Amino Acids (2012) 43:845–855
DOI 10.1007/s00726-011-1141-6

ORIGINAL ARTICLE

Beneficial effect of taurine on hypoxia- and glutamate-induced endoplasmic reticulum stress pathways in primary neuronal culture

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Received: 29 August 2011 / Accepted: 25 October 2011 / Published online: 13 November 2011

Abstract Stroke (hypoxia) is one of the leading causes of mortality in the developed countries, and it can induce excessive glutamate release and endoplasmic reticulum (ER) stress. Taurine, as a free amino acid, present in high concentrations in a range of organs in mammals, can provide protection against multiple neurological diseases. Here, we present a study to investigate the potential protective benefits of taurine against ER stress induced by glutamate and hypoxia/reoxygenation in primary cortical neuronal cultures. We found that taurine suppresses the up-regulation of caspase-12 and GADD153/CHOP induced by hypoxia/reoxygenation, suggesting that taurine may exert a protective function against hypoxia/reoxygenation by reducing the ER stress. Moreover, taurine can down-regulate the ratio of cleaved ATF6 and full length ATF6, and p-IRE1 expression, indicating that taurine inhibits the ER stress induced by hypoxia/reoxygenation and glutamate through suppressing ATF6 and IRE1 pathways.

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Keywords Taurine · Neuroprotection · Hypoxia · Endoplasmic reticulum stress · Glutamate

Introduction

Stroke is the third cause of mortality and one of the leading causes of serious, long-term disability in developed countries. Of all strokes, 87 percent are hypoxic, and 13 percent are hemorrhagic. Glutamate is the main excitatory neurotransmitter in the mammalian nervous system. However, excessive levels of extracellular glutamate are excitotoxic and lead to neuronal death. Cerebral hypoxia and/or ischemia result in a decrease of oxygen and glucose which in turn induce the release of glutamate at the presynaptic level. The high levels of glutamate and the subsequent activation of glutamatergic postsynaptic receptors are the main components of a cascade of sequential molecular events that culminates the death of neurons (Choi and Rothman 1990; Nicholls and Attwell 1990; Nilsson and Lutz 1991; Rothman and Olney 1986).

There is increasing evidence that ER stress plays a crucial role in hypoxia/ischemia-induced cell dysfunction (Azfer et al. 2006; DeGracia and Montie 2004; Kumar et al. 2001; Paschen et al. 1998). The ER is an important subcellular organelle that is responsible for intracellular calcium homeostasis, protein secretion and lipid biosynthesis (Anelli and Sitia 2008; Ma and Hendershot 2004; Pizzo and Pozzan 2007). Hypoxia triggers the accumulation of unfolded proteins in the ER, leading to the unfolded protein response (UPR) and ER-associated protein degradation (ERAD) (Kaufman 1999). The UPR coordinates processes of modified gene transcription and protein translation in order to reduce ER stress, whereas the ERAD pathway up-regulates protein degradation, thereby eliminating

accumulated misfolded or unfolded proteins. Both steps help to reestablish the cell's physiological state. The proximal signaling pathways that are initiated in response to the UPR include activation of double-stranded RNA dependent protein kinase-like endoplasmic reticulum kinase (PERK), transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1), which in turn activate distinct signaling cascades mediating the ER stress response (Harding et al. 2000a; Wang et al. 1998; Yoshida et al. 1998). In the neuronal homeostasis, PERK, ATF6, and IRE1 activities are inhibited by binding to glucose regulated protein 78 (GRP78), an ER chaperone. Under ER dysfunction, GRP78 dissociates from PERK, ATF6, and IRE1, inducing the dimerization and phosphorylation of PERK and IRE1, and cleavage of ATF6 (P90) to ATF6 (P50). Activated PERK in turn phosphorylates eukaryotic translation initiation factor 2 subunit α (eIf2α), which suppresses global protein synthesis (Prostko et al. 1993; Ron 2002). Moreover, phosphorylation of eIF2α also leads to the paradoxical increase in translation of ATF4 (Harding et al. 2000a). All of these three pathways up-regulate the transcription factor C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153) (Oyadomari and Mori 2004). Caspase-12 is known to be essential for this ER stress-induced apoptosis (Yoneda et al. 2001).

Taurine, 2-aminoethanesulfonate, is the most prevalent of the amino acids found in the brain, skeletal muscle and cardiac muscles (Huxtable 1992; Sturman 1993). Taurine has been applied to treat multiple neurological diseases including Alzheimer's disease, Huntington's disease, brain ischemia and heart ischemia (Paula-Lima et al. 2005; Tadros et al. 2005; Takahashi et al. 2003; Takatani et al. 2004b). However, the mechanisms underlying the functions of taurine are still not fully understood. Our previous studies show that taurine can protect PC12 cells against ER stress induced by oxidative stress and cortical neurons against ER stress induced by glutamate (Pan et al. 2010a, b). In this study, we investigated the protective effects of taurine in cortical neurons against hypoxia/reoxygenation and glutamate cytotoxicity. Furthermore, we analyzed which ER stress pathway can be inhibited by taurine during hypoxia/reoxygenation and conditions of glutamate exposure.

