for 5 min at 4 °C. The supernatants were then collected into a clean test tube. A 50 % volume of hexane was added to remove lipids via mixing and centrifugation at $10{,}000\times g$ for 5 min at 4 °C. The aqueous layer was filtered through a 0.22-μm syringe RC-membrane filter (Sartorius Stedim Biotech, Goettingen, Germany), and the free amino acids concentrations were assayed by reverse-phase high-performance liquid chromatography (HPLC) (Hitachi, Tokyo, Japan) using an OPA reagent set (Wako, Osaka, Japan). Amino acid standard solution type B and type AN-II (Wako) were used as reference standards.

Immunohistochemical analysis of taurine

Taurine immunohistochemistry using an anti-taurine polyclonal antibody (Millipore, Billerica, MA) was performed as described previously (Lobo et al. 2000) with minor modifications. Briefly, eel testes were sampled and fixed in Davidson's fix solution supplemented with 0.5 % glutaraldehyde, embedded in paraffin, and cut into 5 μm serial sections. The sections were then deparaffinized in xylene, hydrated in a graded ethanol series, and then incubated for 15 min in 0.03 % H_2O_2 in methanol, and then washed by PBS. The sections were incubated in 5 % skimmed milk in PBS (pH 7.4) to reduce non-specific background staining at room temperature for 45 min. This was followed by an overnight incubation at 4 °C with a rabbit anti-taurine antibody diluted 1:1,000. The sections were then immunocytochemically stained using the biotin/streptavidin/peroxidase complex (HRP/DAB) method. The signal was amplified using Intensi/Fire (Diagnostic BioSystems, Pleasanton, CA) according to the manufacture's instruction.

SOD enzymatic activity analysis in cultured testis

To measure the SOD activity levels in the eel testis, cultured testicular fragments were homogenized in sucrose buffer. After adjusting the concentration of protein to 1 mg/ml, a SOD activity assay was performed with the SOD Assay Kit-WST (Dojindo, Kumamoto, Japan) using tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and xanthine. Forty microliters of the resultant solution was used to prepare the samples in accordance with the supplier's protocol. One unit (U) of SOD activity was defined as the amount of protein that inhibited tetrazolium reduction to 50 % of the maximum level. The results were expressed as U/mg protein. Prior to these experiments, we confirmed that taurine used in the experiment did not show any total SOD activity levels using the method described above.

Statistical analysis

All values were expressed as the mean ± SEM. Statistical analysis of these values was performed using one-way ANOVA with the Tukey's multi-comparison test via KaleidaGraph statistical software.

Results

Cloning of eel TauT and Mn SOD from the Japanese eel

The TauT cDNA isolated from the Japanese eel was found to be 2,271-bp long, with an ORF encoding a protein of 625 amino acids (DDBJ/EMBL/GenBank accession number AB674512) and showing an approximately 85 and 75 % similarities to this transporter in other fish and mammalian species, respectively (Supplementary Fig. S1). The deduced amino acid sequence also corresponded closely to that of eel TauT reported previously (Chow et al. 2009). However, some differences were found with previously published sequence, most notably at the C-terminus.

The isolated Japanese eel Mn SOD cDNA fragment was a 1,059-bp fragment encoding predicted amino acid sequence of 221 residues (accession number AB674513). Sequence analysis revealed conserved sequence motifs, such as the Mn SOD signature DVWEHAYY. Database

searches further showed that the deduced amino acid sequence of this clone is highly homologous to Mn SOD across teleosts and mammals (Supplementary Fig. S2).

Free amino acid concentration in eel serum and tissues

The free amino acid composition values of Japanese eel serum (μM), liver, muscle and immature testis (nano-mole/100 mg tissue) were measured using HPLC, and are listed in Table 1. The taurine concentration in the serum, liver, muscle and testis was measured at 90.6 ± 4.97 μM, and at 220.0 ± 4.12, 92.4 ± 5.62 and 360.0 ± 2.61 nano-mole/100 mg tissue, respectively. The concentration of β-alanine, a competitive taurine transport inhibitor, in the serum, liver, muscle and testis was 3.6 ± 1.09 μM, and at 10.6 ± 1.26, 11.9 ± 2.6 and 1.1 ± 0.27 nano-mole/100 mg tissue, respectively.

Localization of taurine in the eel testis

To determine the distribution of taurine in eel testis, we performed immunohistochemistry using anti-taurine antibodies (Fig. 1). Taurine was mainly detectable in the spermatogonia in immature testis, but was only weakly expressed in the somatic cells or the Sertoli cells surrounding spermatogonia, and not at all in connective tissues.

