MECHANISM OF NEUROPROTECTION IN STROKE-RELATED MODELS

by

Chunliu Pan

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This dissertation was prepared under the direction of the candidate's dissertation advisor, Dr. Howard Prentice, Department of Biomedical Science, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Charles E. Schmidt College of Science and was accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

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Stroke is the third leading cause of mortality in the United States, and so far, no clinical interventions have been proved truly effective in stroke treatment. Stroke may result in hypoxia, glutamate release and oxidative stress, etc. The purpose of this dissertation study is to evaluate the neuroprotective effects of four drugs (taurine, G-CSF sulindac and DETC-MeSO) on PC12 cell line or primary cortical neuronal cell culture, and to understand the protective mechanisms underlying in three stroke-related models: hypoxia, excessive glutamate and oxidative stress. In the first part of this dissertation, we studied the neuroprotection of taurine against oxidative stress induced by H_2O_2 in PC12 cells. Our results show that extracellular taurine exerts a neuroprotective function by restoring the expression of Bcl-2 and downregulation of the three Endoplasmic Reticulum (ER) stress markers: GRP78, Bim and CHOP/GADD153, suggesting that ER stress can be provoked by oxidative stress and can be suppressed by taurine. In the second part, glutamate excitotoxicity-induced ER stress was studied with dose and time

as variables in primary cortical neurons. The results demonstrate that glutamate excitotoxicity leads to the activation of three ER stress pathways (PERK, ATF6 and IRE1) by initiating PERK first, ATF6 second and IRE1 pathway last. The third part of this dissertation studied the robust and beneficial protection of taurine in cortical neurons under hypoxia/reoxygenation or glutamate toxicity condition. We found that taurine suppresses the up-regulation of GRP78, Bim, caspase-12 and GADD153/CHOP induced by excessive glutamate or 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 or glutamate through suppressing ATF6 and IRE1 pathways. In the fourth part, the synergistic benefits of the combination of taurine and G-CSF, and the neuroprotective effects of G-CSF, sulindac or DETC-MeSO are studied in cortical neurons. Our results show that G-CSF, sulindac or DETC-MeSO can highly increase the neuron viability by inhibiting ER stress induced by hypoxia/reoxygenation or glutamate toxicity. Furthermore, we proved that G-CSF or sulindac can significantly inhibit the activation of ATF6 or IRE1 pathway stimulated by hypoxia/reoxygenation, and DETC-MeSO can suppress the activation of both PERK and IRE1 pathways in primary neuron cultures. These findings provide promising and rational strategies for stroke therapy.

DEDICATION

This dissertation is dedicated to my parents, my husband and my son.

MECHANISM OF NEUROPROTECTION IN STROKE-RELATED MODELS

LIST OF FIGURES

1. INTRODUCTION

1.1. Stroke

Stroke is the rapidly developing loss of brain functions due to disturbance in the blood supply to the brain, caused by a blocked or burst blood vessel. This can be due to ischemia (lack of glucose and oxygen supply) or due to a hemorrhage [1]. As a result, the affected area of the brain is unable to function, leading to inability to move one or more limbs on one side of the body, inability to understand or formulate speech, or inability to see one side of the visual field [2]. Stroke is the third leading cause of mortality in the United States. Of the approximately 700,000 strokes occurring each year, about 550,000 are first strokes. About 400,000 strokes are ischemic. Stroke is the leading cause of adult disability with more than 4 million stroke survivors in the United States alone. Approximately 90% of stroke survivors are left with some residual deficit [3].

An observational study has shown that more than 60% of patients develop hypoxia within the first 60 hours after stroke [4]. Stroke or ischemia leads to an increase in the extracellular concentrations of excitatory amino acids, especially in glutamate [5]. This elevation of glutamate could be linked to increasing release from neurons, resulting from energy failure, or to reducing clearance of glutamate by glial transporters. An increased generation of free radicals and other reactive species in stroke leads to oxidative stress [6].

Stroke (ischemia) causes a cascade of events that can induce the glutamate release and increase free radical production via several different pathways (shown in Figure 1). The main change affecting neurons during ischemia is the exhaustion of the high-energy phosphate compound, ATP, due to the lack of the substrates for its production, i.e., oxygen and glucose. The energy failure causes membrane depolarization, due to the reduced activity of ATP-dependent ion pumps, such as Na+/K+-ATPase. This impairment in turn compromises transmembrane ionic gradients, causing an influx of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels and uncontrolled release of excitatory amino acids, such as glutamate in the extracellular space [7]. Glutamate is the most common excitatory neurotransmitter. In small amounts, it is indispensable for neuronal function. In excessive amounts, it is a neuronal poison, a toxin, and has been called excitotoxin. The excessive release of glutamate is also mediated by Ca^{2+}- induced stimulation of presynaptic terminals and the disturbance of the uptake and inactivation system of glutamate, which is ATP and voltage dependent. Glutamate activates both N-methyl-d-aspartic acid (NMDA) and non- NMDA-type ionotropic receptors, thereby further increasing the concentration of Ca^{2+} in the cytoplasma [8]. Some glutamate receptors are non-selective cation-permeable ion channels. Initially, over-activation of these channels causes a passive influx of Cl^- (and Na^+) into cells causing osmotic (cytotoxic) edema and rapid death. The final result of ischemia is intracellular Ca^{2+} accumulation from multiple sources inducing the activation of a variety of Ca^{2+}-stimulated enzymes, such as proteases, lipases, nucleases, protein kinases, and nitric oxide synthase (NOS). This is an important mechanism by which the damage of cellular and subcellular structures leading to neuronal death is generated [9]. During the ischemic

phase and early reperfusion, some Ca^{2+}-dependent enzymes, such as phospholipase A2 and cyclooxygenase (COX), produce oxygen free radicals while neuronal nitric oxide synthase (nNOS) generates nitric oxide (NO) [7,10]. The production of free radicals is induced and sustained also by the modulation of general protein synthesis occurring after ischemia. This process includes the activation of transcription of proteins that are likely to play a damaging role, such as COX-2, inducible nitric oxide synthase (iNOS) and nNOS [7]. Free radicals and activated catabolic enzymes destroy structural proteins, membrane lipids, nucleic acids, and other cellular contents, causing neuronal necrosis. DNA damage from endonucleases and mitochondrial injury from free radicals trigger apoptosis.

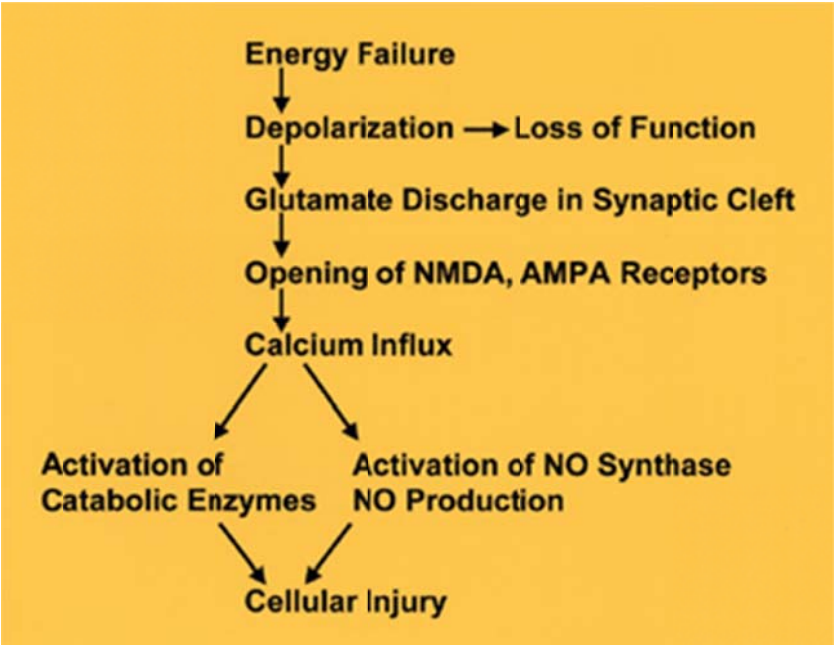

Figure 1. A cascade event induced by ischemia

Since these factors, such as increased excitotoxicity, calcium overload, formation of free radicals and inhibition of protein synthesis are all involved in stroke, it is a reasonable strategy to aim to treat stroke by calcium antagonists, glutamate antagonists and antioxidants, etc. Despite tremendous efforts in stroke research and significant improvements in stroke care within the last decade, therapy is still insufficient. Stroke, exemplified as an undertreated disease, demands an improvement of the existing therapy and a vigorous search for new therapies [11].

1.2. Stroke treatment

Currently, the only FDA approved medical treatment for acute ischemic stroke is intravenous tissue plasminogen activator (tPA), a thrombolytic agent which must be administered within 3 hours of symptom onset, and only after neuroimaging has ruled out intracerebral hemorrhage. Current estimates are that only 1-3% of acute ischemic stroke patients in the US receive tPA [12]. The only other agent of proven utility in acute ischemic stroke is aspirin, which confers only minimal benefit, helping only one of every 110 patients treated [13]. New, effective, widely applicable treatments for acute ischemic stroke are desperately needed. This fueled the interest in the development of neuroprotective therapies.

The concept of neuroprotection mainly came from the studies of the pathology and pathophysiology of ischemic brain injury. It has been well documented that abrupt deprivation of oxygen and glucose to neuronal tissues elicits a series of pathological cascades, leading to spread of neuronal death. Of the numerous pathways identified, excessive activation of glutamate receptors, accumulation of intracellular calcium cations,

abnormal recruitment of inflammatory cells, excessive production of free radicals, and initiation of pathological apoptosis are believed to play critical roles in ischemic damage, especially in the penumbral zone. Thus, it is logical to suggest that if one is able to interrupt the propagation of these cascades, at least part of the brain tissue can be protected.

Recently, taurine, G-CSF and sulindac have been proposed as candidates for treatment of stroke [14-16].

1.2.1. Taurine

Taurine, a sulfur-containing amino acid, is a free amino acid present in high concentrations in variety of organs of most mammals, including brain, heart, kidneys [17]. Taurine mediates many physiological functions, such as neuro-modulation, regulation of calcium-dependent processes, osmoregulation, thermoregulation, membrane stabilization and detoxication, neurotransmission and neuroprotection [18-22]. Taurine is known as an antioxidant to counteract the oxidative stress, which is involved in many diseases, such as chronic lung disease, diabetes, Alzheimer's disease, Parkinson's disease and heart failure [23, 24]. A recent paper revealed that taurine plays an important role in reducing ER stress in C2C12 and 3T3L1 cells [25].

Taurine is a potential neuroprotectant in cerebral ischemia [26]. Its release is markedly enhanced under ischemic conditions. It has the ability to forestall harmful metabolic events evoked by ischemia and hypoxia and to attenuate Ca^{2+} influx during ischemia [27, 28]. Pretreatment with taurine (0.5, 1 or 2 mmol/L) could aid in the recovery of synaptic function in rat hippocampus following a standardized hypoxic insult.

In *vivo* rat brain ischemic models, several studies have documented the neuroprotective effect of taurine (200-300 mg/kg) of cellular damage conditions [29].

1.2.2. Granulocyte colony-stimulating factor (G-CSF)

Granulocyte colony-stimulating factor (G-CSF), a 20-kDa protein, is a member of the cytokine family of growth factors which currently approved for clinical use to routinely treat neutropenia as well as for bone marrow reconstitution and stem cell mobilization [30]. It primarily stimulates proliferation, differentiation and maturation of cells committed to the neutrophilic granulocyte lineage through binding to the specific G-CSF receptor [31]. G-CSF also has been shown to have trophic effects on neuronal cells in *vitro* [32]. Moreover, G-CSF is an effective neuroprotectant in the treatments of a number of neurological diseases including stroke, Parkinson's disease and Alzheimer's disease [33-36]. In addition, apart from its protective role in neurons, G-CSF also dampens systemic inflammatory reactions, which may be of additional benefits in neurodegenerative conditions [37].

Because of its anti-inflammatory and neuroprotective properties and its capacity to mobilize stem cells, G-CSF has the potential to be used in clinical applications, including those following myocardial infarction and stroke. G-CSF can be upregulated by cerebral ischemia in neurons, indicating an autocrine protective response of the injured brain [38].

In addition to its infarct-reducing effects, G-CSF treatment strongly improves post-stroke recovery; this effect is related to sensorimotor and, possibly, cognitive functions that are distinct from the infarct reducing capacity of G-CSF [39, 40]. An

exciting explanation for this is that G-CSF triggers neurogenesis in the adult brain. Furthermore, G-CSF enhances the formation of new blood vessels following ischemia, which would increase restructuring of the infarcted brain [40]. Although speculative, G-CSF might cause bone-marrow-derived stem cells to invade the infracted brain, which could contribute to the enhanced neurogenesis and angiogenesis by secretion of neurotrophines and other trophic factors [41].The protective mechanism of G-CSF after stroke are shown in Figure 2.

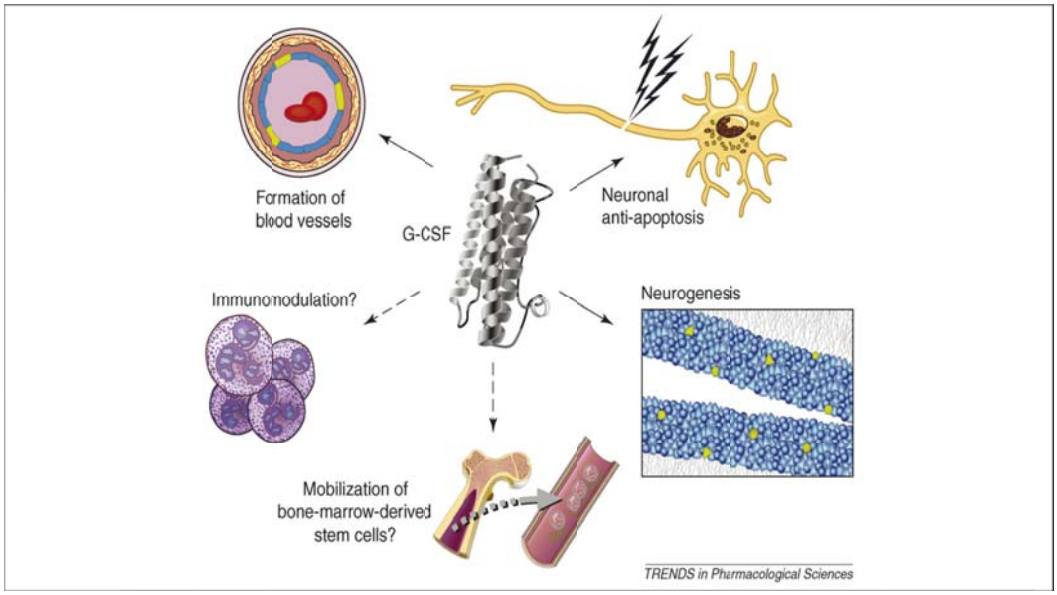

Figure 2. G-CSF induces multiple mechanisms of action after stroke, such as the generation of new neurons (neurogenesis), the prevention of delayed neuronal death (antiapoptotic function) and the formation of new blood vessels (arteriogenesis). Potential protective mechanisms include the mobilization of stem cells from the bone marrow as well as immunomodulatory effects after stroke. Solid arrows indicate proven evidence; broken arrows indicate suspected mechanisms. See reference 41.

1.2.3. Sulindac

Sulindac is a nonsteroidal anti-inflammatory drug that is capable of inhibiting cyclo-oxygenases (COX) 1 and 2 [42]. In addition to its known anti-inflammatory activity there have been numerous studies on the ability of sulindac and its metabolites to act as potential anti-cancer agents, based on their ability to slow the progression of colorectal polyps to colon cancer, as well as their ability to kill colon and other cancer cells [43, 44]. S epimer of sulindac is a substrate for methionine sulfoxide reductase (Msr) A [45], which reduces the S epimer of sulindac to sulindac sulfide, the active COX inhibitor. The Msr system has been shown to be an important cellular protective system against oxidative stress, and may play a role in aging [46, 47]. Recently, sulindac was proven to have highly protective effects on myocardial ischemia due to its properties as a preconditioning agent [14, 48].

1.2.4. S-methyl N, N-diethylthiolcarbamate sulfoxide (DETC-MeSO)

S-methyl-N, N-diethyldithiocarbamate sulfoxide (DETC-MeSO) is an active metabolite of disulfiram, which has an antagonistic effect on brain glutamate receptors in mice [49]. Disulfiram has been used in the treatment of alcoholism for more than 60 years [50]. It has been demonstrated that disulfiram exerts its anti-alcohol effect *in vivo* only after bioactivation to the active metabolite DETC-MeSO [51], and that is a potent and selective carbamoylating agent for sulfhydryl groups in glutamate receptors [49, 52], as shown in Figure 3. DETC-MeSO partially blocks glutamate binding to synaptic membrane preparation from the brains of mice, and DETC-MeSO also prevents seizures in mice induced by glutamate analogs and hyperbaric oxygen [49].

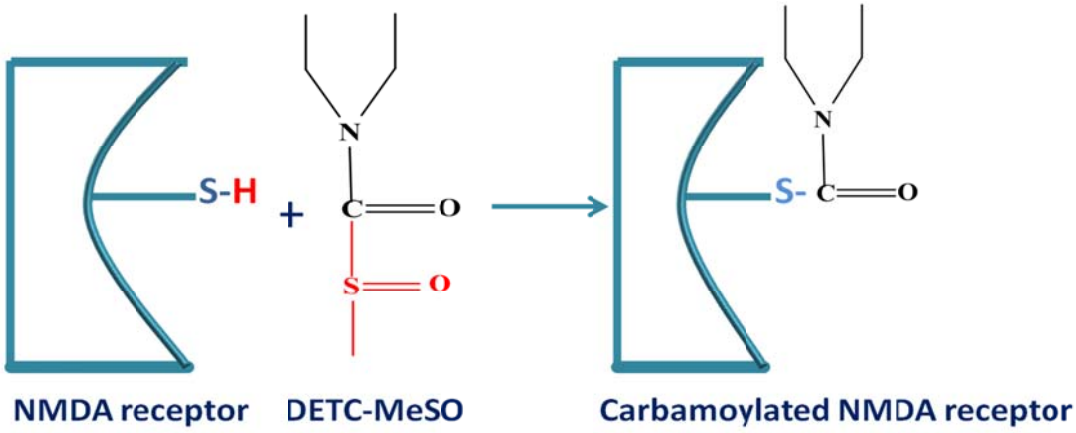

Figure 3. Carbamoylation of NMDA receptors by DETC-MeSO

1.3. Endoplasmic reticulum (ER) stress and its pathways

Endoplasmic reticulum (ER) is an important cell organelle that is responsible for synthesis and folding of proteins destined for secretion, cell membrane, Golgi apparatus, lysosomes and others, intracellular calcium homeostasis, and activation of cell death signaling activation [53]. Physiological or pathological processes that disturb protein folding in the ER lumen are referred to as ER stress, and a set of signaling pathways responding to ER stress are called the Unfolded Protein Response (UPR) [54]. The predominant signaling pathways associated with the ER stress are initiated by the ER membrane-associated proteins, protein kinase R [PKR]-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), which in turn activate distinct signaling cascades mediating the ER stress response [55-57]. Among these three major UPR signal transduction pathways, the IRE-1 and ATF-6 pathways increase the expression of the ER-resident chaperone, glucose-regulated protein 78 (GRP78) [58, 59], and 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) [60]. CHOP/GADD153 regulates expression of several Bcl-2 family members. For example, CHOP decreases expression of anti-apoptotic Bcl-2 [61], but increases expression of the pro-apoptotic Bim [62], thus contributing to cell death. The PERK pathway can also activate caspase-12, which plays an essential role in programmed cell death progression during the pro-apoptotic phase of the ER stress response [63].