Materials

Fetal bovine serum, basal media Eagle, poly-D-lysine, glutamine, glucose and taurine were purchased from Sigma (St. Louis, MO, USA). Neurobasal medium was purchased from Invitrogen (Carlsbad, CA, USA). Mouse anti-actin, rabbit anti-CHOP/GADD153, rabbit anti-caspase-12 antibodies, secondary mouse and rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p-eIF2α and mouse anti-eIF2α were purchased from Cell Signaling Technology (Boston, MA, USA). Mouse anti-ATF6 was purchased from Imgenex (San Diego, CA, USA). Rabbit Anti-ATF4, Rabbit anti-p-IRE1 and rabbit anti-IRE1 were purchased from Abcam (Cambridge, MA, USA). Adenosine 5′-triphosphate (ATP) Bioluminescent Assay kit was purchased from Promega (Madison, WI, USA). RIPA buffer was purchased from Thermo Scientific (Rockford, IL, USA). Pregnant Sprague–Dawley rats were purchased from Harlan (Indianapolis, IN) and housed in the animal care facility at Florida Atlantic University. The procedures for the care and use of rats, in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals, were approved by the Institutional Animal Care and Use Committee of Florida Atlantic University.

Primary cortical neuronal cell culture

Primary cortical neuronal cell cultures were prepared by a previously described protocol (Chen et al. 2001). Briefly, the pregnant rats were killed after isoflurane exposure, embryos at 16–18 days were removed and brains were isolated from the fetuses and kept in basal media eagle (BME) supplemented with 2 mM glutamine, 26.8 mM glucose, and 20% heat-inactivated fetal bovine serum. This medium is referred to as growth media eagle (GME). The cortices were then dissociated by passing the tissue through a 14-G cannula. Cells were centrifuged at 300 g/min for 5 min at room temperature. The obtained pellet was resuspended in GME and plated on appropriate tissue culture plates pre-coated with 5 ug/ml of poly-D-lysine. Cells were maintained for 1 h in a humidified incubator (37°C, 99% humidity and 5% CO_2) before the incubation medium was replaced with serum-free neurobasal medium supplemented with 2% B27 and 500 uM glutamine. The cells were maintained in an incubator for 12–18 days until they were ready for handling.

Hypoxia/reoxygenation

To generate hypoxic conditions, 14-day cultured neurons in 6 or 96 well plates were placed in a hypoxia chamber with oxygen levels maintained at 0.3–0.4%. The level of oxygen was continuously monitored using an oxygen electrode. Primary cortical neuronal cultures in the presence or in the absence of taurine were subjected to 20 h of hypoxia. Reoxygenation was performed by removing cultured plates from the hypoxic chamber and transferring

them into normal culture incubator remaining for another 20 h.

Glutamate toxicity

For glutamate-induced toxicity, neurons at 14 days in vitro were preincubated with different concentrations or 10 mM taurine for 1 h. Then the neurons were treated with 100 uM glutamate for another 1 h or 10 min.

Measurement of cell viability

Cells were measured using the ATP assay. ATP solution was added to assigned wells of 96 well plates and cells were incubated for 10 min. Then the levels of ATP were quantified in a luciferase reaction. The luminescent intensity was measured using a luminometer (SpectraMax, Molecular Devices) after transferring the lysate to a standard opaque-walled multi-well plate.

Western blot analysis

Primary cortical neuronal cultures were lysed in RIPA buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1% (v/v) mammalian protease inhibitor cocktail and 1% (v/v) phosphatase inhibitor cocktail from Sigma and Thermo Scientific, respectively. Proteins in cell lysates were separated on a SDS-PAGE. After proteins were transferred to a nitrocellulose membrane, the membrane was then blocked in blocking buffer (20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20, 5% milk) for 1.5 h at room temperature. After blocking, the corresponding primary antibody was incubated for 1 h, followed by 1-h incubation with the corresponding HRP-conjugated secondary antibody at room temperature. Extensive washes with blocking buffer were performed between each step. The protein immuno-complex was visualized using ECL detection reagents purchased from Thermo Scientific. Quantitative Western blot results were obtained by densitometric analysis using Image J.

Statistical analysis

All data were expressed as the mean ± SEM. The statistical significance of the data was determined with one-way ANOVA combined with Dunnett post hoc test for comparison between groups. Differences of $P < 0.05$ were considered statistically significant. At least three independent replicates were performed for each experiment.

Results

Taurine demonstrates robust neuroprotective activity against hypoxia/reoxygenation on primary neuronal cultures

In order to determine the appropriate concentration of taurine in cultures, cortical neurons were exposed to hypoxia and reoxygenation in the presence or in the absence of 1–10 mM taurine as shown in Fig. 1a. After hypoxia and reoxygenation, ATP levels for neurons without taurine treatment dropped to about 49% (percentage of control). Taurine treatment dramatically increases the cell viability. The presence of 1 mM taurine clearly improved the cell viability to greater than 70%. With increasing taurine concentrations up to 10 mM cell viability increased to 85%.

Taurine inhibits the expression of CHOP and caspase-12 induced by hypoxia/reoxygenation

To determine the effect of taurine on ER stress induced by hypoxia/reoxygenation, we preincubated 10 mM taurine for 1 h, followed by hypoxia and reoxygenation. The expression of CHOP was measured by Western blot analysis, as shown in Fig. 1b. The expression of CHOP was up-regulated after exposure to hypoxia/reoxygenation. Western blot analysis shows that the levels of both caspase-12 and cleaved caspase-12 are highly up-regulated after hypoxia/reoxygenation (Fig. 1c). Taurine significantly reduced the expression of CHOP, caspase-12 and cleaved caspase-12, demonstrating that taurine has the ability to inhibit the apoptosis induced by ER stress in hypoxia/reoxygenation.

