Obstructive sleep apnea hypopnea syndrome (OSAHS) in children is associated with multiple system morbidities. Cognitive dysfunction as a result of central nervous system complication has been reported in children with OSAHS. However, the underlying mechanisms are poorly understood. Endoplasmic reticulum stress (ERS)-related apoptosis plays an important role in various diseases of the central nervous system, but very little is known about the role of ERS in mediating pathophysiological reactions to cognitive dysfunction in OSAHS. Chronic intermittent hypoxia (CIH) exposures, modeling OSAHS, across 2 and 4 weeks in growing rats made more reference memory errors, working memory errors and total memory errors in the 8-Arm radial maze task, increased significantly TUNEL positive cells, upregulated the unfolded protein response in the hippocampus and prefrontal cortex as evidenced by increased phosphorylation of PKR-like endoplasmic reticulum kinase, inositol-requiring enzyme I and some downstream products. A selective inhibitor of eukaryotic initiation factor 2a, salubrinal, prevented C/EBP-homologous protein activation in the hippocampus and prefrontal cortex throughout hypoxia/reoxygenation exposure. Our findings suggest that ERS mediated cellular apoptosis may be one of the underlying mechanisms of cognitive dysfunction in OSAHS children. Further, a specific ERS inhibitor Salubrinal should be tested for neuroprotection against CIH-induced injury.

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that CIH exposure during a critical period of neuronal development can lead to substantial deficits in spatial memory in this model.

Recently, endoplasmic reticulum (ER), mediating cell stress responses have been linked to cell apoptosis. ER functions can be disturbed by different insults, such as accumulation of unfolded proteins and changes in calcium homeostasis (Boyce and Yuan, 2006; Verkhratsky, 2005). The unfolded protein reaction (UPR) signaling is orchestrated by three different pathways, each of which is initiated by an eponymous distinct sensor anchored in the ER as a transmembrane protein and termed the protein kinase dependent on RNA (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6) (Lupachyk et al., 2013). Disturbed ER functions induce expression of chaperones, attenuate protein translation, and activate ER-associated degradation (Krasikiewicz and FitzGerald, 2012). However, when this stress is insurmountable, the ER may take on the role of executioner, activating three pathways, including C/EBP-homologous protein (CHOP), c-Jun N-terminal kinase (JNK) and cysteine-containing gaspartate-specific proteases (caspase-12).

It has been demonstrated that ER played a central role in both adaptive responses to injury from ischemia–reperfusion challenges, and the change of hypoxia/reoxygenation in OSAHS is similar to that of ischemia–reperfusion. However, little is known about the role of ER responses in mediating pathophysiological reactions in OSAHS, so the aim of this study was to test the hypothesis that ER induced-damages to the hippocampus and prefrontal cortex in the growing rats play important roles in the pathogenesis of CHI associated neurocognitive dysfunction.

An adaptive response to endoplasmic reticulum stress (ERS) is the phosphorylation at Ser51 of the subunit alpha of eukaryotic initiation factor 2 (eIF2a), thereby blocking translation initiation and synthesis of membrane and secreted proteins critical in brain function. Induction of p-eIF2a is a key step in the ER stress response that will be demonstrated in this study by administration of salubrinal (Sal), a small molecule that increases p-eIF2a by inhibiting its dephosphorylation. In addition, we would like to confirm whether Sal could adapt inner-cellular stress to protect against ER stress-induced cell injury.

Materials and methods

Animal model of OSAHS and experimental groups

This study was approved by the Ethics Committee of Wenzhou Medical University. A total of sixty four male Sprague–Dawley rats (80–100 g; Shanghai Laboratory Animal Center, Shanghai, China) were randomly divided into eight groups that the number of each is eight by the method of random number table: 2-week-IH group (2IH), 4-week-IH group (4IH), 2-week-control group (2C), 4-week-control group (4C), 2-week-Salubrinal group (2SAL), 4-week-Salubrinal group (4SAL), 2-week-dimethylsulfoxide (DMSO) group (2DMSO), and 4-week-DMSO group (4DMSO). Rats except in control groups were laid in intermittent hypoxia cabin, an automated nitrogen/oxygen gas delivery system (Scientific Research Center of Wenzhou Medical College, Zhejiang, China) to deliver hypoxia/reoxygenation, using our previously described protocol (Cai et al., 2010). Two durations of chronic intermittent hypoxia (IH) (2 and 4 weeks) were studied.