Active taurine uptake in the eel testis

To confirm whether there is an active taurine uptake into the eel testis, in vitro organ cultures of this tissue were established. After the day six of culture with increasing concentrations of taurine (0, 0.8, 80, 8,000 μM) the taurine levels in the tissue were measured by HPLC at 174.3 ± 4.57, 230.9 ± 4.08, 532.1 ± 35.0, 1,056.5 ± 28.8 (nano-mole/100 mg testis), respectively. The taurine level in the testis just prior to culture was 218.8 ± 12.4 (nano-mole/100 mg testis) and that in the taurine-treated groups (80 and 8,000 μM) was significantly higher than in the control groups. Taurine uptake was found to be blocked significantly by the administration of β-alanine, which is a competitive taurine transport inhibitor (Fig. 2).

Expression of TauT in different eel tissues

RT-PCR analysis of TauT was performed in various eel tissues (brain, gill, heart, liver, spleen, muscle and testis;

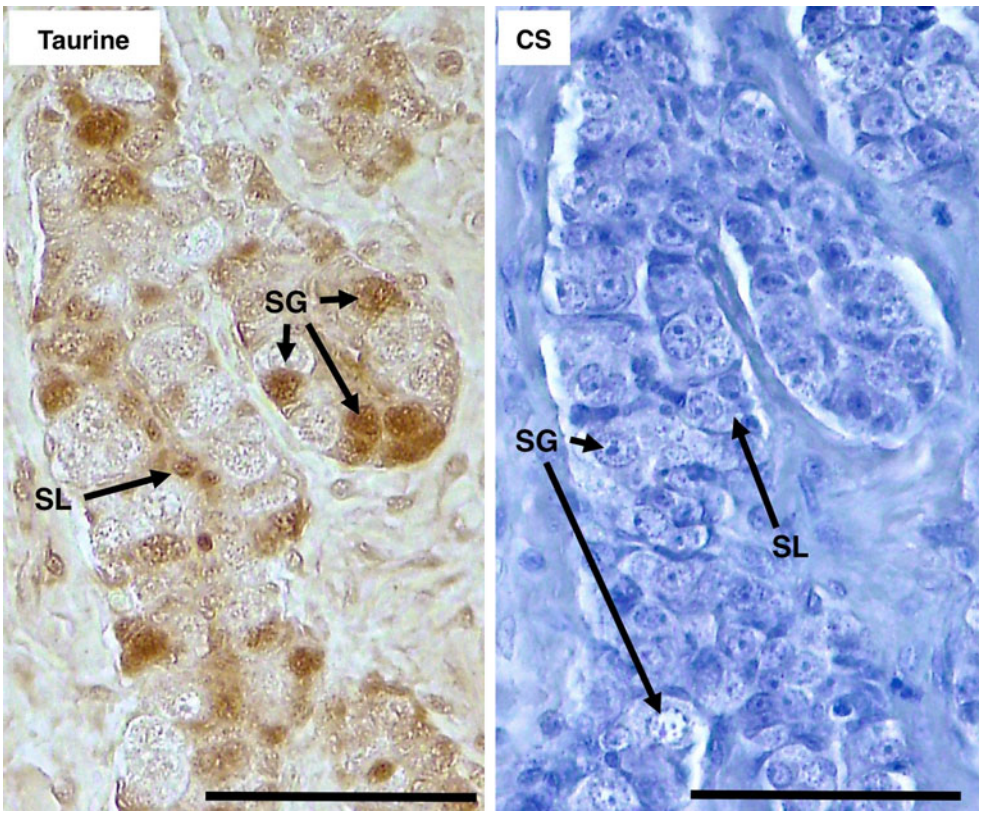

Fig. 1 Localization of taurine in the eel testis assessed by immunohistochemistry (peroxidase method with DAB colorization). Anti-taurine, testis immunostaining with anti-taurine antibodies. Brown staining indicates positive immunoreactivity. CS, counterstaining with hematoxylin. *SG* spermatogonia, *SL* Sertoli cell or somatic cell. *Bar* 50 μm