ATF6 and IRE1 pathways were inhibited by taurine under hypoxia/reoxygenation, but there was no effect on the PERK pathway

It is well established that there are three ER stress-induced signaling pathways: PERK, ATF6 and IRE1 pathways. Since taurine can protect neurons against ER stress induced by hypoxia, we aimed to further identify which signaling pathway is involved in the protective process. Both p-eIF2α and ATF4 are highly expressed after hypoxia/reoxygenation, increased by approximately 1.7- and 3.0-fold over control. After treatment with taurine, followed by hypoxia/reoxygenation, however, the levels of p-eIF2α in cortical neurons are similar to that of hypoxia/reoxygenation alone (Fig. 2a), indicating that taurine does not inhibit the initiation of the PERK pathway under this condition. Similarly, the expression of ATF4 for neurons treated with taurine and hypoxia/reoxygenation (~3.0 times that of control) does not change compared to that of hypoxia/reoxygenation alone (Fig. 2b).

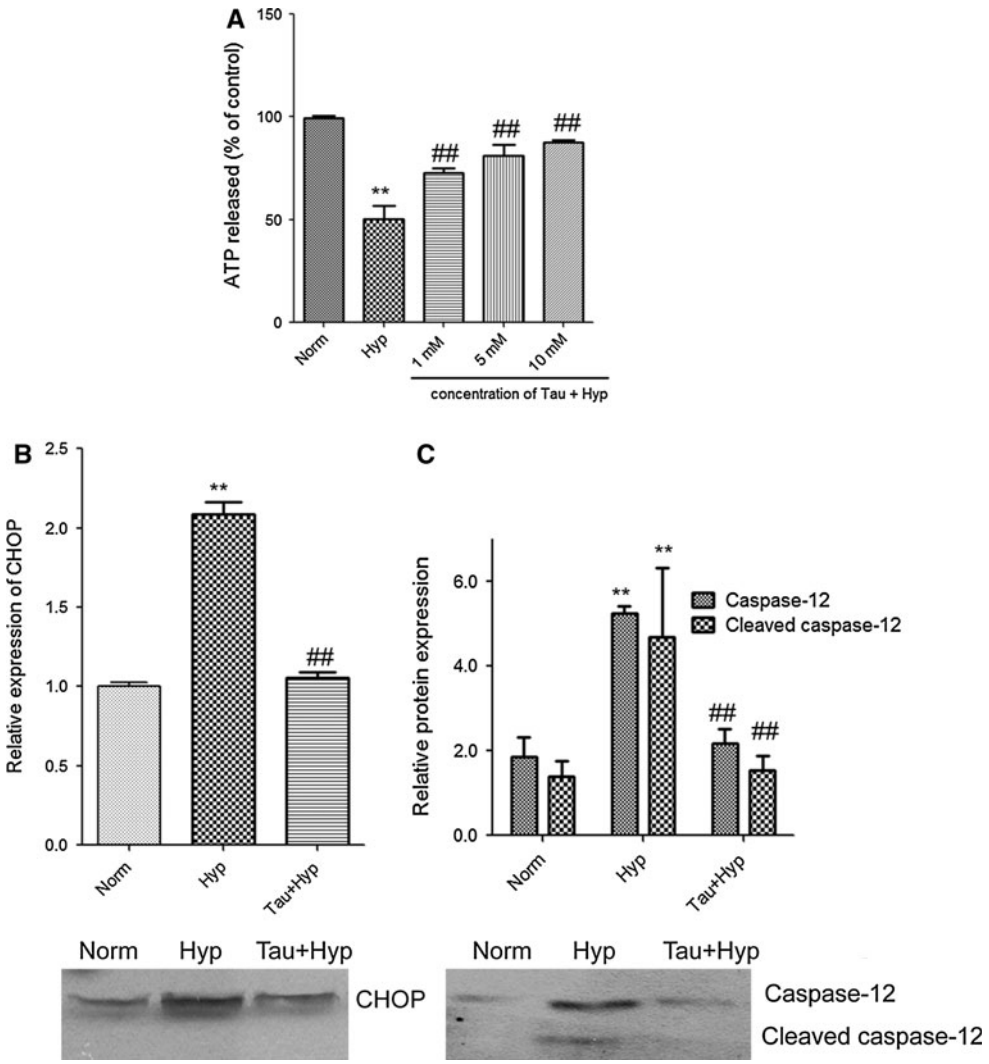


Fig. 1 Neuroprotective effects of taurine against ER stress induced by hypoxia/reoxygenation. Primary neuronal culture was supplied with different concentration of taurine. *Norm* normoxia; *Hyp* hypoxia (0.3% O_2) for 20 h, reoxygenation for 20 h; Tau + Hyp: neurons were treated with 1, 5 and 10 mM or only 10 mM taurine for 1 h, then hypoxia for 20 h, reoxygenation for 20 h. **a** Dose-dependent neuroprotection of taurine against hypoxia/reoxygenation. Cell viability was measured by ATP assay. Control values were fixed at 100%. The values for Hyp and Tau + Hyp were normalized relative to the control values and represent mean ± SEM of 5 preparations. **b** CHOP expression analyzed by Western blot. The bar graphs reflect the densitometric data from the experiment of CHOP Western blot results with arbitrary units. **c** Caspase-12 expression analyzed by Western blot. The bar graphs reflect the densitometric data from the experiment of caspase-12 and cleaved caspase-12 Western blot results with arbitrary units. The values in bar graph represent mean ± SEM, $n = 3$, $^{**}P < 0.01$ versus Norm and $^{\#\#}P < 0.01$ versus Hyp

This result indicated that taurine has no observable effects on PERK pathway activation.