The mechanism of ER stress pathways are shown in Figure 4 [64]. Dissociation of GRP78 from PERK initiates the dimerization and autophosphorylation of the kinase and generates active PERK. Once activated, PERK phosphorylates eukaryotic initiation factor 2 (eIF2), which leads to inhibition of general (eIF2α-dependent) protein translation. Inhibition of protein translation aids cell survival by decreasing the load of nascent proteins arriving at the ER. The phosphorylation of PERK enables translation of ATF4, which occurs through an alternative, eIF2α-independent translation pathway. ATF4, being a transcription factor, translocates to the nucleus and induces the transcription of genes required to restore ER homeostasis. The transcription factor C/EBP homologous protein (CHOP), whose induction strongly depends on ATF4, is well known to promote apoptotic cell death. Therefore, activation of PERK is initially protective and crucial for survival during even mild stress. However, activation of PERK also leads to the induction of CHOP, which is an important element of the switch from pro-survival to pro-death signaling.

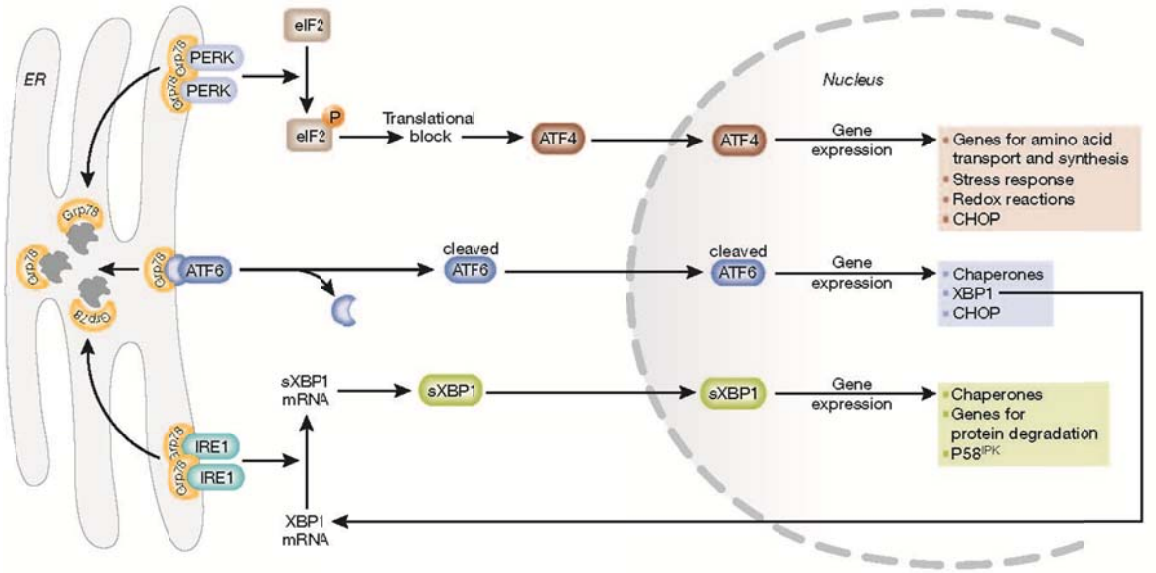

Figure 4. The mechanism of ER stress response. Upon aggregation of unfolded proteins, GRP78 dissociates from the three endoplasmic reticulum (ER) stress receptors, pancreatic ER kinase (PKR)-like ER kinase (PERK), initiating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1), allowing their activation. Activated PERK inhibits general protein synthesis by phosphorylating eukaryotic initiation factor 2α (eIF2α). Then eIF2α enables translation of ATF4, which occurs through an alternative, eIF2α-independent translation pathway. ATF4, as a transcription factor, translocates to the nucleus and induces the transcription of genes required to restore ER homeostasis. ATF6 is activated by limited proteolysis after its translocation from the ER to the Golgi apparatus. Active ATF6 is also a transcription factor and it regulates the expression of ER chaperones and X box-binding protein 1 (XBP1), another transcription factor. To achieve its active form, XBP1 must undergo mRNA splicing, which is carried out by IRE1. Spliced XBP1 protein (sXBP1) translocates to the nucleus and controls the transcription of chaperones, the co-chaperone and PERK-inhibitor P58IPK, as well as genes involved in protein degradation. This concerted action aims to restore ER function by blocking

further build-up of client proteins, enhancing the folding capacity and initiating degradation of protein aggregates. See reference 64.

After the dissociation of GRP78, ATF6 translocates to the Golgi apparatus where it is cleaved into its active form by site-1 and site-2 proteases. Active ATF6 then moves to the nucleus and induces genes with an ER stress response element (ERSE) in their promoter [65]. So far, the identified targets of ATF6 include ER chaperone proteins such as GRP78, GRP94, protein disulphide isomerase, and the transcription factors CHOP and X box-binding protein 1 (XBP1).

IRE1 is a dual-activity enzyme, having a serine–threonine kinase domain and an endoribonuclease domain. On activation, the endonuclease activity of IRE1 removes a 26-nucleotide intron from the XBP1 mRNA, previously induced by ATF6. Although the IRE1–XBP1 axis seems to have pro-survival effects through the induction of ER chaperones, overexpression of IRE1 resulted in apoptotic cell death [66]. Active IRE1 has been shown to recruit the adaptor molecule TNF-receptor-associated factor 2 (TRAF2). The IRE1–TRAF2 complex forms during ER stress can recruit the apoptosis-signal-regulating kinase (ASK1), which is a mitogen-activated protein kinase kinase kinase (MAPKKK) that has been shown to relay various stress signals to the downstream MAPKs JNK and p38 [67]. Activation of JNK has also been reported in response to ER stress and was shown to be IRE1- and TRAF2-dependent [68]. Activation of JNK is a common response to many forms of stress and is known to influence the cell-death machinery through the regulation of Bcl-2 family proteins [69]. For example, phosphorylation of Bcl-2 by JNK, which occurs primarily at the ER, suppresses the anti-

apoptotic activity of Bcl-2. Besides Bcl-2, JNK also phosphorylates BH3 (Bcl-2 homology domain 3)-only members of the Bcl-2 family such as Bim, which enhances their pro-apoptotic potential (Figure 5).

All in all, the cell returns to normal functioning by IRE1-XBP1 pathway or, if the stress persists, IRE1 triggers apoptosis by recruiting ASK1 and JNK. During ER stress, all three arms of the UPR induce transcription of CHOP. A study examining the mechanism of CHOP-induced apoptosis identifies numerous target genes including Bcl-2 and GADD34. GADD34 is a protein phosphatase 1 (PP1)-interacting protein that causes PP1 to dephosphorylate eIF2α and thus releases the translational block [70]. Expression of GADD34 correlates with apoptosis induced by various signals, and its overexpression can initiate or enhance apoptosis [71]. CHOP is known to repress Bcl-2 gene expression, which increases the proportion of pro-apoptotic Bcl-2 proteins in the cell and enables their activation.

JNK is known to regulate Bcl-2 proteins by phosphorylation. First of all, JNK is able to phosphorylate Bcl-2 localized to the ER. Of the BH3-only subfamily, p53-upregulated modulator of apoptosis (PUMA), Noxa and Bim have been reported to have a role in ER stress. Bim exists in several isoforms, including a short form (BimS) and two longer forms BimL and BimEL with the two longer forms being constitutively expressed. The pro-apoptotic effects of BimL and BimEL are restrained in unstressed cells by their binding to the dynein motor complex. Phosphorylation by JNK releases Bim from this inhibitory association and allows it to exert its pro-apoptotic effects [72] (Figure 5).

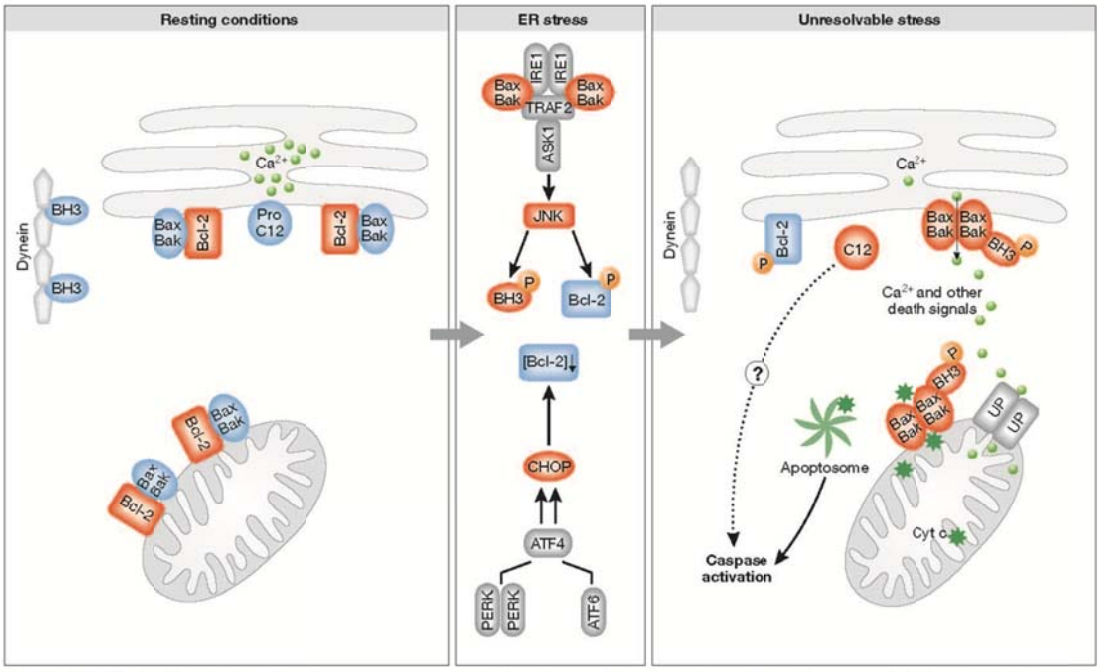

Figure 5. The Bcl-2 family of proteins in resting cells and in endoplasmic reticulum stress condition. In resting conditions, the pro-apoptotic Bax and Bak (Bax/Bak) are kept inactive by interaction with Bcl-2 both on the mitochondrial and endoplasmic reticulum (ER) membranes, whereas Bim (BH3) is inhibited by binding to cytoskeletal dynein. Severe ER stress leads to activation of c-Jun N-terminal kinase (JNK) and induction of C/EBP homologous protein (CHOP; initiation phase). Both JNK and CHOP eliminate the anti-apoptotic effects of Bcl-2; CHOP blocks expression of Bcl-2, whereas JNK phosphorylates it. JNK also phosphorylates Bim, which leads to its release from the cytoskeleton and to its activation (commitment phase). Collectively, these changes allow activation of Bax and Bak, transmission of the signal from the ER to the mitochondria and execution of death (execution phase). Caspases are activated possibly on the ER membrane itself, as well as in the apoptosome, after transmission of the death signal to

mitochondria and the release of cytochrome c. Blue labels show inactive molecules, whereas red labels indicate active molecules, with the rounded shapes representing the pro-apoptotic molecules and rectangles representing the anti-apoptotic molecules.ATF6, activating transcription factor 6; IRE1, inositol-requiring enzyme 1; PERK, pancreatic ER kinase (PKR)-like ER kinase; TRAF2, TNF-receptor-associated factor 2; UP, uniporter.

1.4. Stroke and ER stress

ER stress plays an important role in stroke. Several recent studies have highlighted the importance of ER stress in the pathogenesis of neuronal cell injury during and after cerebral ischemia [73-75]. It was shown that ischemia induces all three arms of the UPR: PERK, ATF6, IRE1, and their downstream targets CHOP, caspase-12, PUMA, etc in heart [76, 77]. The ER stress involved in cerebral ischemia is not yet well understood.

This research work focuses on the mechanisms underlying neuronal apoptosis or necrosis under different conditions including too much glutamate presence, hypoxia and oxidative stress and explores the therapeutic strategies for the cerebral vascular dysfunction. Therefore, in this study I tested that the protective effects of these four drugs (taurine, G-CSF, sulindac and DETC-MeSO) against excitotoxicity induced by glutamate, hypoxia or oxidative stress induced by H_2O_2 in PC12 cells or primary cortical neuronal culture; and which signal pathways are key elements or reasons responsible for protective effects of these four drugs. To explore the specific mechanism underlying, this study focused on ER stress and related pathways induced by three cell apoptotic inducers which

are involved in pathologic consequences of stroke: hypoxia, glutamate toxicity and oxidative stress. Furthermore, taurine, G-CSF, sulindac and DETC-MESO, recently having been shown effectively in ischemia, are investigated for their protective effects under the three cell apoptosis inducers and the neuroprotective mechanism related to the ER stress.

2. MATERIALS AND METHODS

2.1. Materials

F-12K media, trypsin-EDTA solution, horse serum and rat phenocromocytoma PC12 cell line were purchased from ATCC (Manassas, VA, USA). Fetal bovine serum, poly-D-lysine, taurine, sulindac, Penicillin-Streptomycin and other chemicals were purchased from Sigma (St. Louis, MO, USA). Mouse anti-actin, rabbit anti-Bax, rabbit anti–Bcl-2, rabbit anti-GRP78, rabbit anti-CHOP/GADD153 antibodies, and secondary mouse and rabbit antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-Bim antibody was purchased from assay designs (Ann Arbor, Michigan, USA). Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit and 3, (4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide (MTT) assay kit were purchased from Promega (Madison, WI, USA) and ATCC (Manassas, VA, USA) respectively. RIPA buffer was purchased from thermo scientific (Rockford, IL, USA).

Fetal bovine serum, basal media Eagle, poly-D-lysine, glutamine and glucose were purchased from Sigma (St. Louis, MO, USA). Neurobasal medium was purchased from Invitrogen (Carlsbad, CA, USA). Rabbit anti-caspase-12 and rabbit anti-GADD34 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-p-eIF2α, mouse anti-eIF2α, mouse anti-GAPDH, rabbit anti-HIF2α and rabbit

anti-cytochrome c 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, rabbit anti-XBP-1, rabbit anti-calnexin, rabbit anti-PUMA and rabbit anti-Hsp27 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.

2.2. Cell culture

2.2.1. PC12 cell line culture

PC12 cells were maintained at 37^{o}C/5% CO_2 in F12-K medium supplemented with 2.5% (v/v) fetal bovine serum (FBS), 15% (v/v) heat-inactivated horse serum (HS) and 1% (v/v) penicillin-streptomycin solution. All experiments were performed on undifferentiated cells plated in 96-well plates at a density of approximately 5×10^4 cells/ml for the ATP assay, 1×10^5 cells/ml for the MTT assay and in 60mm petri dishes at 5×10^5 cells/well for western blot for 24 hours before starting the experiments. The 96-well plates or petri dishes were precoated with poly-D lysine before plating.

2.2.2. Primary cortical neuronal cell cultures

Primary cortical neuronal cell cultures were prepared by a previously described protocol [121]. Briefly, the pregnant rats were sacrificed 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 300g/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 hour in a humidified incubator (37^{o}C, 99% humidity and 5% CO_2) before the incubation medium was replaced with serum-free neurobasal medium supplemented with 2% B27 and 500μM glutamine. The cells were maintained in an incubator for 12 -18 days until they were ready for handling.

2.3. Stroke-related models

2.3.1. Oxidative stress model

For H_2O_2-induced oxidative stress, PC12 cell line was treated with a proper concentration of H_2O_2 for cell death induction.

2.3.2. Glutamate excitotoxicity

For glutamate-induced toxicity, neurons at 14 days in *vitro* were preincubated with a proper concentration of taurine for one hour. Then the neurons were treated with assigned concentration of glutamate for proper time.

2.3.3. Hypoxia/reoxygenation

To generate hypoxic conditions, 14 days-cultured neurons in 6 or 96 well plates were placed in a hypoxia chamber with oxygen levels maintained at 0.3-0.4 % (Figure 6). The level of oxygen was continuously monitored using an oxygen electrode. Primary cortical neuronal cultures in the absence or presence of taurine were subjected to 20 hours of hypoxia. Reoxygenation was performed by removing cultured plates from the hypoxic chamber and transferring them into normal culture incubator remaining for another 20 hours.

Figure 6. Hypoxia chamber

2.4. Measurement of Cell Viability

2.4.1. ATP assay

PC12 Cells or primary cortical neuronal cells in 96-well plates were treated with or without drugs (taurine, G-CSF, DETC-MESO and sulindac) for 1 hour, and then cells were exposed to 100-500μM H_2O_2, or assigned glutamate concentration or under hypoxia reoxygenation condition for proper time to induce cell death. ATP solution (Promega) was added to each well and cells were incubated for 10 minutes, then the amount of ATP was quantified through a luciferase reaction. The luminescence intensity was determined using a luminometer (SpectraMax, Molecular Devices) after transferring the lysate to a

standard opaque walled multi-well plate. The ATP content was determined by running an internal standard and expressed as a percentage of untreated cells (control).

2.4.2. MTT assay

PC12 Cells in 96 well plates were treated with 25mM Taurine for 1 hour and then cells were exposed to 250 μM H_2O_2 for 4 hours to induce cell death. Subsequently, 10ul MTT reagent (ATCC) was added to each well and cells were incubated for 4 hours until a purple precipitate was visible. Then 100ul detergent reagent was added and the solution was left at room temperature in the dark for 2 hours. The absorbance was detected with a microtiter plate reader at 570nm.

2.5. Western Blot Analysis

PC12 cell line or 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 hours at room temperature. After blocking, the corresponding primary antibody was incubated for one hour, followed by one hour 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 processing and Analysis in Java (Image J).

2.6. Statistical Analysis

All data were expressed as the mean±SEM. The statistical significance of the data was determined with t-test or 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.

3. RESULTS

3.1. Taurine protects PC12 cells against ER stress induced by oxidative stress

Recently, it has been suggested that oxidative stress and ER stress are closely linked events, although the molecular pathways that couple these processes are poorly understood [78]. Moreover, GRP78 was shown to protect neurons against excitotoxicity and to suppress oxidative stress [79]. Here, I demonstrated that taurine exerts a protective function against ER stress induced by oxidative stress in PC 12 cells.

3.1.1. Dose-dependent toxicity of H_2O_2 in PC12 cells

The PC12 cells were exposed to different concentrations of H_2O_2 in a range of 100-500 μM for 4 hours, then the ATP assay was performed. As expected, the survival of PC12 cells decreased with the increasing of concentrations of H_2O_2 from 76% at 100 μM H_2O_2 to 18% at 500 μM H_2O_2 as demonstrated in Figure 7. We chose the optimal doses of 250 μM H_2O_2 for cell viability test. At 250 μM H_2O_2, about 45% of survival PC12 cells was observed.