Sal (Ellisville, USA) was initially solubilized in DMSO (Sigma, Helsinki, Finland) to make a 20 mM stock solution, which was diluted with buffered saline to 100 μM aliquots, also 0.48 mg of Sal in 1 ml of DMSO and 99 ml of buffered saline. The final concentration of DMSO was 1%. 100 μM Sal was administered at the dose of 1 mg/kg intraperitoneally 30 min before CHI daily (Sokka et al., 2007). An equal volume of DMSO (1%, 20.8 ml/kg/d) was injected in DMSO group rats.

Intermittent hypoxia exposure

The CIH model was established according to Wang et al. (2011) with modifications. A steel cabin (60 × 22 × 16 cm) for generating intermittent hypoxia and air control was created and an automated nitrogen/oxygen gas delivery system was used to deliver hypoxia/reoxygenation, using our previously described protocol (Cai et al., 2010). Briefly, in this system O2 concentration could be reduced to a nadir of 9% ± 1.5% in 30 s by infusion of 99.99% nitrogen with the pressure kept at 0.3 KPa, stabilized at that level for 30 s, and then gradually increased to 21.0 ± 0.5% over the next 12 s by infusion of 99.50% oxygen (25 l/min) into the cabin. The computer controlled the infusion of oxygen and nitrogen. This cycle was repeated every 90 s over 7.5 h (from 8:00 to 15:30) during the animals’ diurnal sleep period for certain days according to the experimental design. Ambient temperature was kept at 22–24 °C. The rats in IH group, SAL group and DMSO group were exposed to CIH for 2 weeks and 4 weeks. The control groups were placed in the cabin filled with compressed air for 2 weeks as 2C group or for 4 weeks as the 4C group respectively. The O2 concentration was kept at 21.0 ± 0.5% in the control cabin.

Test of the chronic intermittent hypoxia cabin

The CIH cabin validation was performed before this experiment. 10 rats were anesthetized with 35 mg × kg–1 sodium pentobarbital (Sigma, USA) by intraperitoneal injection, and the carotid artery was catheterized. The catheter with heparin anticoagulation was inserted in the left carotid artery, sutured in place. When all rats recovered from surgery, 5 rats were selected randomly and placed individually into the IH cabin, while the left 5 rats were placed into the control cabin. Then, the experimental protocol was carried out for 2 h.

The arterial blood samples were drawn in 22.5 s interval during a single IH cycle, continuous blood for 5 times, every <3 s, with the initial nitrogen gas input as the first sample respectively. Arterial blood samples (0.5 ml) were collected in a 5-gauge needle at the end of each sequential condition and immediately analyzed using a blood-gas analyzer (GEM Premier 3000; America).

8-Arm (4-arm baited) radial maze test

The eight-Arm radial maze with four arm-baited was used to assess the spatial memory. We used a technique similar to that described in detail by Andoh et al. (2009) previously. The maze made of plexiglass was positioned in a testing room (room temperature of 25 °C, a humidity of 50 ± 5% and 12 h light/dark cycle). The central area was 30 cm in diameter and the arms were 50 cm long, 10 cm high, and 12 cm wide. A food cup (20 mm in diameter and 1 cm in depth) was located at the distal end of each arm. Prior to the experiments, the rats were restricted from chow for 2 days until their body weights were reduced to 80–85% of the baseline weight. First, an acclimation trial was conducted according to our previous experiment (Cai et al., 2010). After adaptation, all rats were trained once daily following CIH at 19:00 PM for 10 days. During the trial periods, 45 mg food pellets were located only in the food cups of arms 1, 3, 5 and 7 while the remaining 4 arms were empty. The rat was placed in an opaque box (20 × 20 × 20 cm) in the central platform. 15 s later, the box was taken away and the rat was permitted to seek food freely through the maze.