We next examined the effect of taurine on the ATF6 pathway in cortical neurons induced by hypoxia/reoxygenation. Treatment with taurine considerably reduced the level of cleaved ATF6. The ratio of cleaved ATF6 to ATF6 in neurons treated with taurine dramatically declined by ~50% relative to neurons under hypoxia/reoxygenation without taurine as shown in Fig. 2c. These results demonstrate that taurine can prevent the activation of the ATF6 pathway in hypoxia/reoxygenation.

To determine if taurine affects the IRE1 pathway induced by hypoxia/reoxygenation, we tested the expression of p-IRE1 in rat cortical neurons with and without taurine treatment under hypoxia/reoxygenation conditions by Western blot analysis (Fig. 2d). The results show that phosphorylated IRE1 is highly expressed in cortical neurons under hypoxia/reoxygenation. Taurine reverses the expression of p-IRE1 to its normal condition, demonstrating that taurine significantly inhibits the IRE1 pathway in ER stress induced by hypoxia/reoxygenation.

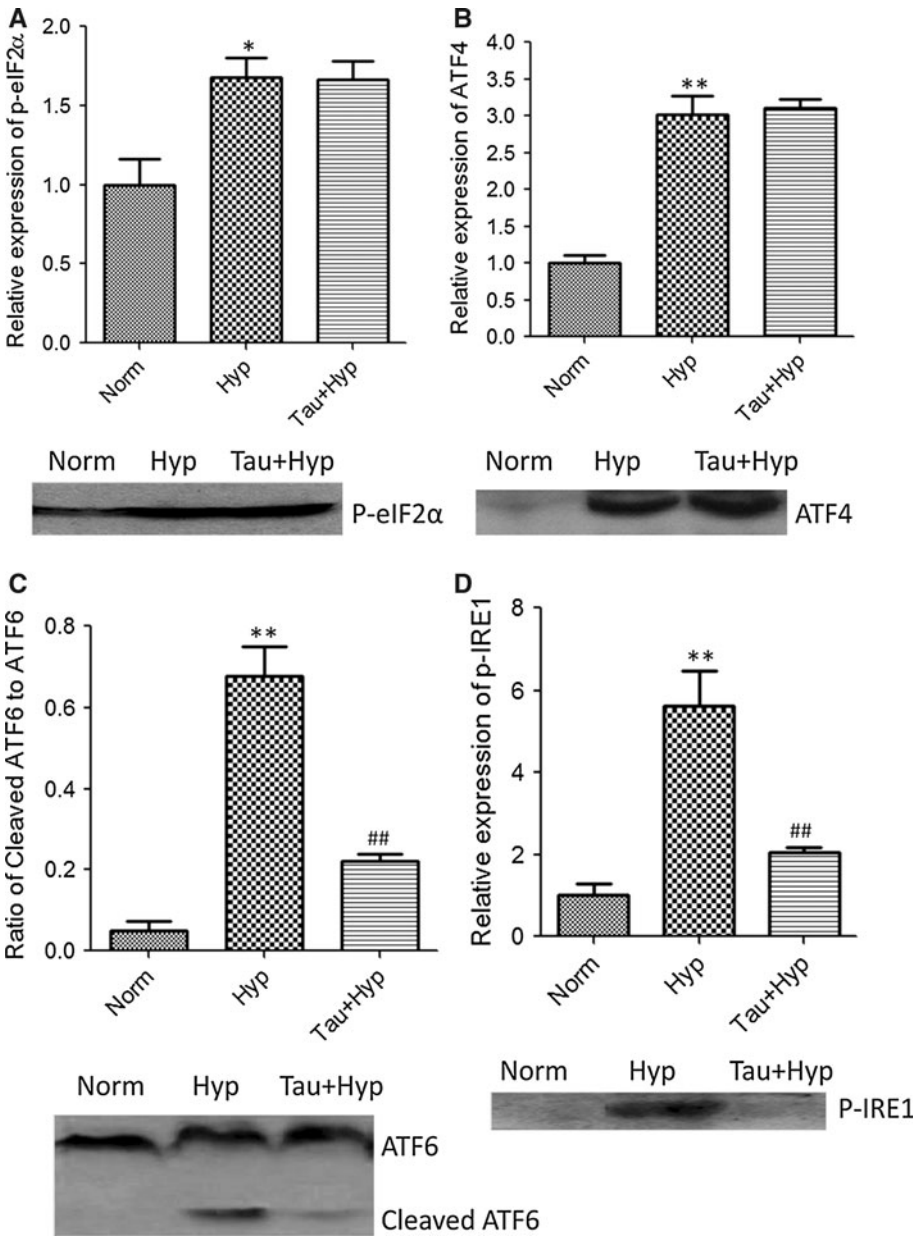


Fig. 2 Taurine does not alter activity of the PERK pathway, but inhibits the ATF6 and IRE1 pathway after hypoxia/reoxygenation. *Norm* normoxia; *Hyp* hypoxia (0.3% O_2) for 20 h, reoxygenation for 20 h; *Tau* + *Hyp* neurons were treated with 10 mM taurine for 1 h, then hypoxia for 20 h, reoxygenation for 20 h. **a** P-eIF2α expression analyzed by Western blot. The bar graphs reflect the densitometric data from the experiment of P-eIF2α Western blot results with arbitrary units. **b** ATF4 expression analyzed by Western blot. The bar graphs reflect the densitometric data from the experiment of ATF4 Western blot results with arbitrary units. **c** ATF6 expression analyzed by Western blot. The bar graphs represent the ratio of cleaved ATF6 to ATF6 using the densitometric data from the experiment of ATF6 Western blot results with arbitrary units. **d** P-IRE1 expression analyzed by Western blot. The bar graphs reflect the densitometric data from the experiment of P-IRE1 Western blot results with arbitrary units. The values in bar graph represent mean ± SEM, $n = 3$, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus Norm; $^{\#\#}P < 0.01$ versus Hyp