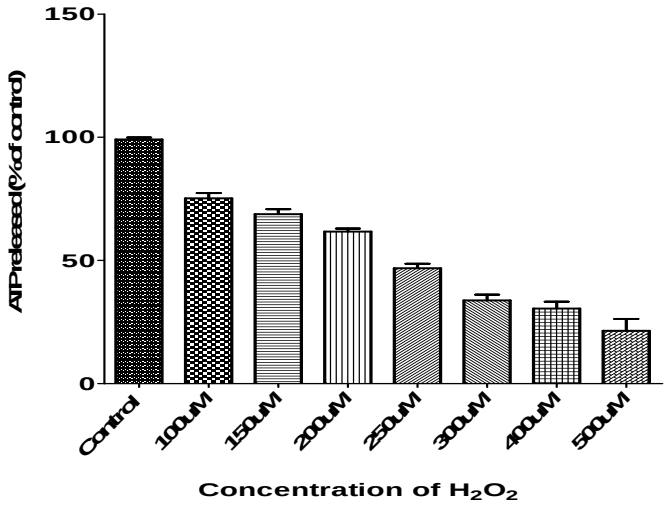

Figure 7. Dose-dependence of H_2O_2-induced cell injury in PC12 cells measured by ATP assay. PC12 cells were exposed to H_2O_2 for 4 hours.

3.1.2. Taurine protects PC12 cells against H_2O_2-induced oxidative stress

In our previous research, it was revealed that preincubation with 25 mM taurine resulted in maximal recovery from neuronal injury induced by glutamate [80]. So, we chose 25mM as the optimal concentration of taurine. To determine the protective effect of 25mM taurine on 250 μM H_2O_2-induced oxidative stress, cell viability was examined using the ATP assay and MTT assay, respectively, as shown in Figure 8 (A and B). Lane 2 in Figure 8 A and B showed that 250 μM H_2O_2 significantly decreased the survival of PC12 cells about 45-50%. The protective effect of taurine is up to 75-80%, as shown in Figure 8 (lane 2 in A, lane 2 in B) after treatment with 25mM taurine for 1 hour, following exposure to 250 μM H_2O_2 for 4 hours.

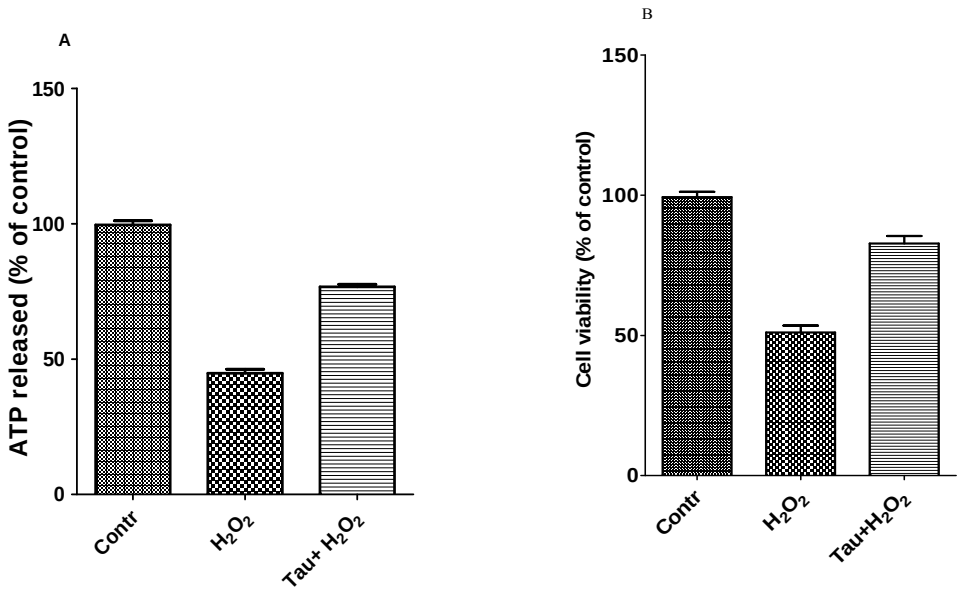

Figure 8. Effect of taurine on H_2O_2-induced cell injury in PC12 cells. A, Cell viability was measured by ATP assay; B, Cell viability was measured by MTT assay. 25mM taurine was preincubated for 1 hour following by 250 μM H_2O_2 treatment for 4 hours.

3.1.3. Extracellular taurine executes protection of PC12 cells against H_2O_2-induced oxidative stress

Taurine uptake across the cell membrane was found to be dependent on Na^+/Cl^- dependent TauT or H^+ - coupled PAT1 transporters [81, 82]. In order to investigate whether intracellular or extracellular taurine has a protective effect, β- alanine, the analogue of taurine was utilized as a competitive inhibitor of the taurine transporters [83]. The PC12 cell viability was tested by ATP assay, as shown in Fig. 9. To compare the PC12 cell viabilities of treatment with taurine and β- alanine, we treated neurons with 25mM β- alanine, which was at the same concentration as taurine. Cell survival was

similar with or without β- alanine, indicating that β- alanine afforded no protective effects against the toxicity of H_2O_2 (Figure 9, lane 2 and lane 5). The ATP released of the 25mM taurine preincubation after exposure to H_2O_2 was similar with that of 25mM taurine and 25mM β- alanine, which means that extracellular taurine protected the PC12 cells against oxidative stress induced by H_2O_2 (Figure 9, lane 3 and lane 7). PC12 cell survivals indicated no significant difference among with or without 25mM taurine or 25mM β- alanine or taurine + β- alanine, suggesting that 25mM alanine has no effect on the PC12 cells, at least for the ATP released (Figure 9 lane 4, 6 and 8).

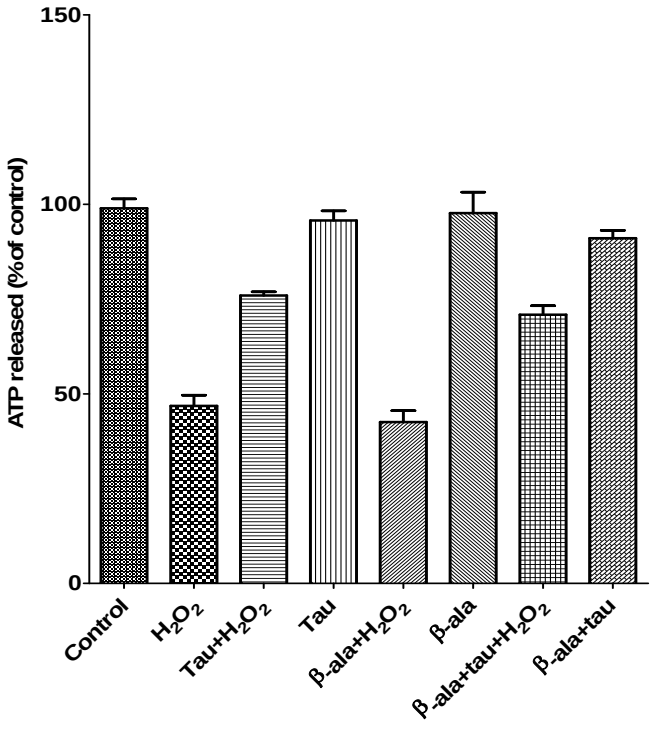

Figure 9. Effects of β-alanine (β-ala), an analog of taurine (tau) on cell viability in PC12 cells. PC12 cells were preincubated with 25mM taurine, 25mM β-alanine or

combination of 25mM taurine and 25mM β-alanine for 1 hour following treatment with or without 250 μM H_2O_2.

3.1.4 Taurine restored the expression of Bcl-2 and had no significant effect on the expression of Bax

Bcl-2 and Bax are two proteins which belong to the Bcl-2 family that modulate cell survivals. It has been demonstrated that cell survival is modulated at least in part by the Bcl-2 family of proteins: apoptosis-inhibiting gene products, Bcl-2 and Bcl-xL and apoptosis-accelerating gene products, Bax and Bad [84, 85]. The level of Bcl-2 was decreased but Bax was slightly increased in PC12 cells under treatment with 250 μM H_2O_2 for 4 hours (Figure 10, lane 2). Nevertheless, the Bcl-2 level was restored after preincubation with taurine, but there was no significant change in the Bax level (Figure 10, lane 3).

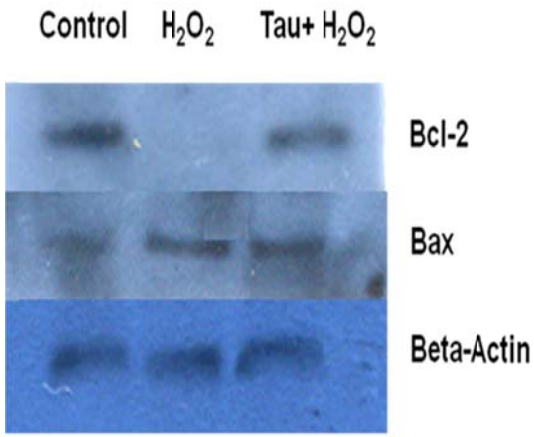

Figure 10. Western blot analysis of expression of Bcl-2 and Bax. PC12 cells were treated with or without 25mM taurine before treatment with or without 250 μM H_2O_2 for 4 hours. Beta-actin is used as a control.

3.1.5 Taurine reversed the H_2O_2-induced up-regulation of GRP78, CHOP/GADD 153 and Bim in PC12 cells

Western blot analysis showed that UPR regulator GRP78 was up-regulated after treatment with H_2O_2 for 4 hours, indicating that ER stress was induced by oxidative stress (Figure 11 lane 2). Taurine suppressed the expression of GRP78 protein in PC12 cells after exposure to H_2O_2 (Figure 11 lane 3).

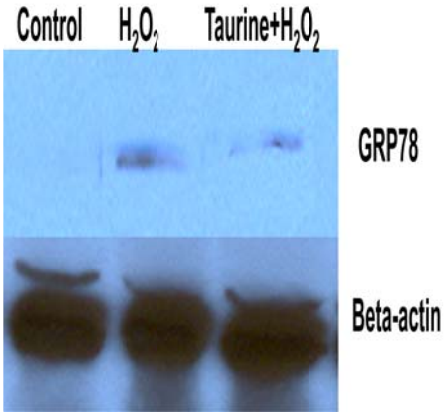

Figure 11. Western blot analysis of expression of ER stress marker GRP78. PC12 cells were treated with or without 25mM taurine before treatment with or without 250 μM H_2O_2 for 4 hours. Beta-actin is used as a control.

To determine if oxidative stress-induced apoptosis was also activated by the ER stress pathways, we examined expression of the ER stress apoptotic factor, CHOP. CHOP expression is minimal and barely detectable under normal homeostatic conditions, and upregulation of CHOP has been reported to signal the activation of ER stress-mediated apoptosis [86]. Here, we characterized the processing of CHOP/GADD153 in PC12 cells by Western blotting of whole cell lysates after treatment with H_2O_2 for 2.5

and 4 hours, as shown in Figure 12. Western blot analyses revealed a slight level of CHOP expression at 2.5 hours exposed to H_2O_2, and pronounced CHOP expression at 4 hours of treatment by H_2O_2 alone. There was a remarkable reduction in CHOP protein expression in PC12 cells treated by taurine, following by H_2O_2 treatment, compared to cells exposed to H_2O_2 only (Figure 11). Recently, ER stress was shown to up-regulate Bim protein level through CHOP/GADD153 mediated direct transcriptional induction [87]. Our results demonstrated that two longer isoforms of Bim, Bim_{EL} and Bim_L in PC12 cells treated with H_2O_2 were highly expressed compared to controls without any treatment. Taurine suppressed the expression of Bim_{EL} and Bim_L, especially for PC12 cells treated with H_2O_2 for 2.5 hours, as shown in Figure 12.

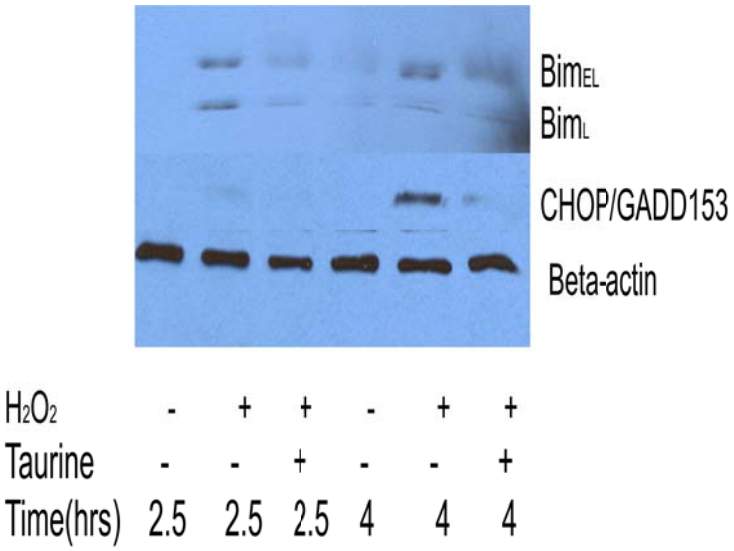

Figure 12. Western blot analysis of expression of Bim and CHOP/GADD153. PC12 cells were treated with or without 25mM taurine before treatment with or without 250 μM H_2O_2 for 2.5hours or 4 hours, respectively. Beta-actin is used as a control.

3.2. Glutamate toxicity and endoplasmic reticulum stress

Glutamate is the major excitatory neurotransmitter in the brain of mammals responsible for learning, memorizing and cognitive function. It is released to the synaptic cleft and binds to glutamate receptors able to propagate the action potential and signal cascade. Synaptic transmission and glutamate levels in the synaptic cleft are regulated by glutamate transporters. It has been well established that excessive glutamate in nervous system becomes toxic to neurons. Glutamate was first demonstrated as a powerful "neurotoxin" in late 1950s by the results that glutamate injection into mouse retina was proved to cause the retinal degeneration [88]. Many neurological diseases, such as ischemia, amyotrophic lateral sclerosis, epilepsy, trauma and hepatic encephalopathy, have been found to initiate the massive glutamate release and accumulation in the extracellular space, which turn out to be able to overstimulate the glutamate receptors, elevate intracellular free calcium ions concentration, then lead to dysfunctions in both mitochondria and endoplasmic reticulum [89-94].

Glutamate uptake from extracellular spaces into cytoplasm of neurons and astrocytes is mediated by glutamate transporters, which are responsible for maintaining glutamate concentration in synaptic cleft low enough to prevent glutamate receptors desensitization and/or overstimulation. Under normal conditions, glutamate transport is coupled with sodium and potassium, and the process can concentrate glutamate in cells up to 10000-fold compared to the synaptic clefts. To date, there are five high-affinity glutamate transporters that differ in their regional and cellular expression: glutamate astrocyte-specific transporter (GLAST [also termed excitatory amino acid transporter 1 (EAAT1)]), glutamate transporter 1 (GLT-1 [or referred as excitatory amino

acid transporter 2 (EAAT2)]), excitatory amino acid carrier 1 (EAAC1 [or referred as excitatory amino acid transporter 3 (EAAT3)]), excitatory amino acid transporter 4 (EAAT4), excitatory amino acid transporter 5 (EAAT5) [95-97]. Glutamate transporter dysfunction leads to extracellular glutamate increase by its "reverse" operation occurring in some pathologic conditions, such as cerebral ischemia [98]. Once glutamate is released, postsynaptic response occurs through two main glutamate receptors: ionotropic glutamate receptors and metabotropic glutamate receptors. It was proposed that there are three types of ionotropic glutamate receptors named after their preferred agonists: N-methyl-D-aspartate receptor (NMDAR), 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) proprionate receptor (AMPAR) and kainate receptor [99, 100]. Activation of ionotropic glutamate receptors results in permeability to sodium, potassium and calcium ions. Metabotropic glutamate receptors couple to G-protein to modulate synaptic transmission through intracellular second messengers cascade. To date, eight metabotropic glutamate receptors (mGlu1-8) have been cloned [101]. Overstimulation of metabotropic glutamate receptors leads to free calcium release from internal calcium pools [102].

The mitochondrion and endoplasmic reticulum (ER) are two significant intracellular sources of calcium stores. Intracellular free calcium accumulation in neurons disturbs the calcium levels in both mitochondria and ER organelles and may lead to mitochondrial dysfunction and ER stress by prolonged overstimulation of glutamate receptors [103,104]. Mitochondrial dysfunction in neurons results in some tragic consequences: inhibition of mitochondrial calcium ion uptake, depolarization of mitochondrial membrane potential, depletion of ATP, some enzyme over-expression, production of reactive oxygen species and collapse of mitochondrial structure. These

results finally cause the neuronal necrosis or apoptosis [104, 105]. ER is a very important cell organelle which serves as a protein factory for protein synthesis and folding [106]. ATP, Ca^{2+}, and an oxidizing environment are required for protein properly folding to allow disulphide-bond formation In ER [107]. Although there are numerous papers for investigating mitochondrial dysfunction induced by excessive glutamate, only a few papers investigated ER stress resulting from glutamate challenges [103, 108,109]. Thus, in this part, we investigated the effects of glutamate neurotoxicity-induced ER stress on rat primary neuronal cell culture.

3.2.1. Glutamate excitotoxicity is dose-dependent in primary neuronal cell culture.

To evaluate the glutamate toxicity in primary neuronal cell culture, neurons were exposed to different concentration of glutamate ranging from 10 μM to 2 mM for 1 hour, as shown in Figure 13. Cell viability decreases with the elevation of glutamate concentration and ATP levels at 10 μM glutamate significantly dropped to approximately 93%, compared to control neurons. With glutamate concentration increasing up to 2 mM cell viability decreased to about 24% of the control neurons. Cell survival at 2 mM glutamate is very close to that at 1 mM, as shown in Figure 13 lane 7 and 8. The cell viability at 100 μM glutamate reduces to 46% of the controls. Thus, 100 μM glutamate was employed for neuronal excitotoxicity at different time points.

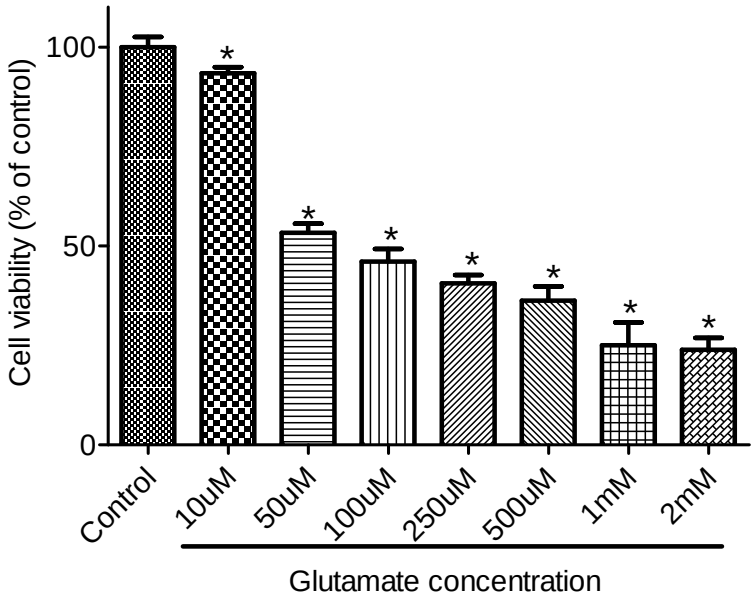

Figure 13. Glutamate toxicity is dose-dependent in primary neuronal cell culture. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1mM or 2mM glutamate for 1hour, respectively.