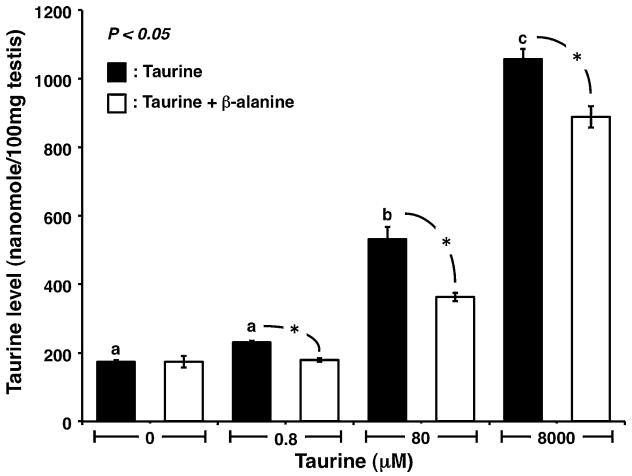

Fig. 2 Taurine levels (nano-mole/100 mg testis) in eel testis cultured with taurine or with a combination with taurine and *β*-alanine (80 μM) ($n = 6$ per group). Values are the mean ± SEM. The *different letters* and *asterisk* indicate significant differences ($P < 0.05$)

Fig. 3a). Western blot analysis of this transporter in the same tissues revealed bands with an equivalent molecular mass of 70 kDa, which was consistent with the predicted molecular weight (69.9 kDa). The TauT protein showed clear expressed in the gills, heart, liver, spleen and testis, but was only weakly detectable in the brain and muscle (Fig. 3b).

Localization of TauT in the eel testis

To determine the distribution of TauT at different germ cell stages in the eel testis, we performed immunohistochemical analysis using anti-TauT antibodies. High expression was detected in spermatogonia, but TauT showed much weaker levels at other stages of germ cell development including the spermatocytes, spermatids, and spermatozoa (Fig. 4).

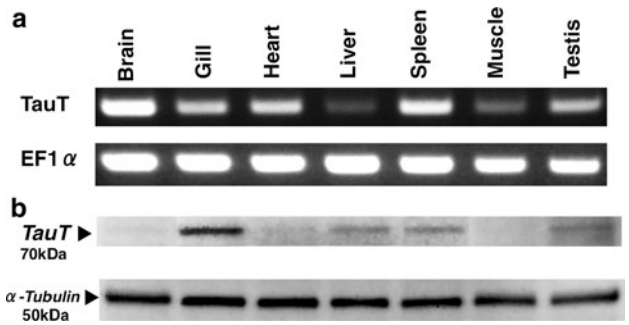

Fig. 3 RT-PCR analysis of eel TauT in the brain, gill, heart, liver, spleen, muscle and testis. EF1α was used as an internal control (**a**). Results of Western blotting analysis in the same eel tissues using a commercial human anti-TauT antibody. α-Tubulin was used as a reference protein (**b**)

Effects of taurine on the SOD activity in the eel testis

To investigate the direct effects of taurine on SOD activity in testis, in vitro testicular organ cultures were performed. Testicular fragments from the eel were cultured with increasing concentrations of taurine (0.8, 8, 80 or 800 μM) for 6 days, and the total SOD activities in these cultures were assayed. This activity in the taurine-treated groups was significantly higher than in the control groups in a dose-dependent manner with the peak found at 80 μM of taurine treatment. *β*-Alanine administration (80 μM) was found to significantly inhibit the increases in SOD activity induced by taurine (Fig. 5).

Expression of Cu/Zn SOD and Mn SOD in cultured eel testis

Taurine treatment increased the total SOD activity in cultured testis as mentioned above. Three types of SODs have been identified in mammals: Cu/Zn SOD, Mn SOD and EC SOD. To our knowledge, functional EC SOD has not been identified in teleosts. Therefore, we investigated the action of taurine on the expression of Cu/Zn SOD and Mn SOD. The semi-quantitative PCR analysis revealed that taurine did not affect mRNA expression of Cu/Zn SOD or Mn SOD (Fig. 6a). However, Western blot analysis showed that the protein expression of Cu/Zn SOD was remarkably higher in taurine-treated groups. On the other hand, remarkable difference was not observed in the expression level of Mn SOD (Fig. 6b).

Discussion

Taurine is found at high concentrations in the plasma, tissues and seminal fluids of both mammals and fish (Billardo and Menezo 1984; Sturman et al. 1988; Karlsson et al. 2006). Our present analysis reveals that taurine is present in the serum, liver muscle and testis Japanese eel (*A. japonica*) at far higher concentrations than any other free amino acids. These results suggested that taurine plays important physiological roles in eel tissues.