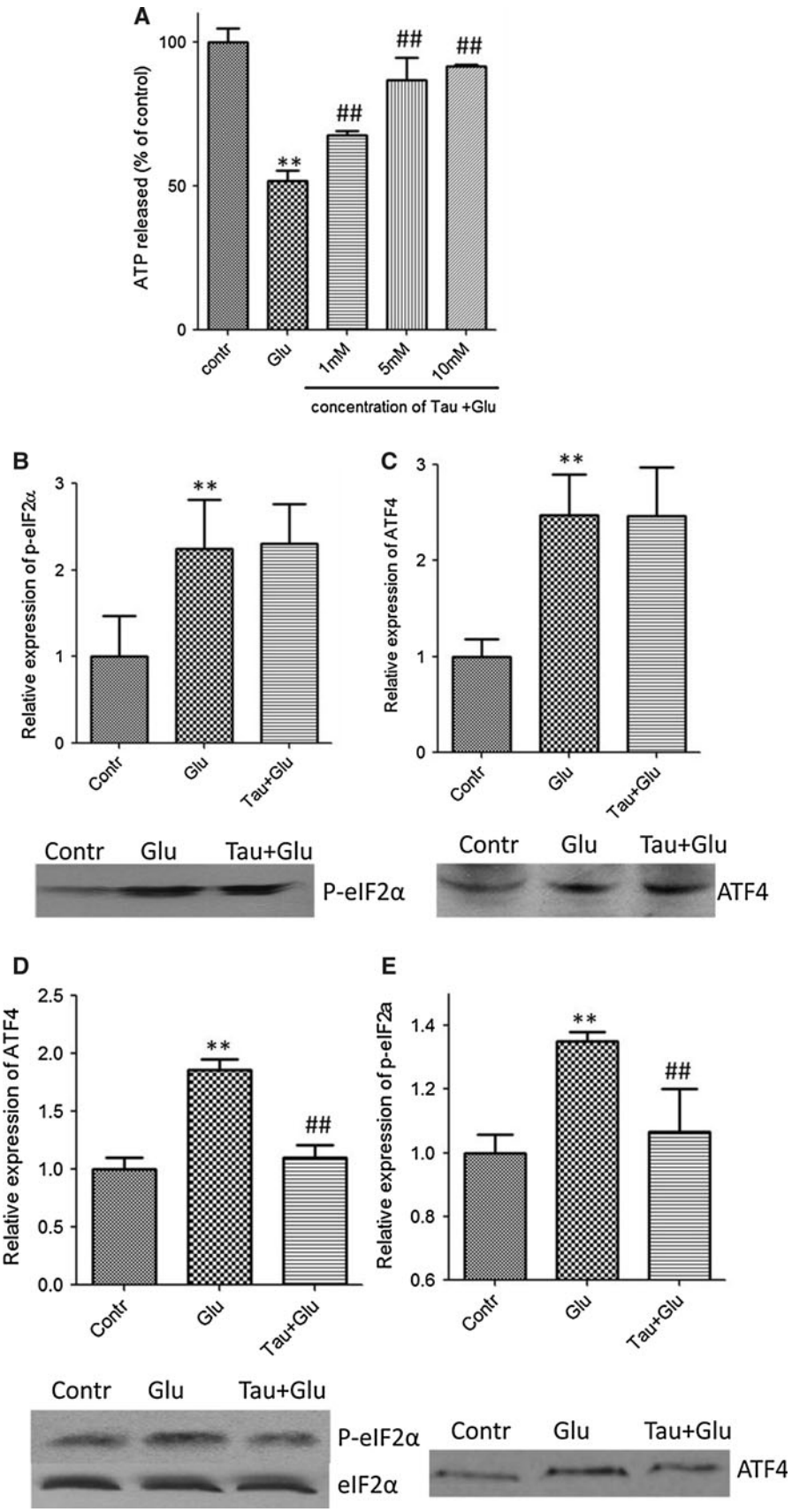
Taurine strongly suppresses the toxicity of glutamate in primary cortical neuronal cultures

As shown in Fig. 3a, taurine protection is dose-dependent in cortical neurons exposed to glutamate. Taurine can significantly augment the level of cellular viability following exposure to glutamate at concentrations as low as 1 mM. As compared to controls, taurine at 10 mM increased cell viability, by greater than 90%. Higher levels of taurine did not produce any further increase. Thus, we chose the 10 mM taurine as the optimal concentration for the following Western blot analysis.

Here, we further investigated the effects of altered concentrations of taurine on protection of neurons against glutamate, and specifically focused on the effects of taurine on three ER stress pathways (PERK, ATF6 and IRE1 pathways) induced by glutamate.