3.2.2. The expression of Bcl-2 family proteins (Bcl-2, PUMA and Bax) changes with different concentration of glutamate

Bcl-2 family proteins are well-known regulators of neuronal apoptosis, and some key components are involved in ER stress-induced apoptosis. The anti-apoptotic factor Bcl2, pro-apoptotic factors Bax (Bcl2-associated X protein) and PUMA (p53 up-regulated modulator of apoptosis) all belong to Bcl-2 family. The expressions of Bcl-2, Bax and PUMA at different concentration of glutamate were measured by western blot in primary neuronal cell culture, as shown in Figure 14 and 15. The results show that Bcl-2

expressions are down-regulated with the elevation of glutamate concentration. When glutamate concentration is in a range between 10 μM and 2 mM, the expressions of PUMA have little changes until exposure to 1mM glutamate. PUMA in neurons after 1 or 2mM glutamate exposure is highly expressed compared with the control neurons. Bax expressions are increasing gradually with the increment of glutamate concentrations, and reach the highest level at 500 μM glutamate exposure in the range of 100-500 μM concentration.

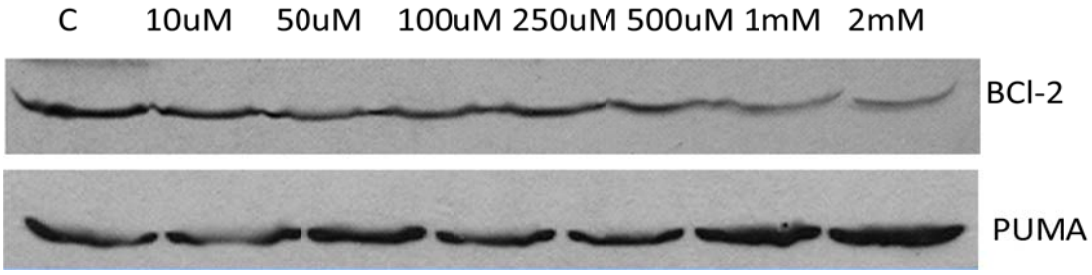

Figure 14. The expressions of Bcl-2 and PUMA are dose-dependent after exposure to glutamate in neurons. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1mM and 2mM glutamate for 1hour.

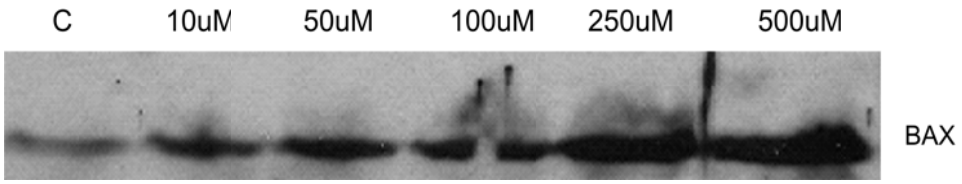

Figure 15. The expressions of Bax are dose-dependent after exposure to glutamate in neurons. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM and 500 μM glutamate for 1hour.

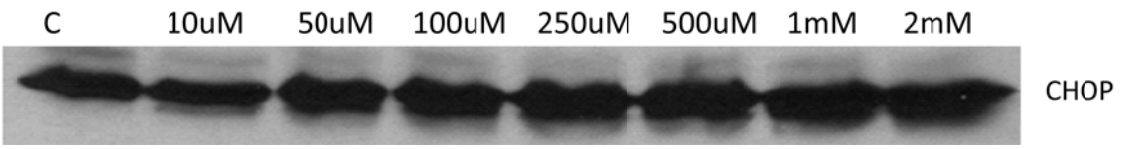

Figure 16. Upregulation of ER stress marker CHOP expressions at different dose of glutamate. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1mM and 2mM glutamate for 1hour.

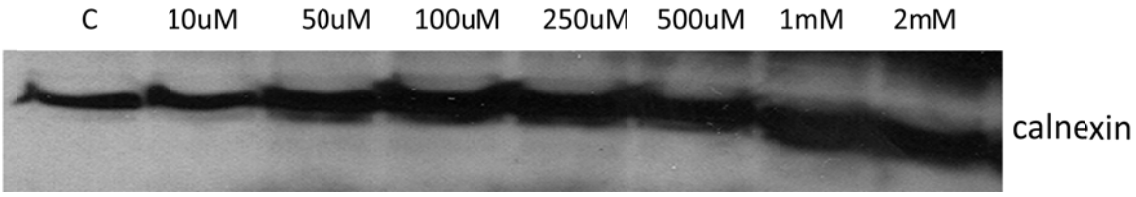

Figure 17. Upregulation of calnexin expressions at different dose of glutamate. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1mM and 2mM glutamate for 1hour.

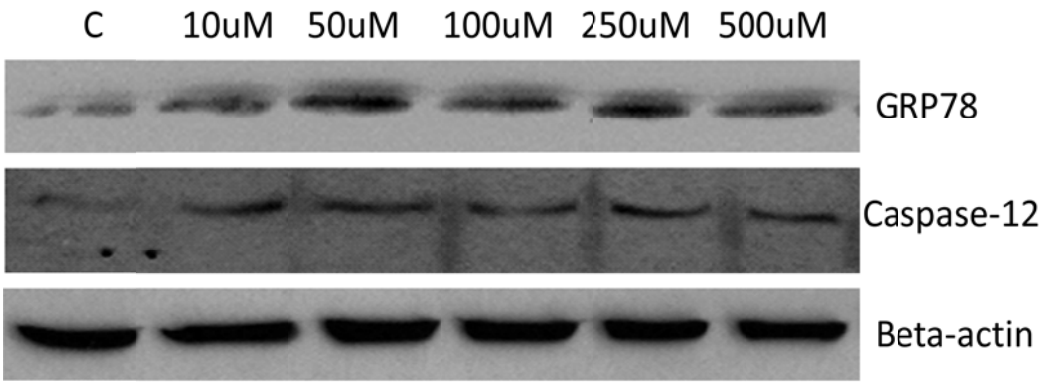

Figure 18. Glutamate-induced upregulation of GRP78 and Caspase-12 expression. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM and 500 μM glutamate for 1hour.

3.2.3. ER stress can be induced by glutamate toxicity in cortical neurons

CHOP is a bZIP-containing transcription factor and one of the most highly upregulated proteins during prolonged ER stress. Calnexin is a 90-kDa type I integral ER transmembrane chaperone that transiently binds newly synthesized monoglucosylated and misfolded glycoproteins, promoting their folding and oligomerization [110]. The expressions of CHOP and Calnexin in neurons exposed to different concentration of glutamate are shown in Figure 16 and 17, respectively. Glutamate toxicity causes overexpression of both CHOP and Calnexin at concentration as low as 50 μM. Glucose-regulated protein 78 (Grp78) is one of the most abundant and best-characterized ER chaperones and can be up-regulated by the three pathways after ER stress. Caspase-12 is an ER-membrane -associated protein, which belongs to caspase family. GRP78 and caspase-12 are also upregulated after exposure to different concentration of glutamate, as shown in Figure 18. All these results demonstrate that ER stress can be induced by glutamate toxicity.

3.2.4. Glutamate activatesthe three ER stress pathways: PERK, ATF6 and IRE1 pathways.

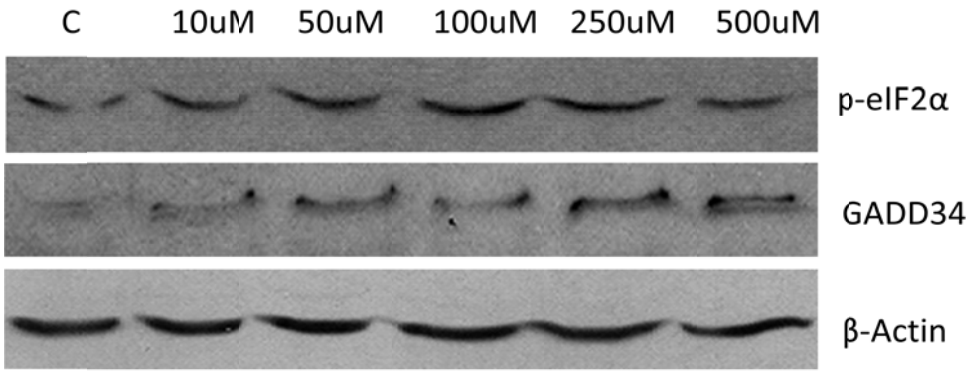

Figure 19. PERK pathway is activated by different concentration of glutamate. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM and 500 μM glutamate for 1hour.

It has been proposed that there are three pathways induced by ER stress: PERK, ATF6 and IRE1 named by the three ER membrane -associated proteins. Since glutamate can promote ER stress in primary neurons, the three ER stress pathways may be activated during the glutamate toxicity. The expression of P-eIF2α, a downstream protein of PERK pathway is upregulated with the exposure of different concentration of glutamate, as shown in Figure 19. In the PERK pathway, phosphorylation of eIF-2α by p-PERK promotes the expression of GADD34, which then assembles an eIF-2α phosphatase that functions in a negative feedback loop to reverse eIF-2α phosphorylation and suppress unfolded protein response [111]. The expressions of GADD34 increase with the increment of glutamate concentration, as shown in Figure 19. P-eIF2α expression is reduced at 500 μM glutamate exposure compared to those in 250 μM glutamate. This

may be because the overexpression of GADD34 in neurons reverses the phosphorylation of eIF-2α, which results in down-regulation of expression of p-eIF2α in high concentration of glutamate. The expression of both cleaved ATF6 and p-IRE1 are up-regulated with the increment of glutamate concentration, as shown in Figure 20 and 21. These results show that glutamate can stimulate the three ER stress pathways induced at different dose points.

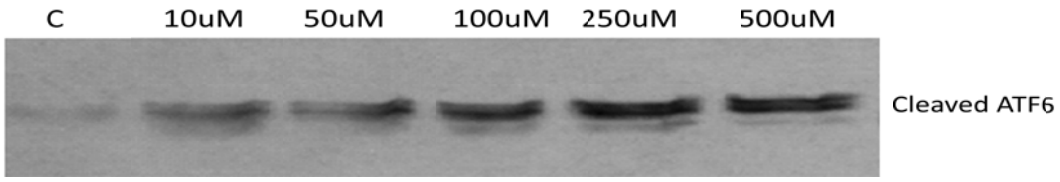

Figure 20. ATF6 pathway is activated by different concentration of glutamate. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM and 500 μM glutamate for 1hour.

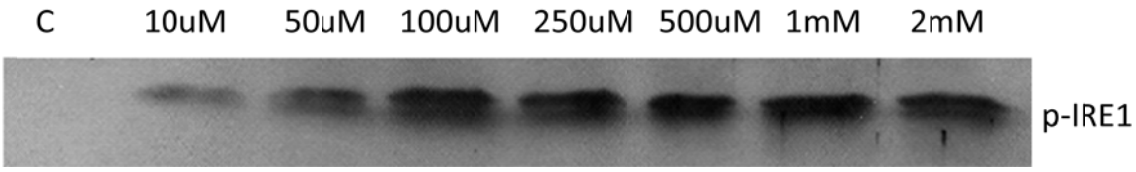

Figure 21. IRE1 pathway is activated by different concentration of glutamate. Neurons were treated with 10 μM, 50 μM, 100 μM, 250 μM, 500 μM, 1mM and 2mM glutamate for 1hour.

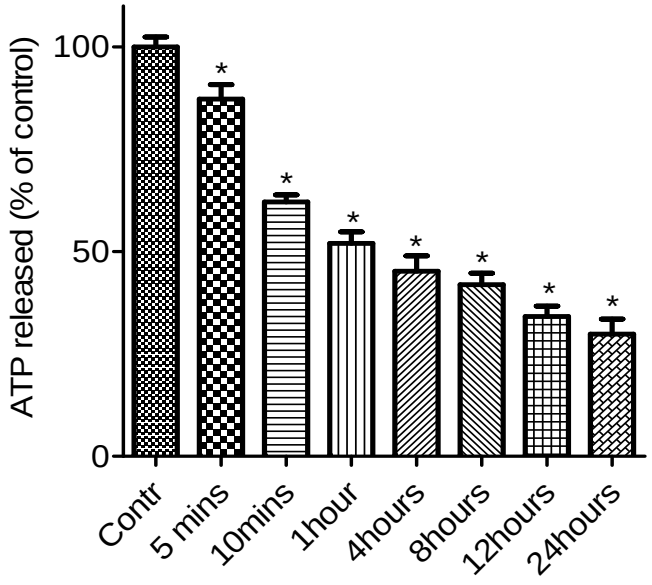

Figure 22. Aggravation of glutamate toxicity in a prolonged time. Neurons were exposed to 100 μM glutamate for 5 mins, 10 mins, 1 hour, 4 hours, 8hours, 12 hours and 24 hours. *P<0.05 as compared to control neurons.

3.2.5. Glutamate toxicity is exacerbated in a prolonged time

Neuronal cell injury is aggravated with the prolonged time exposure to 100 μM glutamate. The cell viability measured by ATP assay is shown in Figure 22. The results demonstrate that 100 μM glutamate at 5 mins causes unambiguous neuron death. The cell viability at 5 mins is about 87% of control. After one day exposure to glutamate, only 30% of control neurons can survive.

3.2.6. The occurring sequences of the ER stress pathways after exposure to glutamate in primary neuronal cell culture

Expression of ER chaperon GRP78 is significantly increased at 5 mins after exposure to glutamate. It decreases gradually with the prolonged time, as shown in Figure 23. P-eIF2α, a downstream protein of PERK pathway is overexpressed at 5 mins after glutamate addition, and then it goes down gradually with the prolonged time points, as shown in Figure 24.

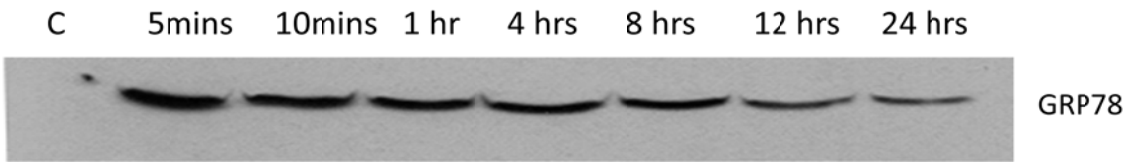

Figure 23. Representative GRP78 expression changes with the prolonged-time glutamate exposure. Neurons were exposed to 100 μM glutamate for 5 mins, 10 mins, 1 hour, 4 hours, 8 hours, 12 hours and 24 hours.

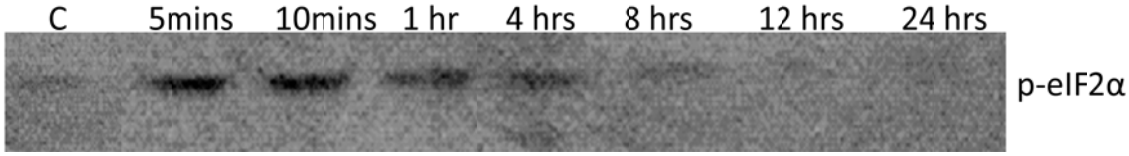

Figure 24. Representative p-eIF2α expression changes with the prolonged-time glutamate exposure. Neurons were exposed to 100 μM glutamate for 5 mins, 10 mins, 1 hour, 4 hours, 8 hours, 12 hours and 24 hours.

The expression of cleaved ATF6 represents the activation of ATF6 pathway. Cleaved ATF6 expression is upregulated at 10 mins relative to the control neurons, reaching its highest level at one hour. The results are shown in Figure 25. P-IRE1 and XBP-1, downstream proteins regulated by IRE1 pathway are highly expressed at 1 hour and then downregulated with the prolonged time. The quantitative results from 3 independent western blots results prove that PERK pathway is activated first, then ATF6 pathway, IRE1 pathway is last, as described in Figure 26.

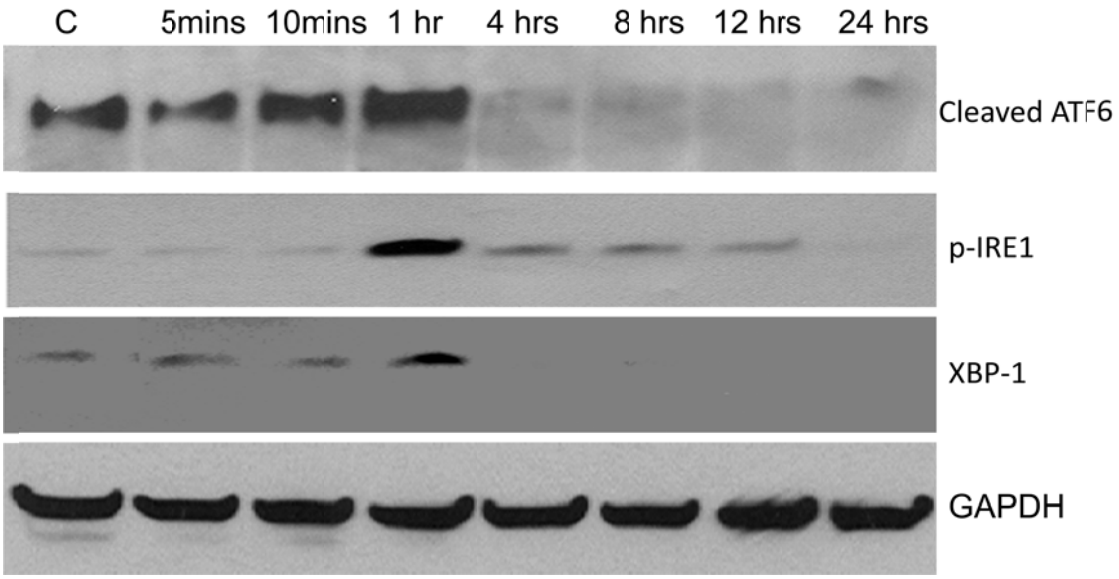

Figure 25. Representative cleaved ATF6, p-IRE1, XBP1 and GAPDH expression changes with the prolonged-time glutamate exposure. Neurons were exposed to 100 μM glutamate for 5 mins, 10 mins, 1 hour, 4 hours, 8 hours, 12 hours and 24 hours.