The performance of the rat was assessed by the number of error choices. A correct choice occurred if the rat entered into an unvisited arm, followed by food acquisition in the trial. An error was defined as the hind legs of the rats enter an incorrect arm. Entry into the never-baited arm was regarded as a reference memory error (RME), while re-entry into the arms in which the bait had already been acquired was considered as a working memory error (WME). RME plus WME was called as the total error (TE). The trial ended when the rat acquired...
all bait or 5 min had elapsed. After CHI for 2 weeks or 4 weeks, the 8-Arm radial maze test was performed once again as detailed above to assess spatial memory in the rats.

Collection of the specimens

After 8-Arm radial maze test was completed, 64 rats were anesthetized with 35 mg x kg⁻¹ sodium pentobarbital (Sigma, USA) by intra-peritoneal injection. The hippocampus and prefrontal cortex tissues were rapidly dissected. The right side of hippocampus and prefrontal cortex tissues were fixed in 4% paraformaldehyde for TUNEL assay, while the rest were frozen in liquid nitrogen, and then collected rapidly and stored at −80 °C for western and real time PCR analysis.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

A TUNEL assay (in situ cell death detection kit, fluorescein, Roche) was performed to detect apoptosis according to the manufacturer’s instruction. Briefly, tissues were processed for paraffin embedding, cut in 3-mm-thick sections on a microtome. Tissue sections, after dewaxing, were blocked by 3% hydrogen peroxide for 15 min and rinsed with PBS. After treatment with TUNEL assay mixture for 30 min and POD for 60 min, the sections were incubated with DAB (3,3-diaminobenzidine tetrahydrochloride, Sigma-Aldrich), counterstained with Novocastra hematoxylin dye (Leica Microsystems, Vienna, Austria), and mounted in Eukitt (Sigma-Aldrich). Cells with yellow-brown granules in the nucleus were considered to be apoptotic cells. Total TUNEL positive stained cells in the CA1 area of hippocampus and in prefrontal cortex were respectively counted in three randomly chosen views per section and five sections per sample under the light microscope with 400 × magnification. The Image-Pro Plus software was used for image analysis. An apoptotic index (AI) was calculated as the number of TUNEL-positive cells/total cells × 100 to be used to assess the apoptosis. Six animals in each group were analyzed, and the mean AI was determined.

Quantitative PCR

RNA was prepared from hippocampal and prefrontal cortical tissue and cDNA synthesized using 50 U of SuperScript II reverse transcriptase and components given by the vendor (Invitrogen, America). Quantitative PCR was performed using LightCycler (Roche, America) and the following primers: BiP: forward 5′-GGCGATCAAGTGGAAAGAAA-3′, reverse 5′-TCTGACACCCAGCAACG-3′; ATF4: forward 5′-CTCAAGATGCGTCTATGGGA-3′, reverse 5′-GGGGAGCGTCAAGAAGTGA-3′; CHOP: forward 5′-GGCGTCCCGAGAAGACCA-3′, reverse 5′-TGAGATATAGGTGCCCTCCC-3′; XBP-1 s: forward 5′-GAACGACGTTAAAGACGCG-3′, reverse 5′-AGGCGACAGTGCAGATCCTG-3′; TRAF2: forward 5′-ACCTGTGATGCGTGGAGCA-3′, reverse 5′-GGTTCTGTAGGCTGGAGACT-3′; EDEM: forward 5′-CCATATCATCTATGTCGCTAGC-3′, reverse 5′-TCAAATCAAAGCACCCTCGTG-3′; ERSE: forward 5′-CTCTAAAGAACCTTGAGGCCCAAGTC-3′, reverse 5′-CTATATTCCATCTGTTTCTCT-3′; c-ATF6: forward 5′-CCAGCTTCAGATGGAACGG-3′, reverse 5′-GAGACACAAACTGGACGCAG-3′; β-actin: and forward 5′-CACAGTGGCCCATCTATGTA-3′, reverse 5′-CCATCTCCGTCGTAAGCT-3′.