Taurine moderately inhibits the PERK pathway at 10 min after induction by glutamate but has no effect at later time points

We have shown that expression of p-eIF2α and ATF4 did not alter with taurine treatment in hypoxia/reoxygenation, which suggested that taurine may not be acting on the PERK pathway. Because hypoxia can induce the release of glutamate in neurons, we aimed to investigate whether taurine would influence the PERK pathway by measuring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◀ **Fig. 3** Neuroprotective effects of taurine against ER stress induced by 100 uM glutamate. Taurine can inhibit the PERK pathway against glutamate toxicity in the short-term, but not over a long-time frame. *Contr* control; *Glu* 100 uM glutamate treatment; *Tau* + *Glu* neurons were treated with 1, 5 and 10 mM or only 10 mM taurine for 1 h, then exposed to 100 uM glutamate for 1 h or 10 min. **a** Dose-dependent neuroprotection of taurine against 100 uM glutamate for 1 h. Cell viability was measured by ATP assay. Control values were fixed at 100%. The values for Glu and Tau + Glu were normalized relative to the control values and represent mean ± SEM of 5 preparations. **b** P-eIF2α expression analyzed by Western blot. Cells were submitted with 10 mM taurine for 1 h, then exposed to 100 uM glutamate for another 1 h. The bar graphs reflect the densitometric data from the experiment of P-eIF2α Western blot results with arbitrary units. **c** ATF4 expression analyzed by Western blot. Cells were submitted with 10 mM taurine for 1 h, then exposed to 100 uM glutamate for another 1 h. The bar graphs reflect the densitometric data from the experiment of ATF4 Western blot results with arbitrary units. **d** P-eIF2α and eIF2α expressions analyzed by Western blot. Cells were submitted with 10 mM taurine for 1 h, then exposed to 100 uM glutamate for 10 min. The bar graphs reflect the densitometric data from the experiment of P-eIF2α Western blot results with arbitrary units. **e** ATF4 expression analyzed by Western blot. Cells were submitted with 10 mM taurine for 1 h, then exposed to 100 uM glutamate for 10 min. The bar graphs reflect the densitometric data from the experiment of ATF4 Western blot results with arbitrary units. The values in bar graph represent mean ± SEM, $n = 3$, $^{**}P < 0.01$ versus Contr and $^{\#\#}P < 0.01$ versus Glu

the expression of p-eIF2α and ATF4 after neurons were subjected to glutamate excitotoxicity. As shown in Fig. 3b, c, the levels of p-eIF2α and ATF4 were examined by Western blot after neurons were treated with taurine for 1 h, then exposed to 100 uM glutamate for another 1 h. Glutamate induced the up-regulation of both p-eIF2α and ATF4 levels, by comparison to control neurons. Quantitation of the Western blot by densitometric scanning showed approximately a 2.2-fold increase in p-eIF2α and 2.5-fold increase in ATF4 expression after glutamate treatment alone. Comparing with and without taurine treatment following glutamate exposure, p-eIF2α and ATF4 levels demonstrated no significant change; indicating taurine may not affect the PERK pathway in ER stress induced by exposure to glutamate for 1 h (lanes 2 and 3 of Fig. 3b, c).

Glutamate-induced neuronal dysfunction and cell death may occur rapidly through glutamate receptors, even in a few minutes (Cheng et al. 1999). To determine if taurine has an effect on the PERK pathway during ER stress in short-term excitotoxicity, we applied 100 uM glutamate to treat the neurons for 10 min after cultures were exposed to taurine. Within 10 min of excitotoxicity induced by glutamate, there was a moderate increase in both p-eIF2α and ATF4 production relative to control, about 0.8- and 0.35-fold, respectively (lanes 1 and 2 of Fig. 3d, e). Intriguingly, taurine treatment prevented the expression of p-eIF2α and ATF4, indicating that the PERK pathway is inhibited by taurine in short-term glutamate treatment (Fig. 3d, e lane 3).

Taurine shows significant inhibitory effects on both the ATF6 and IRE1 pathways under ER stress induced by glutamate

Taurine did not inhibit the PERK pathway in prolonged exposure to glutamate (1 h), which is similar as neurons exposed to hypoxia/reoxygenation. We next examined if ATF6 and IRE1 pathways were affected by taurine followed by glutamate exposure for 1 h. After cultured neurons were treated with 10 mM taurine for 1 h followed by 100 uM glutamate for 1 h, the ATF6, cleaved ATF6 and p-IRE1 were analyzed by Western blot, as shown in Fig. 4. The ratio of cleaved ATF6 to ATF6 expression increased by glutamate excitotoxicity was noticeably reduced by taurine treatment (Fig. 4a). In addition, as shown in Fig. 4b, the p-IRE1 level induced by glutamate toxicity was substantially and considerably decreased by taurine treatment, from greater than fivefold to ~1.5-fold relative to control culture. These results suggest that both ATF6 and IRE1 pathways were blocked or at least partially attenuated by taurine.

Discussion

Taurine, a major intracellular free amino acid, is known to be able to protect against tissue damage in a variety of diseases (Birdsall 1998). Several protective mechanisms of taurine have been proposed. Taurine was first shown to be capable of improving osmotic status and calcium homeostasis in cell damage caused by hypoxia or glutamate excitotoxicity (Chang et al. 2004; Idrissi and Trenkner 1999; Michalk et al. 1997).As an antioxidant, taurine protects tissues against reactive oxygen species generation from hypoxia or from Mn-superoxide dismutase inhibition in the myocardial mitochondria (Chen et al. 2009). In addition, taurine is also a GABA agonist and may increase GABA levels by increasing GABA synthesis and by GABAA receptor activation (Paula-Lima et al. 2005; Tadros et al. 2005). Several papers provided the evidence that taurine exerts protective effects through prevention of mitochondrial dysfunction (Chang et al. 2004; Chen et al. 2009; Hansen et al. 2010). We recently demonstrated that ER stress inhibition may also be involved in taurine protection mechanisms under conditions of glutamate excitotoxicity (Pan et al. 2010b). However, details of the relevant signaling pathways remain to be elucidated. In this paper, ATP levels are significantly enhanced by taurine relative to hypoxia/reoxygenation or glutamate treatment alone, indicating high cell viability and protective effects upon treatment with taurine in these two toxic conditions. Taurine was shown to regulate mitochondrial protein synthesis, enhance electron transport chain activity, and thereby