Taurine was principally detectable in spermatogonia and weakly in the Sertoli cells or somatic cells in eel testis. In rat, it has been reported that taurine is present in rat Leydig cells, vascular endothelial cells, and other interstitial cells within the seminiferous tubules, but not in the germ cells at any spermatogenic stage and or the Sertoli cells (Lobo et al. 2000). However, there has been no other reported information to date concerning the taurine distribution in the immature and maturating testis in other vertebrates.

In our present study, we investigated the levels of active taurine uptake by the eel testis using an in vitro testicular

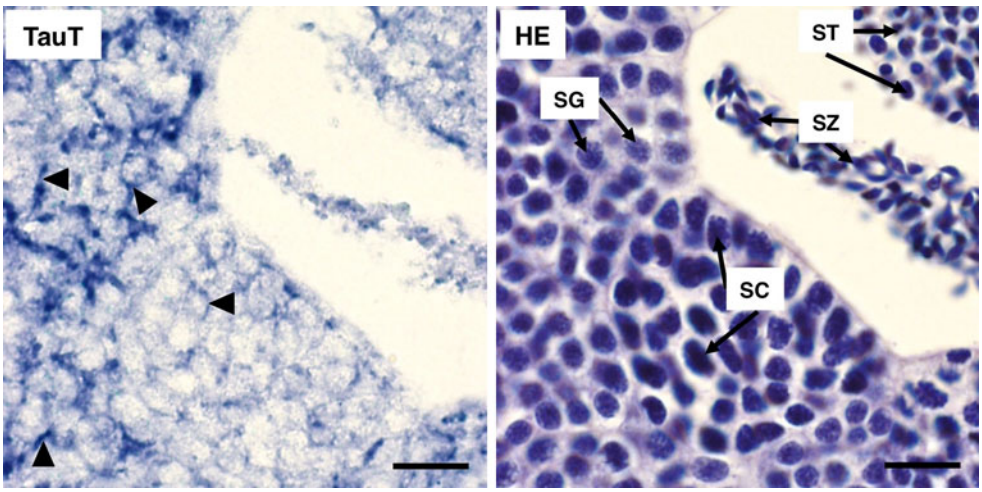

Fig. 4 Localization of TauT in matured eel testis assessed by immunohistochemistry (alkaline phosphatase method with NBT/BCIP colorization). TauT, eel testis immunostained with anti-TauT antibodies. HE, Testis section stained with hematoxylin and eosin. *SG* spermatogonia, *SC* spermatocyte, *ST* spermatid, *SZ* spermatozoa. *Arrowheads* indicate areas of positive immunoreactivity. *Scale bar* 10 μm

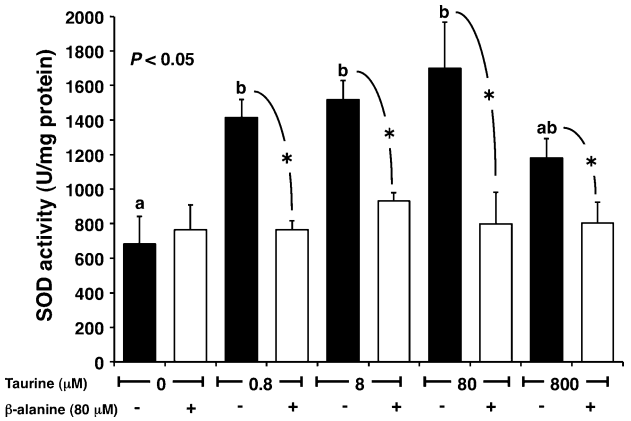

Fig. 5 Total SOD activity assay in cultured eel testis ($n = 6$ per group). Values are the mean ± SEM. *Different letters* indicate significant differences among the taurine-treated groups (*black columns*). *Asterisk* indicates significant differences between cultured testis in the presence of taurine with or without β-alanine ($P < 0.05$)