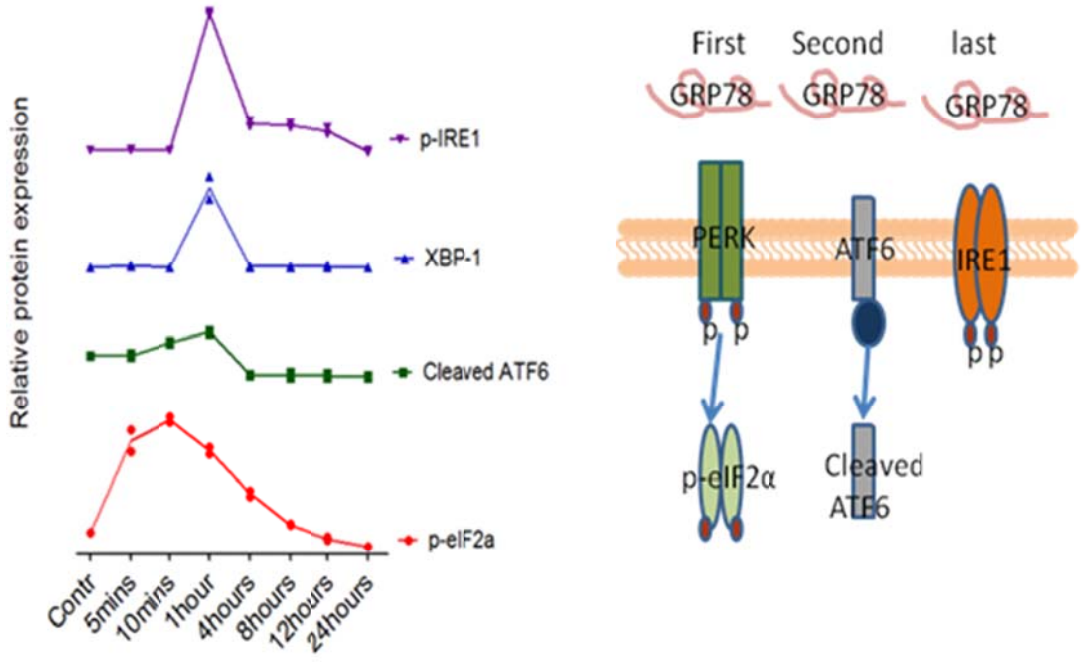

Figure 26. Quantitative results for expressions of p-eIF2α, cleaved ATF6, XBP-1 and p-IRE1 from 3 independent western blot results. The results show that PERK pathway is initiated first, ATF6 second and IRE1 pathway last.

Cytochrome C is a small heme protein found loosely attaching to inner membrane of mitochondria. The expressions of cytochrome c in rat cortical neurons treated with 100 μM glutamate at different time points are shown in Figure 27. The results demonstrate that cytochrome c is overexpressed after 1 hour exposure to glutamate.

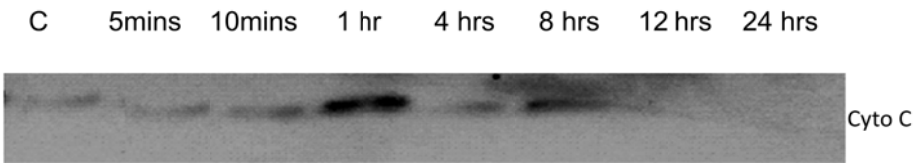

Figure 27. The expression of cytochrome c changes with the prolonged-time glutamate exposure. Neurons were exposed to 100 μM glutamate for 5 mins, 10 mins, 1 hour, 4 hours, 8 hours, 12 hours and 24 hours.

3.3. Beneficial effects of taurine on hypoxia-induced endoplasmic reticulum stress pathways in primary neuronal culture

There is increasing evidence that ER stress plays a crucial role in hypoxia/ischemia–induced cell dysfunction [112-115]. Our previous studies show that taurine can protect PC12 cells against ER stress induced by oxidative stress. Here, we investigated the protective effects of taurine in cortical neurons against hypoxia/reoxygenation-induced toxicity. Furthermore, we identified which ER stress pathway can be inhibited by taurine during hypoxia/reoxygenation process.

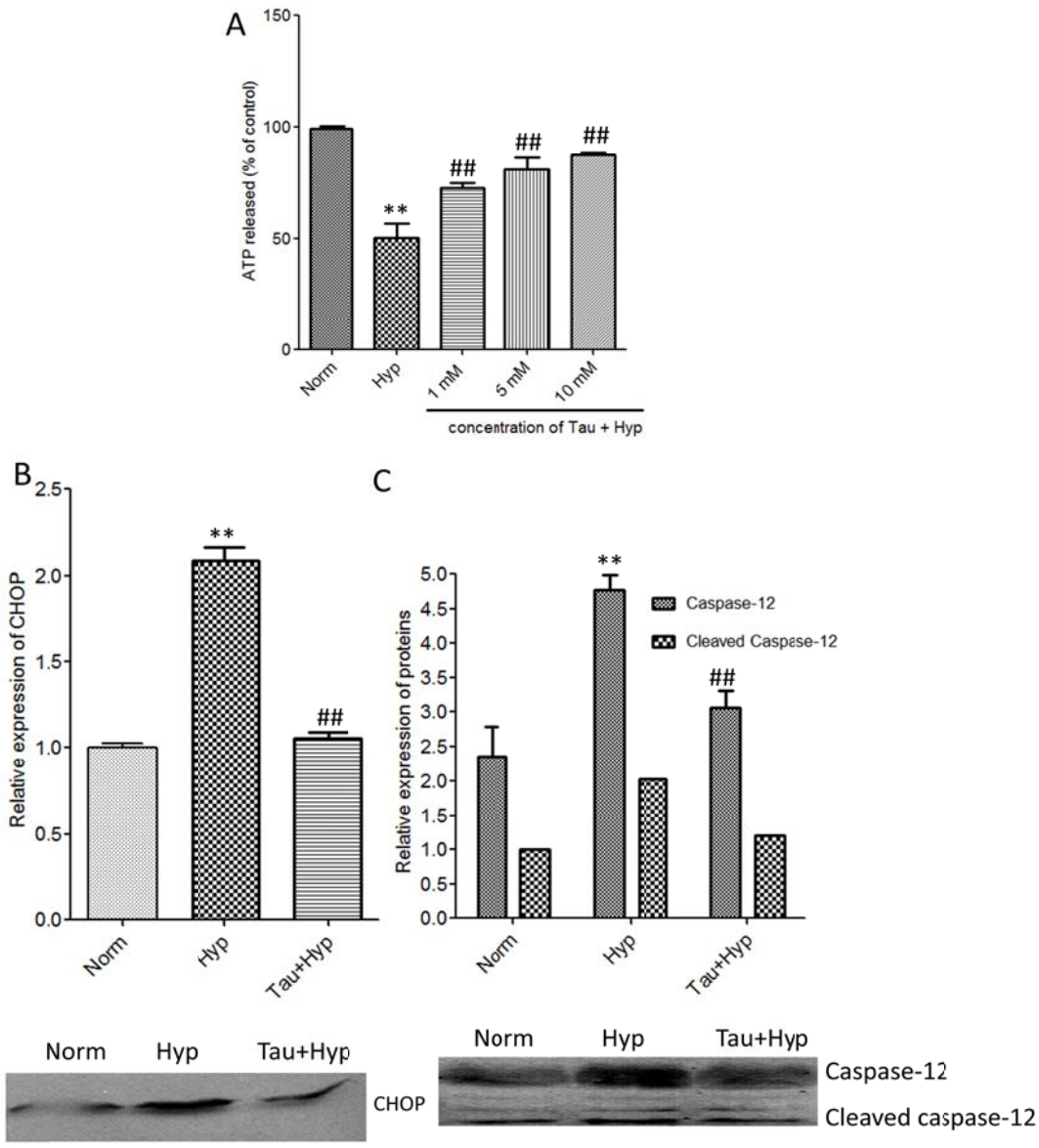

Figure 28. Neuroprotective effects of taurine against ER stress induced by hypoxia/reoxygenation. Primary neuronal culture was supplied with different concentration of taurine. Norm: normoxia; Hyp: hypoxia (0.3 % O_2) for 20 hours, reoxygenation for 20 hours; Tau+Hyp: neurons were treated with 1, 5 and 10 mM or only 10 mM taurine for 1 hour, then hypoxia for 20 hours, reoxygenation for 20 hours.

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, Tau+Hyp were normalized relative to the control values and represent mean±SEM of 5 preparations. B: CHOP expression analysed by Western blot. The bar graphs reflect the densitometric data from the experiment of CHOP Western blot results with arbituary units. C: Caspase-12 expression analysed by Western blot. The bar graphs reflect the densitometric data from the experiment of caspase-12 and cleaved caspase-12 Western blot results with arbituary units. The values in bar graph represent mean±SEM, n=3, $^{**}P<0.01$ vs. Norm and $^{\#\#}P<0.01$ vs. Hyp.

3.3.1. Taurine demonstrates robust neuroprotective effects on primary neuronal cultures against hypoxia/reoxygenation

In order to determine the appropriate concentration of taurine in cultures, cortical neurons were exposed to hypoxia and reoxygenation in the presence or absence of 1-10 mM taurine as shown in Figure 28A. 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 1mM taurine clearly improved the cell viability to greater than 70% (% of control neurons). With taurine concentration increasing up to 10 mM cell viability increased to 85% of the controls.

3.3.2. 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 hour, followed by hypoxia and reoxygenation. The expression of CHOP was measured by Western blot analysis, as shown in Figure 28B. 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 (Figure 28C). 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.

3.3.3. 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. 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. The phosphorylation of elF2α, a down-stream PERK pathway, specifically promotes the translation of the transcription factor ATF4, leading to the translational attenuation [64]. Both p-eIF2α and ATF4 are highly expressed after hypoxia/reoxygenation, and increased by approximately 1.7- and 3.0- fold over control cultures. After treatment with taurine, followed by hypoxia/reoxygenation, however, the levels of p-eIF2α in cortical neurons is similar to that of hypoxia/reoxygenation alone

(Figure 29A), 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 (approximately 3.0 times that of control) does not change in comparison with that of hypoxia/reoxygenation alone (Figure 29B). These results 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. After dissociation of GRP78, ATF6 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) [116]. 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 approximately 50% relative to neurons under hypoxia/reoxygenation without taurine as shown in Figure 29C. These results demonstrate that taurine can prevent the activation of the ATF6 pathway in hypoxia/reoxygenation.

To determine if taurine can affect 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 (Figure 29D). 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.

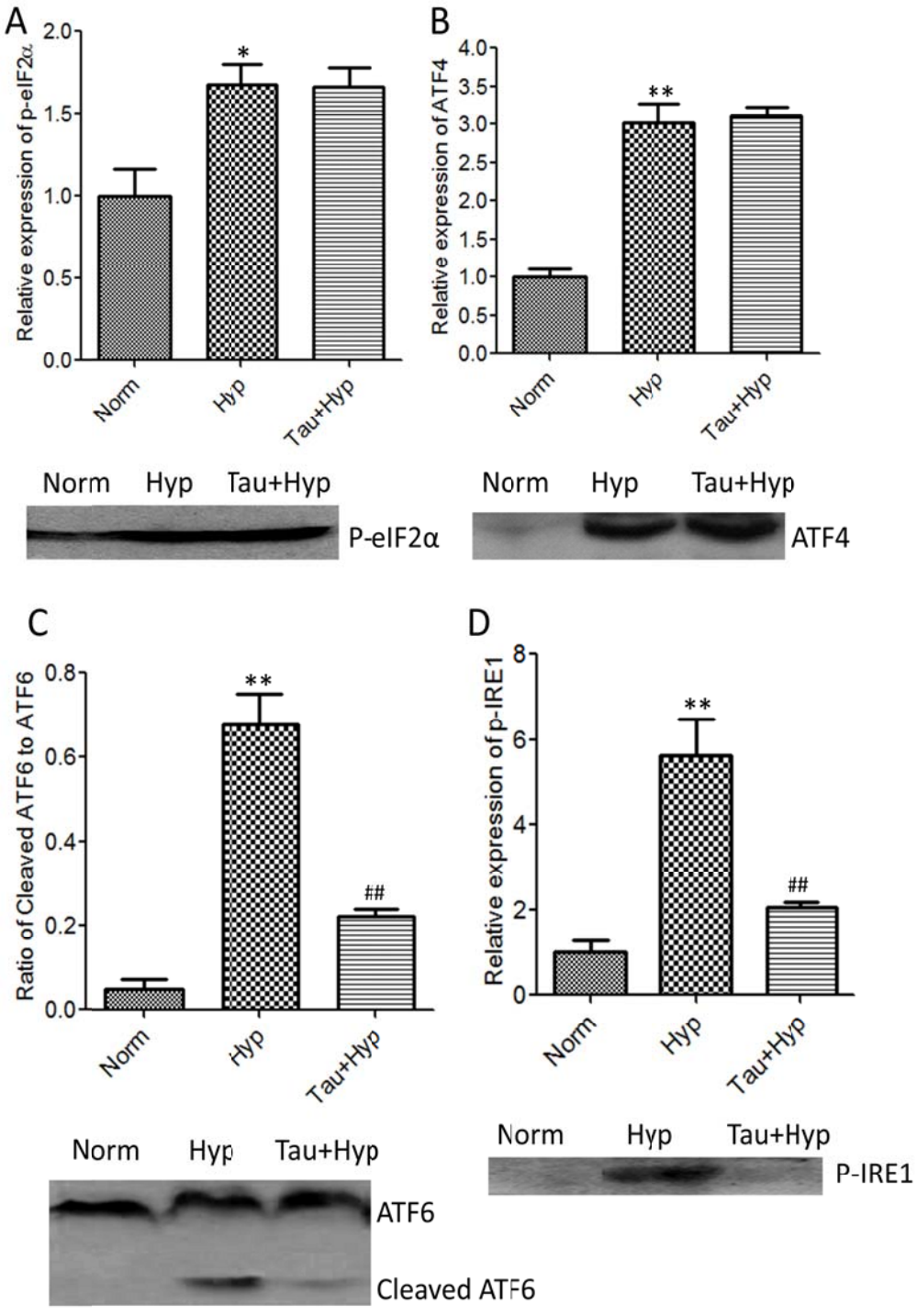
A
Relative expression of p-eIF2α
2.0
1.5
1.0
0.5
0.0
*
Norm
Hyp
Tau+Hyp
B
Relative expression of ATF4
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
Norm
Hyp
Tau+Hyp
Norm Hyp Tau+Hyp
P-eIF2α
Norm Hyp Tau+Hyp
ATF4
C
Ratio of Cleaved ATF6 to ATF6
0.8
0.6
0.4
0.2
0.0
**
##
Norm
Hyp
Tau+Hyp
D
Relative expression of p-IRE1
8
6
4
2
0
**
##
Norm
Hyp
Tau+Hyp
Norm Hyp Tau+Hyp
ATF6
Cleaved ATF6
Norm Hyp Tau+Hyp
P-IRE1

Figure 29. Taurine does not change activity of the PERK pathway, but inhibits the ATF6 and IRE1 pathways after hypoxia/reoxygenation. Norm: normoxia; Hyp: hypoxia (0.3 % O_2) for 20 hours, reoxygenation for 20 hours; Tau+Hyp: neurons were treated with 10 mM taurine for 1 hour, then hypoxia for 20 hours, reoxygenation for 20 hours. A: Western blot analysis of P-eIF2α expression. The bar graphs reflect the densitometric data from the experiment of P-eIF2α Western blot results with arbituary units. B: Western blot analysis of ATF4 expression. The bar graphs reflect the densitometric data from the experiment of ATF4 Western blot results with arbituary units. C: Western blot analysis of ATF6 expression. The bar graphs represent the ratio of cleaved ATF6 to ATF6 using the densitometric data from the experiment of ATF6 Western blot results with arbituary units. D: Western blot analysis of P-IRE1 expression. The bar graphs reflect the densitometric data from the experiment of P-IRE1 Western blot results with arbituary units. The values in bar graph represent mean±SEM, n=3, $^{*}P<0.05$ and $^{**}P<0.01$ vs. Norm; $^{\#\#}P<0.01$ vs. Hyp.

3.4. Beneficial effects of taurine on glutamate-induced endoplasmic reticulum stress pathways in primary neuronal culture

We demonstrated previously that glutamate can induce ER stress and lead to 3 ER stress pathway activation. Here, we 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) induced by glutamate.

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

As shown in Figure 30A, taurine protection is dose-dependent in cortical neurons exposed to glutamate. Taurine can significantly augment the level of cellular viability after 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.