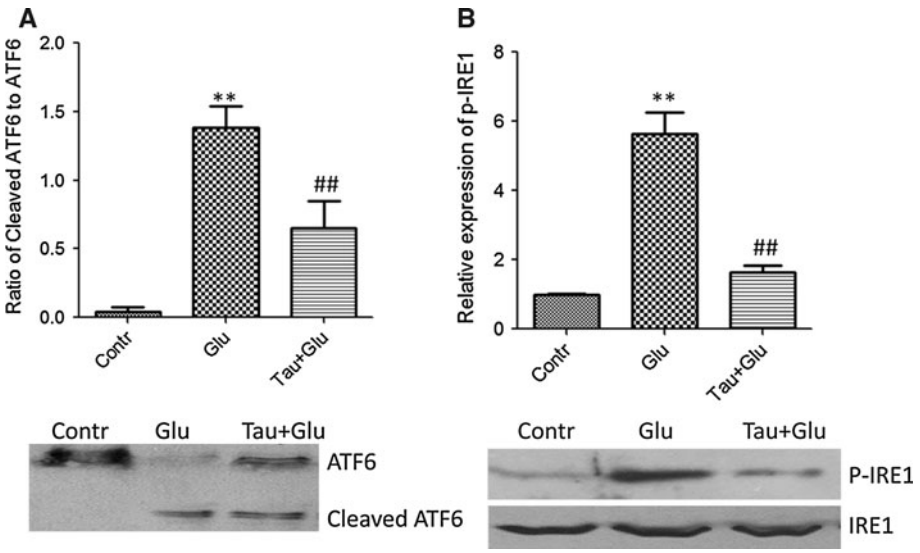

Fig. 4 Taurine inhibits the ATF6 and IRE1 pathways after exposure to glutamate for 1 h. *Contr* control; *Glu* 100 uM glutamate; *Tau* + *Glu* neurons were treated with 10 mM taurine for 1 h, followed by 100 uM glutamate for another 1 h. **a** ATF6 expression analyzed by Western blot. The bar graphs represent the ratio of cleaved ATF6 to ATF6 using the densitometric data from the experiment of ATF6 Western blot results with arbitrary units. **b** P-IRE1 and IRE1 expressions analyzed by Western blot. The bar graphs reflect the densitometric data from the experiment of P-IRE1 Western blot results with arbitrary units. The values in bar graph represent mean ± SEM, $n = 3$, $^{**}P < 0.01$ versus Contr $^{\#\#}P < 0.01$ versus Glu

increase the ATP levels and protect against the excessive superoxide generation (Jong et al. 2011; Schaffer et al. 2009). The activation of an ER localized kinase Akt is attenuated by ER stress (Yung et al. 2007). Taurine, as one of prominent osmolytes, is proven to exert preventative effects on ischemia-induced apoptosis by up-regulation of Akt phosphorylation (Takatani et al. 2004a). Therefore, taurine may affect the pathways related to ER stress. Thus, the studies presented here demonstrated that taurine has a beneficial effect on the protection of cortical neurons against ER stress resulting from hypoxia/reoxygenation. Furthermore, we investigated the effect of taurine on specific ER stress pathways during hypoxia/reoxygenation or glutamate treatment.

Accumulating evidence shows that ER stress plays a crucial role in the mechanisms underlying the pathological events of hypoxic or ischemic cell damage. ER stress responses involve apoptotic signals when the ER stress is severe and prolonged. The proapoptotic factor CHOP is expressed at low levels under physiological conditions, but is strongly induced in ER stress under hypoxic conditions (Nemetski and Gardner 2007; Oyadomari and Mori 2004; Paschen et al. 1998; Szegezdi et al. 2006; Tajiri et al. 2004). We have demonstrated a substantial induction of CHOP levels after exposure to hypoxia/reoxygenation, as shown in Fig. 1b, and this increase was prevented by administration of taurine. Taurine decreases caspase-8 and caspase-9 expression induced by ischemia in the mouse hypothalamic nuclei, indicating that taurine participates in the regulation of the death receptor-mediated and mitochondrial apoptotic pathway (Taranukhin et al. 2008). Caspase-12, which was identified as the first ER-associated member of the caspase family, is activated by ER stress, and this novel caspase is implicated in the cell death-executing mechanisms of ER stress (Nakagawa et al. 2000; Yoneda et al. 2001). We have analyzed the expression of caspase-12 in the presence or in the absence of taurine after treatment with hypoxia/reoxygenation, and demonstrated that the caspase-12 or cleaved caspase-12 expression was clearly reduced by taurine following hypoxia/reoxygenation relative to "no drug" conditions. The results indicating suppression of both CHOP and caspase-12 by taurine treatment provide substantial evidence that taurine can contribute to an effective inhibition of ER stress induced by hypoxia/reoxygenation.