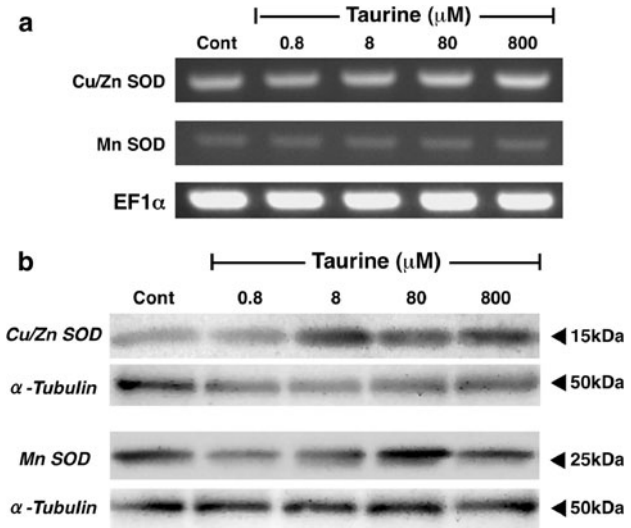

Fig. 6 **a** RT-PCR analysis of Cu/Zn SOD and Mn SOD in cultured eel testis in the presence of increasing concentrations of taurine (0.8, 8, 80 or 800 μM). **b** Western blot analysis of Cu/Zn SOD and Mn SOD in eel testes cultured with increasing concentrations of taurine (0.8, 8, 80 or 800 μM). α-Tubulin was used as a reference protein

organ culture system. A positive uptake was confirmed, and found to be inhibited by administration of β-alanine, a known taurine transporter inhibitor (Allo et al. 1997). Taurine is transported into cells via the sodium/chloride-dependent taurine transporter (TauT), which is found in both mammalian (Ó Flaherty et al. 1997; Liu et al. 1992; Jhiang et al. 1993; Ramamoorthy et al.1994) and fish tissues (Takeuchi et al. 2000; Zarate and Bradley 2007; Kozlowski et al. 2008; Pint et al. 2011). As previously mentioned, previous reports have suggested that TauT is also expressed in eel testis, and thus we attempted to clone the cDNA that encodes TauT from testis of Japanese eels in our current study, which was successfully done. RT-PCR and Western blotting confirmed that TauT is expressed in eel testis but also in the gill, hearts, liver and spleen of this species. However, TauT protein was very weakly expressed in the brain or heart although mRNA level is comparatively abundant. Recently, post-transcriptional regulations such as riboswitch, microRNA or RNA-binding protein have been reported (Ray et al. 2009; Mattick and Makunin 2006; Glisovic et al. 2008). These reports demonstrated that protein levels do not always correlate with mRNA levels. Our results also indicated that the TauT protein level does not always correlate with mRNA levels in eel tissues. Furthermore, immunohistochemistry

revealed that TauT is highly expressed in spermatogonia, but only weakly in other different types of germ cells such as spermatocytes, spermatids and spermatozoa. To our knowledge, our current study is the first report to show the distribution of TauT in the testis at different germ cell stages.

The mechanisms underlying the differential expression profile of TauT at different germ cell stages is still unclear. However, the spermatogonia are highly proliferative, and we have previously reported that taurine promotes spermatogonial proliferation (Higuchi et al. 2011). Hence, our past and present findings together suggested that the demand for taurine is higher in proliferative spermatogonia than in germ cells at other developmental stages. In addition, we also reported in our earlier study that both CDO and cysteine sulfinate decarboxylase, which are essential enzymes in the taurine synthesis pathway, are expressed in Sertoli cells in eel testis (Higuchi et al. 2011). These results indicate that taurine is synthesized in the Sertoli cells and then transported into the germ cells via TauT.

Taurine plays an important role in several essential biological processes such as the development of the central nervous system and the retina, calcium modulation, membrane stabilization, reproduction, and immunity (Schuller-Levis et al. 1990; Huxtable 1992; Sturman 1993). Taurine also functions as an antioxidant and protects against the toxicity of various substances in vivo (Green et al. 1991; Gürer et al. 2001; Balkan et al. 2002; Sinha et al. 2008; Manna et al. 2009). In our present study, we examined the in vitro effects of taurine on the SOD activity levels in testis. Cultured testicular fragments exposed to taurine in the medium showed higher SOD activities than the control groups and this effect was blocked by β-alanine. RT-PCR analysis revealed that taurine does not affect the gene expressions of either Cu/Zn SOD or Mn SOD, but Western blot analysis indicated that taurine induced the protein expression of Cu/Zn SOD only. These data infer that accumulated taurine into testis via TauT promoted Cu/Zn SOD protein synthesis. It has been reported that several hormones such as prolactin, melatonin or growth hormone induce Cu/Zn SOD mRNA expression (Sugino et al. 1998; Antolin et al. 1996; Hauck and Bartke 2000), and that the testicular 65 kDa protein specifically binds to the 5′ UTR of testicular Cu/Zn SOD mRNA and represses its translation in mice (Gu and Hechet 1996). However, there has been no report to date describing the up-regulation of Cu/Zn SOD protein synthesis. To our knowledge, our current study is the first to report that taurine increased the protein expression of Cu/Zn SOD in testis in vitro.