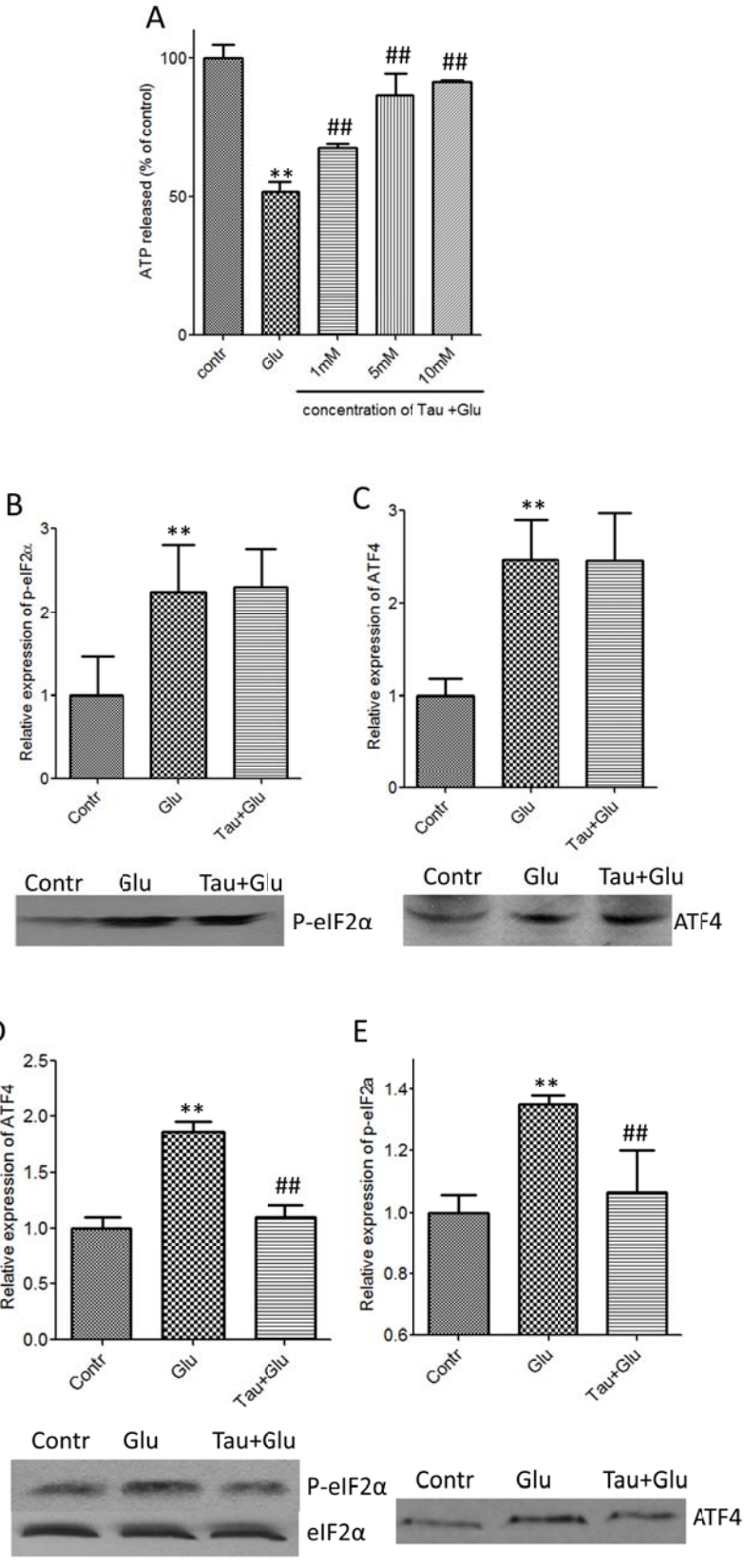
A
ATP released (% of control)
100
50
0
**
##
##
##
contr
Glu
1mM
5mM
10mM
concentration of Tau +Glu
B
Relative expression of p-eIF2α
3
2
1
0
**
Contr
Glu
Tau+Glu
C
Relative expression of ATF4
3
2
1
0
**
Contr
Glu
Tau+Glu
Contr
Glu
Tau+Glu
P-eIF2α
Contr
Glu
Tau+Glu
ATF4
D
Relative expression of ATF4
2.5
2.0
1.5
1.0
0.5
0.0
**
##
Contr
Glu
Tau+Glu
E
Relative expression of p-eIF2a
1.4
1.2
1.0
0.8
0.6
**
##
Contr
Glu
Tau+Glu
Contr
Glu
Tau+Glu
P-eIF2α
eIF2α
Contr
Glu
Tau+Glu
ATF4

Figure 30. Neuroprotective effects of taurine against ER stress induced by 100 μM glutamate. Taurine can inhibit the PERK pathway against glutamate toxicity in a short term, but not over a long time frame. Contr: control; Glu: 100 μM glutamate treatment; Tau+Glu: neurons were treated with 1, 5 and 10 mM or only 10 mM taurine for 1 hour, then exposed to 100 μM glutamate for 1 hour or 10 minutes. A: Dose-dependent neuroprotection of taurine against 100 μM glutamate for 1 hour. Cell viability was measured by ATP assay. Control values were fixed at 100%. The values for Glu, Tau+Glu were normalized relative to the control values and represent mean±SEM of 5 preparations. B: P-eIF2α expression analysed by Western blot. Cells were submitted with 10mM taurine for 1 hour, then exposed to 100 μM glutamate for another 1 hour. The bar graphs reflect the densitometric data from the experiment of P-eIF2α Western blot results with arbituary units. C: ATF4 expression analysed by Western blot. Cells were submitted with 10mM taurine for 1 hour, then exposed to 100 μM glutamate for another 1 hour. The bar graphs reflect the densitometric data from the experiment of ATF4 Western blot results with arbituary units. D P-eIF2α and eIF2α expressions are analysed by Western blot. Cells were submitted with 10mM taurine for 1 hour, then exposed to 100 μM glutamate for 10 minutes. The bar graphs reflect the densitometric data from the experiment of P-eIF2α Western blot results with arbituary units. ATF4 expression is analysed by Western blot. Cells were submitted with 10mM taurine for 1 hour, then exposed to 100 μM glutamate for 10 minutes. The bar graphs reflect the densitometric data from the experiment of ATF4 Western blot results with arbituary units. The values in bar graph represent mean±SEM, n=3, $^{**}P<0.01$ vs. Contr and $^{\#\#}P<0.01$ vs. Glu.

3.4.2. Taurine protects neurons against glutamate excitotoxicity by suppressing the expression of GRP78, CHOP, Caspase-12 and Bim

To investigate if ER stress can be induced by glutamate and then suppressed by taurine, specific ER stress effector proteins were analyzed by western blot. The expression of GRP78 protein was up-regulated in primary neurons after treatment with 100 μM glutamate for 4 hours. However, taurine restored the level of GRP78 to control levels, as shown in Figure 31. Figure 32 shows that the expression of CHOP was up-regulated by glutamate. Taurine treatment restored CHOP expression to the control level (Figure 32). Both Caspase-12 and Bim play an essential role in the progression of programmed cell death during the proapoptotic phase of the ER stress response [117, 118]. Taurine reversed the induction of Caspase-12 and Bim caused by glutamate in primary neurons, as shown in Figure 31 and Figure 32.

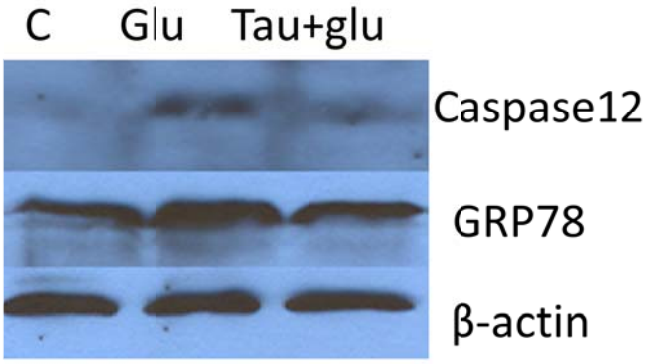

Figure 31. The effects of taurine on expression of GRP78 and caspase-12. Primary cortical neurons were treated with 25mM taurine for 1 hour before exposure to 100 μM glutamate for 4 hours.

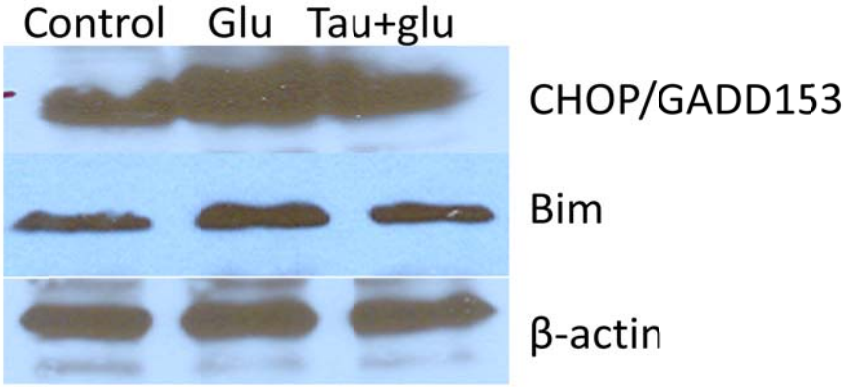

Figure 32. The effects of taurine on expression of CHOP and Bim after exposure to glutamate. Primary cortical neurons were treated with 25mM taurine for 1 hour before exposure to 100 μM glutamate for 4 hours.

3.4.3. Taurine moderately inhibits the PERK pathway at 10 minutes 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, suggesting 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 the expression of p-eIF2α and ATF4 after neurons were subjected to glutamate excitotoxicity. As shown in Figure 29B and C, the levels of p-eIF2α and ATF4 were examined by Western blot analysis after neurons were treated with taurine for one hour, then exposed to 100 μM glutamate for another one hour. 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 changes, indicating taurine may not affect the PERK pathway in ER stress induced by exposure to glutamate for one hour (Figure 30B, lanes 2 and 3; Figure 30C, lanes 2 and 3).

Glutamate-induced neuronal dysfunction and cell death may occur rapidly through glutamate receptors, even in a few minutes [119]. To determine if taurine has an effect on the PERK pathway during ER stress in a short term, we applied 100 μM glutamate to treat the neurons for 10 minutes after cultures were exposed to taurine. Within 10 minutes of exitotoxicity induced by glutamate, there was a moderate increase in both p-eIF2α and ATF4 production relative to the control neurons, about 0.8-fold and

0.35-fold respectively (Figure 30D lanes 1 and 2; Figure 30E lanes 1 and 2). Intriguingly, taurine treatment prevents the expression of p-eIF2α and ATF4, indicating that the PERK pathway is inhibited by taurine in a short term glutamate treatment (Figure 30D and E lane 3).

3.4.4. 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 after one hour exposure to glutamate, which is similar with neurons exposed to hypoxia/reoxygenation. We next examined if ATF6 and IRE1 pathways were affected by taurine after one hour exposure to glutamate. After cultured neurons were treated with 10 mM taurine for one hour followed by 100 μM glutamate for another one hour, the ATF6, cleaved ATF6 and p-IRE1 were analyzed by Western blotting, as shown in Figure 33. The ratio of cleaved ATF6 to ATF6 expression increased by glutamate excitotoxicity was noticeably reduced by taurine treatment (Figure 33A). In addition, as shown in Figure 33B, the p-IRE1 level induced by glutamate toxicity was substantially and considerably decreased by taurine treatment, from greater than 5-fold to approximately 1.5 -fold relative to control cultures. These results suggest that both ATF6 and IRE1 pathways were blocked or partially attenuated by taurine.

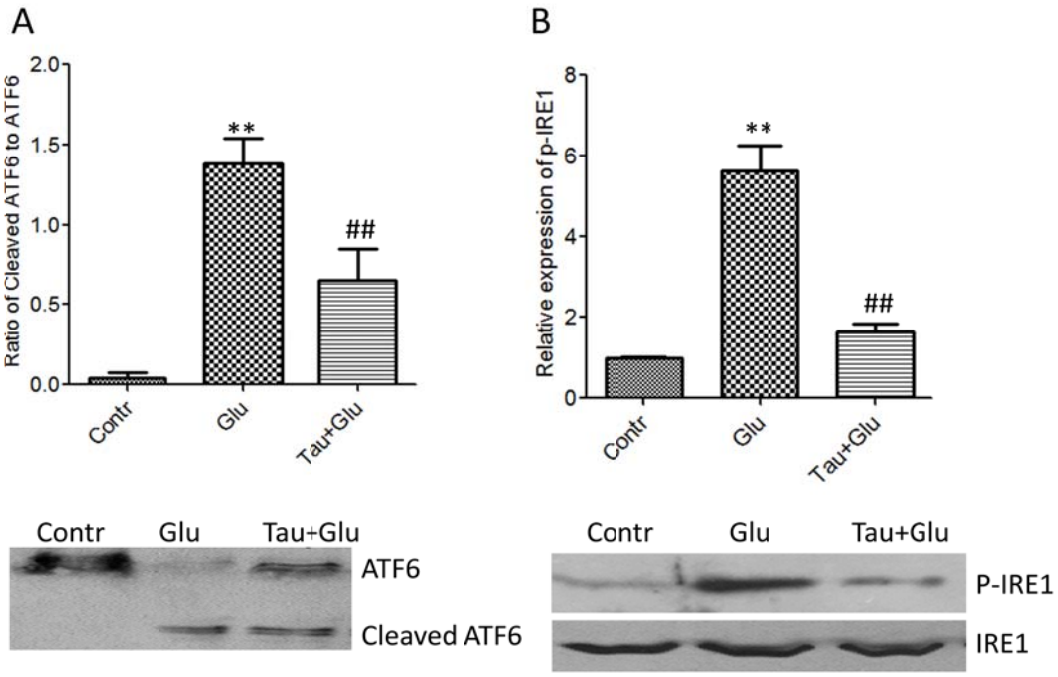

Figure 33. Taurine inhibits the ATF6 and IRE1 pathways after exposure to glutamate for 1 hour. Contr: control; Glu: 100 μM glutamate, Tau+Glu: neurons were treated with 10 mM taurine for 1 h, followed by 100 μM glutamate for another 1 hour. A: ATF6 expression analysed by Western blot. The bar graphs represent the ratio of cleaved ATF6 to ATF6 using the densitometric data from the experiment of ATF6 Western blot results with arbituary units. B: P-IRE1 and IRE1 expressions analysed by Western blot. The bar graphs reflect the densitometric data from the experiment of P-IRE1 Western blot results with arbituary units. The values in bar graph represent mean±SEM, n=3, $^{**}P<0.01$ vs. Contr $^{\#\#}P<0.01$ vs. Glu.

3.5. Granulocyte-colony stimulating factor (G-CSF) enhances cell survivial after glutamate or hypoxia insults

G-CSF is one of the few growth factors currently approved for clinical use for routine treatment of neutropenia [120]. It primarily stimulates proliferation, differentiation and maturation of cells committed to the neutrophilic granulocyte lineage through binding to the specific G-CSF receptor [121]. G-CSF also has been shown to have trophic effects on neuronal cells in *vitro* [122]. Moreover, G-CSF is an effective neuroprotectant in the treatment of a number of neurological diseases including stroke, Parkinson's disease and Alzheimer's disease [123-126]. Apart from its protective role in neurons, G-CSF also dampens systemic inflammatory reactions, which may be of additional benefits in neurodegenerative conditions [121]. Here, we studied the neuroprotective effects of G-CSF on cortical neurons against either glutamate toxicity or hypoxia/reoxygenation. Furthermore, we also tested the neuroprotective effects of combination of G-CSF and taurine on primary neuronal cell cultures.

3.5.1. Protection of G-CSF against glutamate toxicity in primary neuronal cultures

G-CSF was previously shown to exhibit a protective effect in cerebellar granule cells exposed to glutamate toxicity [123]. In the current study, we demonstrated the protective function of G-CSF at a range of concentrations from 10 to 40 ng/ml against excitotoxicity induced by glutamate in primary neuronal cultures (Figure 34). G-CSF treatment resulted in an enhanced cell survival at several concentrations, with the highest protection of 75% occurring at 25 ng/ml.

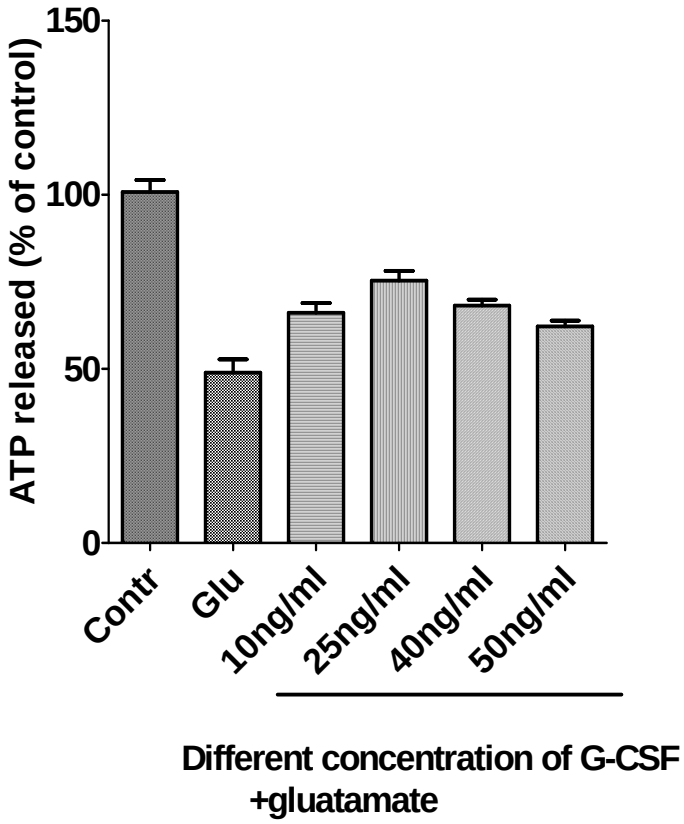

Figure 34. Neuroprotective effect of G-CSF at various concentrations against glutamate toxicity. Neurons were preincubated with 10, 25, 40 and 50 ng/ml G-CSF for 1 hour, and then exposed to 100 μM glutamate for 4 hours. Cell survival was measured by ATP assay.

3.5.2. The protective effects of the combination of taurine and G-CSF in primary neuronal cultures

It has been well established that taurine and G-CSF have many beneficial effects under a variety of conditions of cell damage. Both taurine, an inhibitory neurotransmitter, and G-CSF, a growth factor, possess neuroprotective and neurotrophic properties in *vitro*.

Here, we studied the protective effects of the combination of taurine and G-CSF against excitotoxicity induced by glutamate in rat primary neuronal cultures.

To test whether the combination of taurine and G-CSF promotes protection against glutamate-induced toxicity, we treated cortical neurons with 25 mM taurine plus 25 ng/ml G-CSF for one hour, followed by glutamate treatment for additional four hours. The results are shown in Figure 35B. The combination of taurine and G-CSF increased the neuroprotection against glutamate toxicity to 88% cell survival compared to 75% cell survival from taurine or G-CSF treatment alone, as shown in Figure 35A and Figure 34.

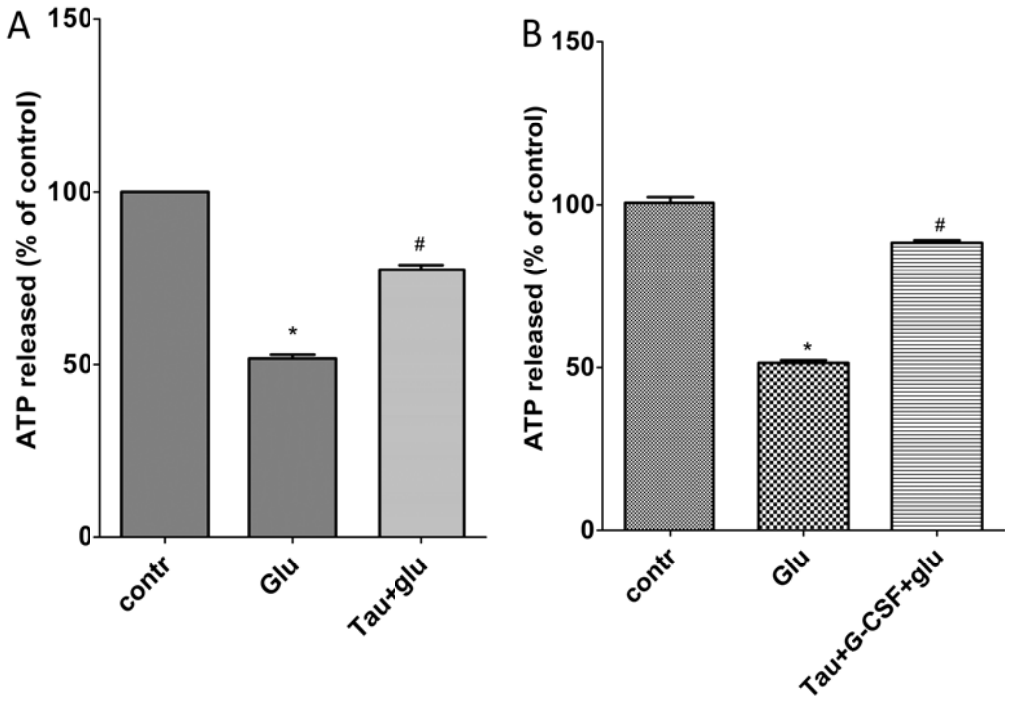

Figure 35. Neuroprotective effects of the combination of taurine and G-CSF at various concentrations against glutamate toxicity. A: neurons were preincubated with 25 mM taurine for1 hour, and then exposed to 100 μM glutamate for 4 hours. B: Neurons was preincubated with 25 mM taurine and 25 ng/ml G-CSF for 1 hour, and then exposed

to 100 μM glutamate for 4 hours. Cell survival was measured by ATP assay. $^{*}P<0.05$ vs. Contr $^{\#}P<0.05$ vs. Glu.