The three ER-resident transmembrane proteins, PERK, ATF6 and IRE1, corresponding to three ER stress-induced signaling pathways, serve as the major proximal sensors of the ER stress response. In this paper, our primary aim is to identify which particular ER stress-induced pathway can be affected by taurine treatment during the process of hypoxia/reoxygenation in the cortical neuronal culture model. Under ER stress condition, PERK has proved to be responsible for repressing global protein synthesis via phosphorylation of the α subunit of eIF2α (Harding et al. 2000a, b; Kumar et al. 2001). Phosphorylation of eIF2α, on the other hand, can also indirectly control gene

transcription by positively regulating the translation of transcription factors as has been shown for mammalian ATF4 (Szegezdi et al. 2006). Since p-eIF2α and ATF4 are two down-stream proteins in the PERK pathway of ER stress, it is appropriate to measure expression levels of these two proteins in order to determine the PERK pathway response in the presence or in the absence of taurine treatment. We found that hypoxia/reoxygenation results in a strong increase in p-eIF2α and ATF4 expression, indicating that the PERK pathway is activated by hypoxia/reoxygenation. However, there are no significant alterations in protein levels of p-eIF2α and ATF4 for neurons in hypoxia/reoxygenation with taurine compared to "no drug" conditions. These results suggest that taurine may have neither suppressed nor facilitated the activation of the PERK pathway, which is responsible for attenuating protein translation in an attempt to restore neurons to homeostasis during ER stress process. Consistent with the above results, taurine was also found to have no effect on the expression of p-eIF2α and ATF4 under glutamate treatment for 1 h compared with no taurine treatment. In short-term exposure to glutamate, taurine appears to be able to delay activation of the PERK pathway as indicated by the results that p-eIF2α and ATF4 levels are moderately reduced by taurine treatment (see in Fig. 3d, e), this may be due to taurine delaying ER stress induction over the short-term. For a longer-term exposure to toxic conditions, neurons stimulate p-eIF2α expression to decrease the levels of unfolded proteins. At this later stage, taurine may have little effect on the PERK pathway. Overall, it is reasonable to conclude that taurine may delay the initiation of the PERK pathway at an early time point, but not change the activation of PERK pathway under chronic stress caused by hypoxia or glutamate.

ATF6 and IRE1 pathways are two branches of ER stress signaling pathways. After dissociation of GRP78, ATF6 (90 kDa, P90) translocates from the ER to the Golgi apparatus where it is cleaved to its active form (cleaved ATF6) by site-1 and site-2 proteases (S1P and S2P) (Chen et al. 2002). Quantifying the ratio of cleaved ATF6 to full length ATF6 have shown that taurine clearly inhibits ATF6 cleavage under the toxic conditions of either hypoxia/reoxygenation or glutamate treatment. Phosphorylation of IRE1 is a characteristic signal of activation of the IRE1 pathway in ER stress. Therefore, levels of p-IRE1 in both hypoxia/reoxygenation- and glutamate-induced cell death were measured to test whether taurine has an effect on the IRE1 pathway. The results indicate that the elevation of p-IRE1 is strongly suppressed by taurine treatment, either from hypoxia/reoxygenation or glutamate toxicity. These findings provide strong evidence that activation of the IRE1 pathway can be inhibited by taurine.

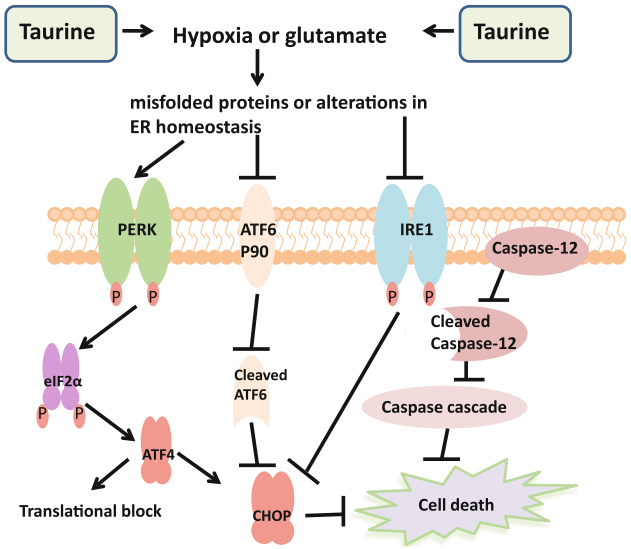

Fig. 5 Scheme for protective effects of taurine against activation of ER stress pathways. After neurons were submitted with glutamate or hypoxia, the homeostasis in neuronal culture is disturbed, which initiates dimerization and autophosphorylation of ER membrane proteins PERK and IRE1. ATF6 (P90) is activated by limited proteolysis after its translocation from the ER to the Golgi apparatus to form cleaved ATF6 (P50). Activated PERK phosphorylates eIF2α, which induces ATF4 expression. ATF4, being a transcription factor, translocates to the nucleus and induces the transcription of genes required to block the translational pathway. All of these three pathways will induce the up-regulation of CHOP. Caspase-12, a specific ER membrane-associated caspase, is proteolysed to cleaved caspase-12, which induces the caspase pathway cascade. Both expression of CHOP and activation of caspase-12 initiate cell death. Taurine treatment greatly inhibits ATF6 and IRE1 pathways but not PERK pathway after hypoxia/reoxygenation or glutamate over a longer time frame

In summary, the present study demonstrated that taurine may exert its protective effect on cortical neurons through suppression of ER stress induced by hypoxia/reoxygenation or glutamate. Furthermore, the effect of taurine treatment on the three ER stress-induced signaling pathways was also investigated on cortical neurons undergoing hypoxia/reoxygenation or glutamate exposure. As depicted in Fig. 5, the study indicates that taurine may significantly inhibit the activation of the ATF6 and the IRE1 pathway, but not the PERK pathway under chronic exposure to hypoxia/reoxygenation or glutamate. In contrast, activation of the initiation of PERK pathway was delayed by taurine under conditions of brief glutamate exposure.

Acknowledgments This work was supported partially by grant from Department of Health, James & Esther King Biomedical Research Program, State of Florida (09KW-11).

Conflict of interest The authors declare that they have no conflict of interests.

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