Oxidative stress has been implicated in germ cell apoptosis and DNA damage (Celino et al. 2009; Paul et al. 2009; Aitken and Krausz 2001). From this perspective, taurine would play an important role in the protective mechanisms operating in germ cells by promoting the expression of Cu/Zn SOD in the cytosol of spermatogonia. Furthermore, it has been shown previously that Cu/Zn siRNA decreases the number of Cu/Zn SOD-positive spermatogonia and increases the level of oxidative damage (Celino et al. 2011). In our current analysis, we show that TauT is highly expressed in spermatogonia, but weakly expressed in spermatocytes, spermatids and spermatozoa. Also, previously, we have reported that Cu/Zn SOD was highly expressed in spermatogonia, and weakly expressed in spermatocytes, spermatids and spermatozoa (Celino et al. 2011). These data collectively suggest that taurine transport via TauT is important for the protection of spermatogonia from oxidative stress, although the mechanism underlying the regulation of TauT expression in the testis remains unclear. Moreover, in our present analysis, taurine was also found to augment the SOD activity levels in cultured eel primary hepatocytes (data was not shown). These results suggested that taurine may also protect other cells from oxidative stress.

The mechanisms by which taurine induces Cu/Zn SOD are unclear but three possibilities exist. The first of these may involve the anti-oxidant properties of taurine. It is well known that taurine removes hypochlorous acid (HOCl) by reacting with it to form chlorotaurine (Timbrell et al. 1995; Cunningham et al. 1998), and that HOCl stress impairs SOD activity (Maalej et al. 2006). However, sulfur-containing amino acids such as cysteine or methionine can also scavenge HOCl, and these amino acids are present at high levels in the L-15 culture medium used in the organ culture (cysteine 0.99 mM, methionine 0.5 mM) compared with the supplemented taurine (0.8–800 μM) concentrations. It is therefore difficult to favor this hypothesis. The second possibility may be that taurine promotes Cu/Zn SOD protein synthesis. It has been reported that several amino acids such as leucine can individually activate signaling pathway to promote protein synthesis and possibly inhibit autophagy-mediated protein degradation (Anthony et al. 2000; Rhoads and Wu 2008). It was further found that small molecules directly bind to non-coding regions of mRNA and regulate gene expression, known as RNA switches or riboswitches (Winkler et al. 2002; Rodionov et al. 2003; Sudarsan et al. 2003; Mandal et al. 2004). These are mainly found in bacteria, fungi and plants, but were recently identified in human cells (Ray et al. 2009). Moreover, two novel taurine-containing modified uridines are present in human and bovine mitochondrial tRNAs (Suzuki et al. 2002). However, whether taurine can bind to mRNAs and modulate gene expression is not certain at present, and will require further study. The third possibility may be an interaction between taurine and zinc. It is reported that taurine stimulates Zn (II) absorption in human fibroblasts and in rainbow trout intestine (Harraki et al. 1994; Glover

and Hogstrand 2002), and Nusetti and co-workers have demonstrated that low zinc concentrations increase taurine uptake and high concentrations inhibit taurine transport (Nusetti et al. 2010). We have also demonstrated previously that zinc modulates eel testicular Cu/Zn SOD activity (Celino et al. 2011). Furthermore, β-alanine is known as competitive taurine transport inhibitor, and we confirmed it in taurine uptake experiment. In addition, β-alanine also inhibited competitively the promotion of spermatogonial proliferation by taurine (Higuchi et al. 2011). By contrast, β-alanine inhibited non-competitively the up-regulation of SOD activity by taurine. These data indicate the possibility that taurine transport may regulate zinc transport and β-alanine may inhibit zinc transport by inhibition of taurine transport. In this regard, to clarify this possible mechanism, it will be necessary in the future to analyze the interaction between taurine, zinc and Cu/Zn SOD.

In conclusion, taurine is present at high levels in various Japanese eel tissues. In the testis, although the mechanism underlying these protective effects are not yet clear, by promoting Cu/Zn SOD activity it may be possible that taurine and taurine transport clearly may play an important role in protecting germ cells from oxidative stress. There are several possible mechanisms by which taurine promotes Cu/Zn SOD activity, and in this regard our current findings may provide a solid basis for directing future studies in not only reproduction but also other fields of biology.

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