3.5.3. G-CSF can highly protect neurons against hypoxia/reoxygenation

In order to examine the neuroprotective effects of G-CSF, neurons were treated with 12.5 and 25 ng/ml G-CSF for 1 hour, then cells were exposed to hypoxia and reoxygenation for 20 hours respectively. G-CSF highly enhances cell survival at concentration as low as 12.5 ng/ml and the cell viability is up to 75% of normoxia group. G-CSF at 25 ng/ml shows very high neuroprotection and the cell viability reaches to approximate 84% of normoxia, as demonstrated in Figure 36A. 25 ng/ml G-CSF was applied to neurons for western blot analysis. GRP78, an ER chaperon, is highly expressed after hypoxia which is significantly reduced by 25 ng/ml G-CSF treatments (Figure 36B). These results prove that G-CSF can suppress the ER stress in neurons induced by hypoxia/reoxygenation. Antiapototic Bcl-2 is prominently restored by G-CSF, indicating that G-CSF prevents cortical neurons from injury, as shown in Figure 36B. HIF2α, a hypoxia inducible factor, is downregulated after treatment with G-CSF compared with neurons exposed to hypoxia/reoxygenation. All these results shown in Figure 36 demonstrate that G-CSF significantly promotes cell survival under hypoxia/reoxygenation insults.

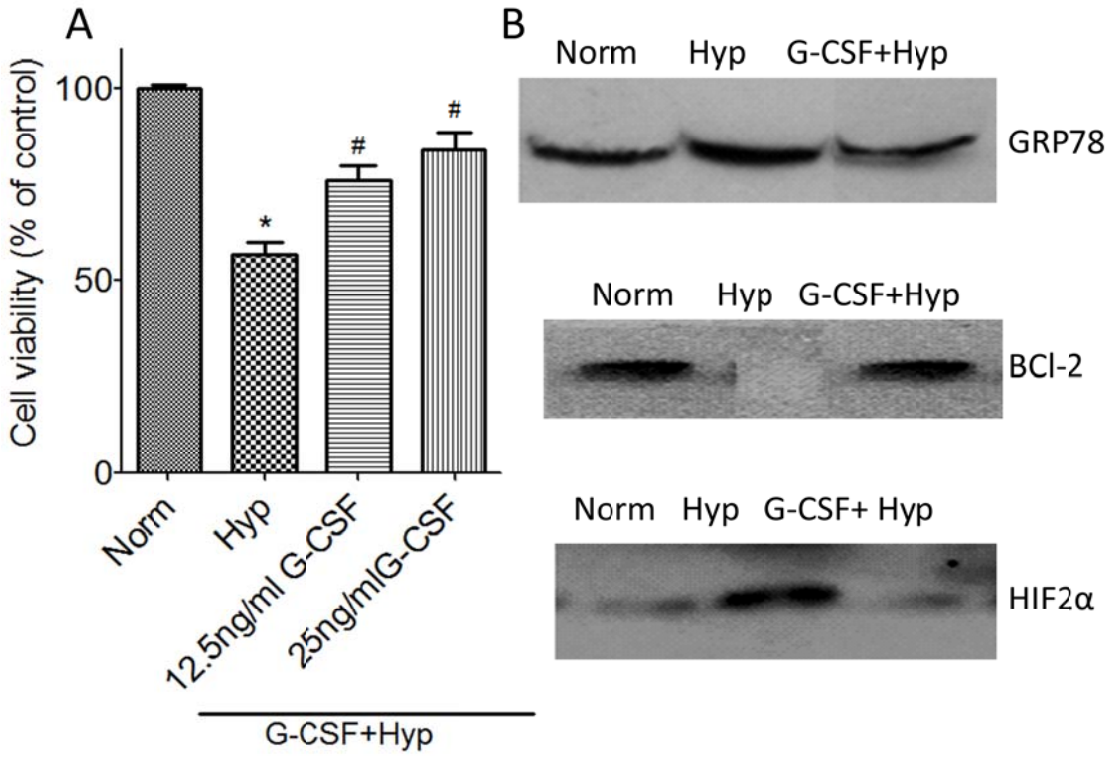

Figure 36. Neuroprotective effects of G-CSF on neurons under hypoxia/ reoxygenation condition. Norm: normoxia; Hyp: hypoxia (0.3 % O_2) for 20 hours, reoxygenation for 20 hours. G-CSF+Hyp: 12.5 or 25 ng/ml G-CSF was preincubated in neurons for 1 hour followed by hypoxia for 20 hours, and reoxygenation for another 20 hours. A: neuroprotection of G-CSF against hypoxia/reoxygenation. Cell viability was measured by ATP assay. Normoxia values were fixed at 100%. The values for Hyp, G-CSF+Hyp were normalized relative to the control values and represent mean±SEM of 4 preparations. The values in bar graph represent mean±SEM. $^{*}P<0.05$ vs. Norm and $^{\#}P<0.05$ vs. Hyp. B: Representitive western blot results of GRP78, BCl-2 and HIF2α. Neurons were treated with 25 ng/ml G-CSF for 1 hour following hypoxia for 20 hours, and reoxygenation for another 20 hours.

3.5.4. G-CSF inhibits both ATF6 and IRE1 pathways, but not PERK pathway in cortical neurons under hypoxia/reoxygenation

In Figure 37, G-CSF significantly decreases both the expression of p-IRE1 and cleaved ATF6 after hypoxia/reoxygenation compared with those hypoxia/reoxygenation neurons. Comparsion with neurons exposured to hypoxia/reoxygenation, the expression of ATF6 is upregulated by G-CSF treatment after hypoxia/reoxygenation. P-eIF2α expression shows no significant changes between before and after G-CSF treatment followed by hypoxia/reoxygenation.

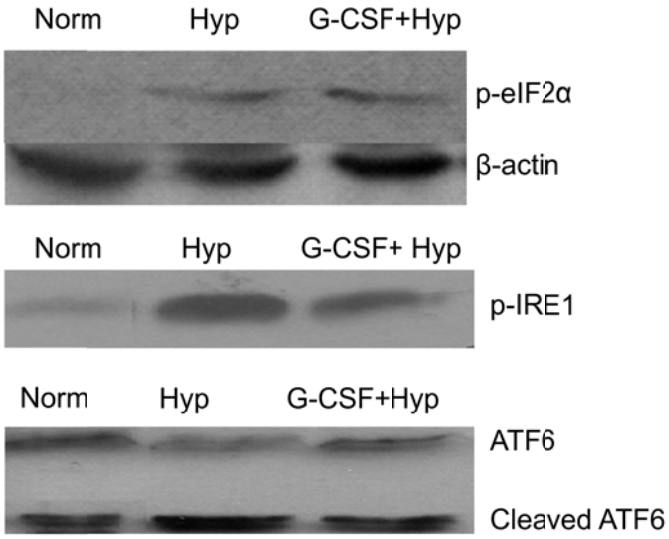

Figure 37. Representative western blot results of p-eIF2α, p-IRE1, cleaved ATF6 and ATF6. Neurons were treated with 25 ng/ml G-CSF for 1 hour following hypoxia for 20 hours, and reoxygenation for another 20 hours.

3.6. Sulindac protects neurons by inhibiting ER stress induced by hypoxia/reoxygenation

Sulindac, as a nonsteroidal anti-inflammatory drug (NSAID), is investigated for preventing recurrence and suppressing colorectal polyps in adenoma number and size in patients with familial adenomatous plyposi [127]. The protective anticancer effect of sulindac has been studied widely [128,129]. Here, we test the neuroprotective effects of sulindac on cortical neurons against hypoxia/reoxygenation-induced cell death and investigate the mechanism of its neuroprotection.

3.6.1. Sulindac shows pronounced neuroprotection in hypoxia/reoxygenation-induced cell injury

After neurons were preincubated with 25 or 50 μM sulindac for one hour followed by hypoxia/reoxygenation, the ATP assay is shown in Figure 38. Sulindac treatment highly increases the cell viability at 25 μM to about 91% of normoxia neurons. Neurons treated with sulindac at 50 μM dropped the cell viability to approximately 64% of nomoxia cultures. These results suggest that the higher concentration of sulindac is harmful to neurons. Therefore, 25 μM sulindac was utilized for western blot analysis.

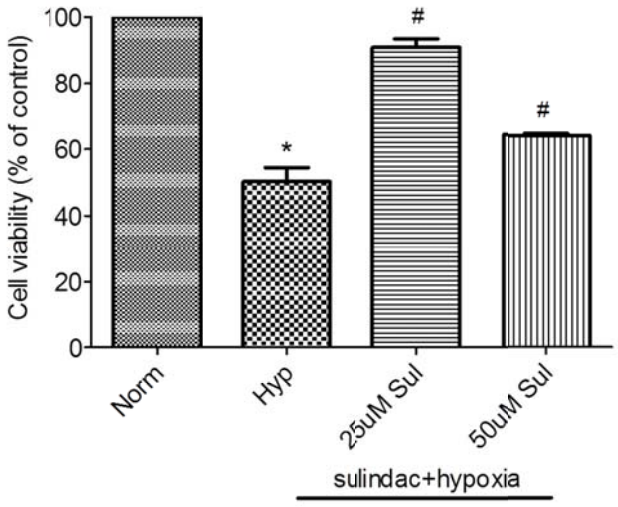

Figure 38. Neuroprotective effects of sulindac on neurons under hypoxia/ reoxygenation condition. Norm: normoxia; Hyp: hypoxia (0.3 % O_2) for 20 hours, reoxygenation for 20 hours. sulindac+Hypoxia: 25 or 50 μM sulindac was preincubated in neurons for 1 hour following hypoxia for 20 hours, and reoxygenation for 20 hours. Cell viability was measured by ATP assay. Normoxia values were fixed at 100%. The values for Hyp, sul+Hyp were normalized relative to the control values and represent mean±SEM of 4 preparations. The values in bar graph represent mean±SEM. $^{*}P<0.05$ vs. Norm and $^{\#}P<0.05$ vs. Hyp.

3.6.2. Sulindac inhibits both ATF6 and IRE1 pathway activation

Comparison to those hypoxia/reoxygeantion neurons, the expression of caspase 12 can be significantly reduced after treatment with 25 μM sulindac followed by hypoxia/reoxygenation as shown in Figure 39. It is demonstrated that sulindac treatment greatly restored the ATF6 expression which is significantly reduced by

hypoxia/reoxygenation. The expressions of cleaved ATF6 and p-IRE1 are downregulated after sulindac treatment, as shown in Fig. 39. All these results show that sulindac can exert its neuroprotection by inhibiting the activation of ATF6 and IRE1 pathways in hypoxia/reoxygenation-induced ER stress.

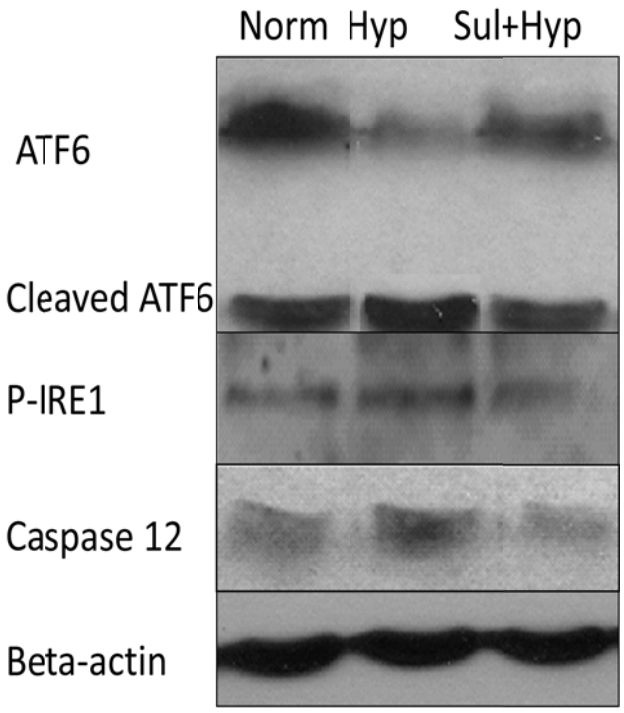

Figure 39. Representative western blot results of p-eIF2α, p-IRE1, cleaved ATF6 and ATF6. Neurons were treated with 25 μM Sulindac for 1 hour followed by hypoxia for 20 hours and reoxygenation for another 20 hours.

3.7. DETC-MeSO protects cortical neurons against ER stress induced by hypoxia/reoxygenation condition

It has been proved that S-methyl-N, N-diethyldithiocarbamate sulfoxide (DETC-MeSO), the active metabolite of disulfiram, is a partial antagonist of brain NMDA

glutamate receptors in mice. It is conceivable that the clinical efficacy of disulfiram in the treatment of alcoholism is due to its effect on glutamate receptors. DETC-MeSO is a potent and selective carbamoylating agent for glutamate receptors. DETC-MeSO partially blocks glutamate binding to synaptic membrane in brain of mice and prevents seizures induced by glutamate analogs and hyperbaric oxygen [49,130,131]. We previously show that glutamate toxicity results in the 3 ER stress pathway activation. It is reasonable to propose that DETC-MeSO may protect neurons against ER stress induced by glutamate release during hypoxia/reoxygenation process.

3.7.1. DETC-MeSO, as a NMDA glutamate receptor antagonist, shows enhanced neuroprotective activity under hypoxia/reoxygenation condition

The protective effects of DETC-MeSO on neurons are shown in Figure 40A. DETC-MeSO treatment significantly increases the cell viability up to 74% of normoxia neurons, which elevates 25%, as compared to those neurons induced by hypoxia/reoxygenation (49% of normoxia). DETC-MeSO, as an antagonist of glutamate receptor, significantly downregulates the expressions of both GRP78 and CHOP. It restores the expression of Hsp27, a heat shock protein, as shown in Figure 40B. All these results prove that DETC-MeSO is a powerful neuroprotective agents against ER stress induced by hyopxia/reoxygenation.

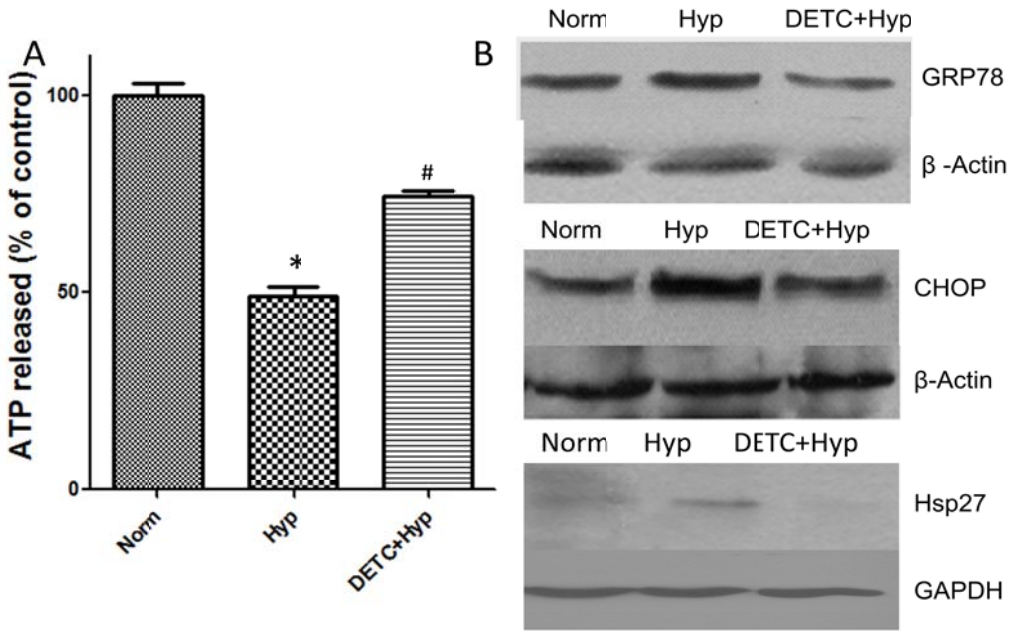

Figure 40. Neuroprotective effects of DETC-MeSO on neurons under hypoxia/ reoxygenation condition. Norm: normoxia; Hyp: hypoxia (0.3 % O_2) for 20 hours, reoxygenation for 20 hours. DETC-MeSO+Hyp: 25 μM DETC-MeSO was preincubated in neurons for 1 hour following hypoxia for 20 hours, and reoxygenation for 20 hours. A: neuroprotection of DETC-MeSO against hypoxia/reoxygenation. Cell viability was measured by ATP assay. Normoxia values were fixed at 100%. The values for Hyp, DETC-MeSO+Hyp were normalized relative to the nomoxia values and represent mean±SEM of 4 preparations. The values in bar graph represent mean±SEM. $^{*}P<0.05$ vs. Norm and $^{\#}P<0.05$ vs. Hyp. B: Representitive western blot results of GRP78, CHOP and Hsp27. Neurons were treated with 25 μM DETC-MeSO for 1 hour following hypoxia for 20 hours, and reoxygenation for another 20 hours.

3.7.2. DETC-MeSO exerts a neuroprotective effect by inhibiting both PERK and IRE1 pathways

As shown in Figure 41, DETC-MeSO restored the expression of p-eIF2α, a downstream protein of PERK pathway, suggesting that DETC-MeSO can significantly inhibit PERK pathway activation. DETC-MeSO treatment also significantly reduces the expression of p-IRE1, as compared to those neurons only performed under hypoxia/reoxygenation condition. These results demonstrate that DETC-MeSO can inhibit ER stress induced by hypoxia/reoxygenation by inhibiting both PERK and IRE1 pathways.

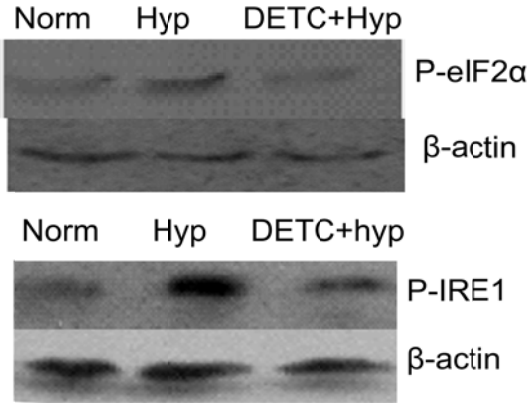

Figure 41. Representative western blot results of p-eIF2α, p-IRE1 and β-actin. Neurons were treated with 25 μM DETC-MeSO for 1 hour following hypoxia for 20 hours, and reoxygenation for another 20 hours.

4. DISCUSSION

4.1. Taurine administration attenuates ER stress induced by oxidative stress

We have presented evidence indicating that 1) extracellular taurine exerted a protective function against oxidative stress induced by H_2O_2 in PC12 cells, 2) Bcl-2 expression in PC12 cells was restored but not Bax after treatment with taurine, 3) H_2O_2 induced ER stress by up-regulation of GRP78, Bim and CHOP/GADD153 and 4) Taurine protected PC12 cells from ER stress induced by H_2O_2 through downregulation of GRP78, Bim and CHOP/GADD153.

As a naturally occurring antioxidant, taurine was investigated to treat oxidative stress trigged by a few ways, such as age-related retinal degeneration, high cholesterol diet, lead poisoning and nitric oxide [132-135]. Although a number of proved that taurine has a protective function against oxidative stress, the mechanism underlying its protection is still not fully understood. Li et al observed that taurine treatment alleviated the oxidative injury of the kidney, improved SOD and GSH-Px activities and prevented mitochondrial membrane injury. They showed that taurine could protect the kidney from oxidative injury through a mitochondrial-linked pathway [136]. Here, we demonstrate the function of taurine as a protectant against oxidative stress induced by H_2O_2 is via the alleviation of ER stress.

It has been confirmed that the overproduction of reactive oxygen species (ROS),

such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), contributes to damages of lipids, proteins, carbohydrates and nucleotides [137]. ER, acting as a protein folding compartment, is susceptible to oxidative stress [138]. In recent years, people noticed that there is cross-link between oxidative stress and ER stress [139, 140]. Hayashi et al found that oxidative ER damage is implicated in ischemic neuronal cell death by investigating changes in activating transcription factor-4 (ATF-4) and CHOP expression [138]. He et al showed that the oxidant tert-butyl hydroperoxide elevates oxidative stress, stimulates accumulation of ROS in the ER and upregulates expression of GRP-78 and GADD153 in human retinal pigment epithelium cells [141]. This gives further evidence of the connection between oxidative stress and ER stress. Interestingly, it was found that ER stress protected renal epithelial cells against oxidative stress by preventing the increase in intracellular Ca^{2+} concentration that normally follows H_2O_2 exposure [142]. Zhang proposed that ROS can target ER-based calcium channels and chaperones, leading to the release of calcium from the ER to the cytosol [139]. Increased cytosolic calcium can stimulate mitochondrial metabolism to produce more ROS. Mitochondrial ROS can further accentuate calcium release from the ER, which leads to the further accumulation of a toxic level of ROS. At the same time, perturbation of ER calcium homeostasis can disrupt the protein folding process, inducing ER stress and the activation of the UPR [139]. In the present study, we provide the supportive evidence that oxidative stress induced by H_2O_2 can contribute to ER stress. Based on the previous findings and the current results, we propose a model for the mechanism of taurine protection against ER stress induced by oxidative stress, as shown in Figure 42.

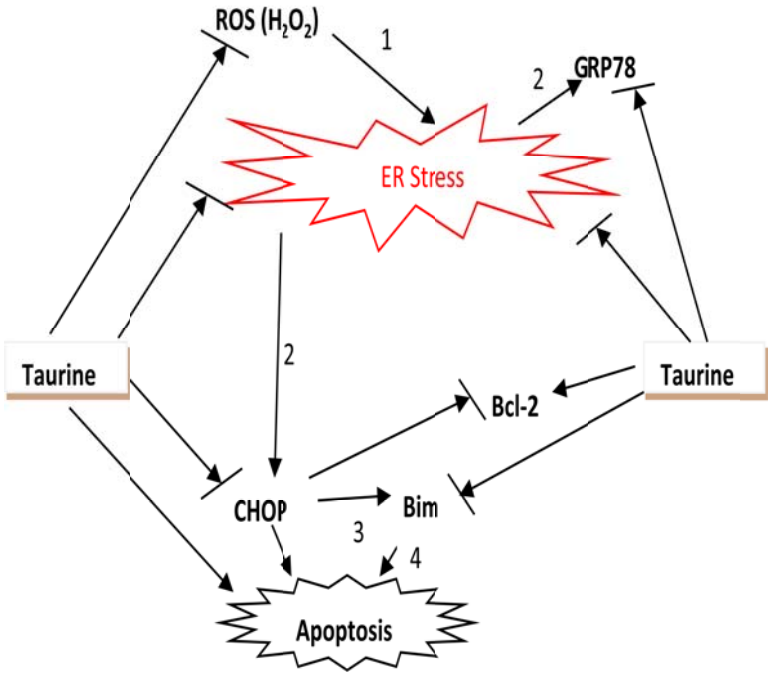

Figure 42. Proposed mechanism of taurine protection against ER stress induced by oxidative stress in PC12 cells. In the first stage, ROS (H_2O_2) induces ER stress, which leads to up-regulation of ER stress marker GRP78 and CHOP/GADD153 in the second stage. In the third stage, CHOP induces apoptosis and up-regulation of BH-3 only proapoptotic protein Bim, inhibits the expression of Bcl-2. In the fourth stage, Bim induced cell apoptosis. Taurine exerts its protection against oxidative stress induced by ROS (H_2O_2). Taurine suppresses ER stress induced by oxidative stress through down-regulating the expression of GRP78 and CHOP. Moreover, taurine protect PC12 cells by up-regulation of Bcl-2 and down-regulation of Bim.

In summary, the results of this section shed a light on the sequential relationship between oxidative stress and ER stress and the mechanism underlying protection by

taurine against oxidative stress. Taurine can protect PC-12 cell lines against oxidative stress through extracellular model, which may be due to either the large amount of taurine surrounding cells or less or no taurine transporters existing in cytoplasmic membrane. Taurine can significantly inhibit oxidative damage-induced ER stress which is triggered by H_2O_2.

4.2. Glutamate excitotoxicity can induce ER stress by activating the three ER stress pathways.

Glutamate, as a powerful neurotoxin, has been proven to be inextricably involved in the neurological disorders such as cerebral ischemia, trauma, seizers and hypoglycemia. Excessive glutamate in neurons leads to activation of glutamate receptors associated with the Ca^{2+} influx from the extracellular space into neurons through voltage-gated calcium channel or some ionotropic glutamate receptor channels, such as NMDA receptors and AMPA receptors. Extracellular glutamate accumulation also stimulates the metabotropic glutamate receptors to produce more inositol 1,4,5-triphosphate (IP3) which binds to its receptor in ER to induce the Ca^{2+} mobilization from ER [143,144]. Intracellular free calcium overload induced by glutamate excitotoxicity leads to uptake of calcium into mitochondria, disturbance of mitochondrial calcium homeostasis, and induction of mitochondrial dysfunction [104,105,145].

ER has a unique and central role among cellular organelles for protein synthesis and folding. Calcium release from ER stimulated by glutamate toxicity interrupts the calcium homeostasis in ER, which may result in ER stress. Recently, a few paper proposed that glutamate excitotoxicity are responsible for ER dysfunction and ER stress

[102,103,146]. However, the mechanism of glutamate-induced ER stress still remains unclear. In this study, we have demonstrated the effects of glutamate on ER stress in changes of dose and time manner.

ER stress initiates the overexpression of CHOP, caspase-12 and 2 ER chaperons: GRP78 and calnexin. Our findings show that the expressions of these 4 ER stress markers are elevated with the increment of glutamate concentration. The classical ER stress response triggers modulation of three distinct signaling pathways: ATF6, PERK and IRE1. Activated PERK phosphorylates the α-subunit of eukaryotic translational initiation factor 2 (eIF2α), leading to translational attenuation and inhibition of global protein synthesis [147]. Phosphorylation of eIF-2α promotes the expression of GADD34, which then assembles an eIF-2α phosphatase that functions in a negative feedback loop to reverse eIF-2α phosphorylation and suppress UPR [111,148]. Our results show that excessive extracellular glutamate increases the expressions of both p-eIF2α and GADD34. After dissociation of GRP78, ATF6 translocates to the Golgi apparatus where it is cleaved into its active form by site-1 and site-2 proteases to generate 50-kDa cytosolic protein fragments that migrate to the nucleus and activate transcription of X box-binding protein 1(XBP-1) [149]. Both cleaved ATF6 and p-IRE1are upregulated after exposure to glutamate in neurons, and gradually increased with the higher glutamate concentration. We also investigated the occurring order of these 3 pathways in glutamate-induced ER stress. We find that PERK pathway was stimulated first, then ATF6 pathway, IRE pathway last.

The pro-apoptotic members of the Bcl-2 family appear to be recruited to the ER surface and to activate caspase-12, whereas the anti-apoptotic members inhibit the

recruitment, although the exact relationship between these factors is still unclear. Thus, it is plausible that Bcl-2 family also is involved during ER stress induced by glutamate. Our results prove that Bcl-2 expression is downregulated, while the expressions of Bax and PUMA are upregulated with the increment of glutamate concentration.

In many cases, apoptosis is initiated via mitochondrial release of cytochrome c, a component of the mitochondrial electron transport chain [150]. Here, we found that cytochrome c reaches its highest expression after 1 hour exposure to glutamate. Cleaved ATF6, p-IRE1 and XBP-1 all have the highest expression after glutamate exposure for one hour. Both ATF6 and IRE1 pathways are responsible for neuronal apoptosis during ER stress. It is quite possible that these few proteins cooperate with each other and work at the same time contributing to the cell death.

In summary, Glutamate excitotoxicity is the final common pathway resulting in neuronal injury for many seemingly unrelated disorders, including ischemia, trauma, seizures, hypoglycemia, hypoxia, and even some neural degenerative disorders. Fully understanding the mechanism under glutamate toxicity is important for neurologists because of its central position in many of the disorders encountered in daily practice. The above results suggest the possible future relevance of therapeutic strategies targeting the ER to rescue neurons from excitotoxicity induced by glutamate.

4.3. Neuroprotective effects of taurine against both hypoxia/reoxygenation- and glutamate-induced toxicity

Taurine, a major intracellular free amino acid, is known to be able to protect against tissue damage in a variety of diseases [151-154]. Several protective mechanisms

of taurine have been proposed. Taurine was firstly shown to be capable of improving osmotic status and calcium homeostasis in cell damage caused by hypoxia or glutamate excitotoxicity [155-157]. As an antioxidant, taurine protects tissues against reactive oxygen species generation from hypoxia or from Mn-superoxide dismutase inhibition in the myocardial mitochondria [157]. In addition, taurine is also a GABA agonist and may increase GABA levels by increasing GABA synthesis and by $GABA_A$ receptor activation [152, 153]. Several papers provided the evidence that taurine exerts protective effects through prevention of mitochondrial dysfunction [157-159]. We recently demonstrated that ER stress inhibition may also be involved in taurine protection mechanisms under conditions of glutamate excitotoxicity [160]. However, details of the relevant signaling pathways remain to be elucidated. 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 [112-114]. 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 [60, 64, 75]. CHOP, an ER stress-associated proapoptotic factor, can be induced by hypoxia and reoxygenation. We have demonstrated a substantial induction of CHOP levels after exposure to hypoxia/reoxygenation, as shown in Figure 29B, and this increase was prevented by administration of taurine. 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 [161,162]. We have analyzed the expression of caspase-12 in the absence or presence 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 been proved to be responsible for repressing global protein synthesis via phosphorylation of the α subunit of eIF2α [55,114]. 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 [64]. 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 absence or presence 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 with "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 hour 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. 31D and E). Overall, it is reasonable to conclude that taurine may delay the initiation of the PERK pathway at an early time point, but does 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. ATF6 (90kDa, P90) can be cleaved into a 50 kDa protein (ATF6 P50) through translocating to the Golgi apparatus. Quantifying the ratio of cleaved ATF6 to full length ATF6 revealed significant differences in hypoxia/reoxygenation or glutamate treatment with taurine, compared with the same conditions but without taurine. The results 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, the 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.

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 Figure 43, our results show that taurine may significantly inhibit the activation of the ATF6 and the IRE1 pathways, 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.

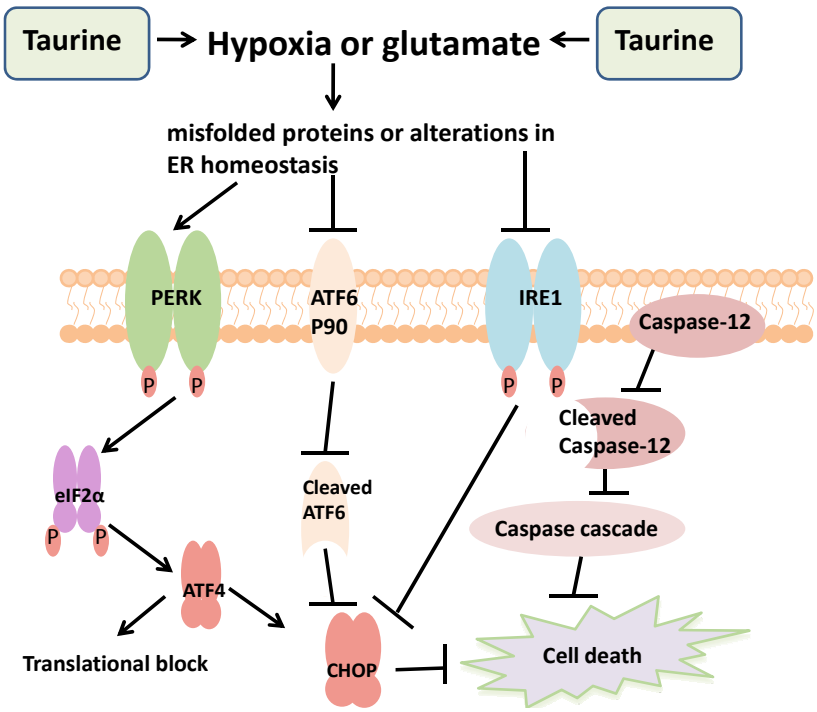

Figure 43. Scheme for protective effects of taurine against activation of ER stress pathways. After neurons were submitted with glutamate or hypoxia, the homeostasis in neuronal culture is disturbed, which initiates dimerization and autophosphorylation of ER membrane proteins PERK and IRE1. ATF6 (P90) 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.

4.4. G-CSF reduces neuronal injury in glutamate or hypoxia/reoxygenation-induced ER stress

We have demonstrated the potent protection by taurine and by G-CSF in an *in vitro* model of primary cortical neuronal cell death induced by glutamate or hypoxia/reoxygenation. Taurine and G-CSF protected primary cortical neurons against glutamate-induced neurotoxicity as determined by measuring cell viability using the ATP assay. On the other hand, we found that the combination of taurine and G-CSF gave a synergistic enhancement of protection against glutamate in primary cortical neurons. We have further shown that the suppression of ER stress is an essential underlying mechanism for G-CSF-induced neuroprotection. Our investigation of the intracellular mechanisms downstream demonstrated a pronounced inhibition of G-CSF in hypoxia/reoxygenation-induced ER stress.

A previous paper reported that the combination of taurine and basic fibroblast growth factor (bFGF) gave an enhanced neuroprotection in granule neurons against glutamate induced excitotoxicity [156]. The investigators showed that neuroprotection was obtained only through the combined action of taurine and bFGF in a cerebellar granule neuron rich culture, but not by these factors alone. Therefore, they believed that taurine can augment bFGF function under certain conditions. Here, we demonstrated that taurine or G-CSF administration showed a neuroprotective effect on neurons. Furthermore, an enhanced protection against glutamate was also observed with a

combination of taurine and G-CSF. The clinical application of taurine was investigated and found to be effective in studies as early as 1974 when it was applied to treatment for refractory epilepsy [163]. Both taurine and G-CSF have been shown to be potential drugs for ischemia or stroke in clinical applications [164, 165]. Since the combinations of taurine and G-CSF have synergistic neuroprotective effects against glutamate excitotoxicity, this strongly suggests that the combination of taurine and G-CSF may be more effective than the individual agents in treatment of neurological diseases, such as stroke.

We demonstrated that both taurine and G-CSF protect primary cortical neurons against glutamate-induced cell death. Interestingly, we found that the combination of taurine and G-CSF results in an enhanced protective effect. Because both taurine and G-CSF are neuroprotective agents that are approved for clinical use, the combined administration of these two factors may constitute a viable therapy with potentially enhanced therapeutic efficacy. Moreover, G-CSF suppressed the hypoxia/reoxygenation-induced ER stress by inhibiting the activation of both ATF6 and IRE1 pathways.

4.5. Sulindac has a beneficial effect on ER stress under hypoxia/reoxygenation condition

Sulindac serves as a nonsteroidal anti-inflammatory drug for a long time due to its reduction to sulindac sulfide by the aldehyde oxidase [166]. Our data provides a direct evidence that sulindac protects neurons against hypoxia/reoxygenation-induced cell injury in primary neuronal cell culture. In addition to the neuroprotective effects of

sulindac, the inhibition of ER stress pathway activation also has been observed in the sulindac treatment for cortical neurons under hypoxia/reoxygenation condition.

The growing evidence indicates that sulindac has strong inhibitory effects on cancer cells, such as glioblastoma, hepatomas, colorectal cancer, and breast and lung cancer [67-169]. Sulindac can enhance the death of cancer cells after they are exposed to oxidative chemicals, under the conditions in which normal cells are either not affected, or show protection [170, 171]. There are a few studies supporting that sulindac can protect cells against free radical or ROS damage. Sulindac has been shown to protect the skin of SKH-1 hairless mice in response to UV light and is found to suppress the depletion of GSH in the hippocampus under conditions of quinolinic acid-induced oxidative stress [172,173]. Sulindac has also been demonstrated to decrease age-related defects in learning and memory in rats [174]. Sulindac is able to protect both rat cardiac myocytes in culture and intact hearts against oxidative stress resulting from ischemia/reperfusion [14]. Hypoxia or ischemia has been proven to induce oxidative stress. We found that chemical agent-induced oxidative stress can result in ER stress in PC12 cell lines. Thus, it seems very reasonable that sulindac shows inhibitory function in ER stress induced by hypoxia/reoxygenation in primary neuronal cell culture. Moreover, our results demonstrated that Sulindac can suppress ER stress induced by hypoxia/reoxygenation through inhibiting the activation of both ATF6 and IRE1 pathways.

4.6. DETC-MeSO attenuates cell death by inhibiting ER stress

DETC-MeSO, a metabolite of the drug disulfiram, is a partial antagonist of glutamate receptors by carbamoylation to sufhydryl group, which is found in surface of

NMDA receptors [49]. We have found the glutamate excitotoxicity on neurons induced ER stress pathway activation. Thus, selective inhibition on glutamate receptor (e.g. NMDA receptor) by DETC-MeSO may also prevent cortical neurons from glutamate- or hypoxia/reoxygenation-induced ER stress.

Glutamate release from presynapic membrane is one of consequences of hypoxic or ischemic cell injury, which has an essential effect on neuronal death. The results show that DETC-MeSO is a very effective agent for protecting cortical neurons from hypoxia/reoxygenation induced cell damage. This may be due to the inhibition of DETC-MeSO on glutamate receptors. Glutamate release triggers activation of glutamate receptors and leads to the injury of surrounding neurons. Therefore, DETC-MeSO may play a dual role in preventing hypoxia-induced cell death, in which it could suppress the glutamate release from the presynaptic sites of neurons and the activation of glutamate receptors postsynaptically on neurons.

DETC-MeSO significantly inhibits ER stress under hypoxic condition in primary neuronal cells, as shown in chapter 3. We also demonstrate that DETC-MeSO inhibits both PERK pathway and IRE1 pathway activation. These results give a clue that DETC-MeSO could be a potential and promising protectant for stroke treatment.

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