Amplification was performed using an initial 10 min step at 95 °C, followed by 50 cycles with 15 s at 95 °C, 5 s at 60 °C, and 4–12 s at 72 °C, and with a final extension for 10 min at 72 °C. Specificity of the product was confirmed by melting-curve analysis. Quantification was done from the product on the reaction kinetics, and expression levels were related to β-actin.

Protein extraction and Western blot

Hippocampal and cortex tissues were lysed using ice-cold radio-immunoprecipitation assay buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 50 mM Tris–HCl, pH 8.0) supplemented with protease inhibitor mixture (Roche, Espoo, Finland). Equal amounts of protein were subjected to SDS-PAGE and blotted onto nitrocellulose filters (Amersham Biosciences, Helsinki, Finland). These were first incubated for 1 h in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 5% skimmed milk and then overnight at 4 °C with primary antibodies: anti-CHOP (1:250; Santa Cruz Biotechnology); anti-phosphorylated (p)-PERK (1:500) and anti-PERK (1:500; both from Santa Cruz Biotechnology); anti-phosphorylated eukaryotic translation initiation factor 2 subunit alpha (anti-p-eIF2α, 1:1000) and anti-eIF2α (1:1000; both from Cell Signaling); and anti-actin (1:5000; Sigma); anti-p-ATF6 (1:800; Cell Signaling); anti-tumor necrosis factor receptor-associated factor2 (anti-TRAF2, 1:800; Cell signal technology); anti-phosphorylated apoptosis signal regulating kinase1 (anti-p-ASK1, 1:800; Cell signal technology); anti-phosphorylated inositol-requiring enzyme 1 (anti-p-IRE-1, 1:800; NOVUS). After washing, the filter was incubated with horseradish peroxidase-conjugated secondary antibodies (1:2500; Jackson ImmunoResearch), followed by detection using enhanced chemiluminescence (Pierce, Helsinki, Finland). Quantification was performed using GelDoc (Bio-Rad, Espoo, Finland).

Statistical analyses

All data were analyzed by the SPSS (Version 18.0) software. The data were expressed as the means ± SEM. All normally distributed data were analyzed by ANOVA followed by the LSD or Dunnett’s T3 test for detecting the significance of differences among the groups. p < 0.05 was considered to be statistically significant.

Results

Blood gas analysis

Blood gas analysis in rats was made in order to validate the CHI cabin. The result showed that over the course of the IH event for 2 h, the PaO2 fluctuated from 44 ± 3 mm Hg to 80 ± 9 mm Hg and SaO2 from 75 ± 3 mm Hg to 95 ± 1 mm Hg, respectively in a cycle of 90 s (Fig. 1A). The PaO2 and SaO2 in control group were exhibited in no significant difference among the five time-point (Fig. 1B). The magnitude of oxygen saturation that was induced in our model of IH was consistent with the degree of hypoxia that occurs in moderate to severe OSAHS (Lee et al., 2009; Morgan, 2009).

Effect of intermittent hypoxia on the spatial memory of rats as determined by 8-Arm radial maze test

IH exposure caused substantial spatial memory impairment as demonstrated by the results obtained from the 8-Arm radial maze test (Fig. 2). There was a significant increase in RME, WME and TE in the rats that had been exposed to IH for 2 and 4 weeks (RME: F = 19.06, P = 0.001; WME: F = 15.90, P = 0.001; TE: F = 46.64, P = 0.001). There were no significant differences between DMSO group and 2IH group [(RME: P = 0.229; WME: P = 0.471; TE: P = 0.122)]. We also found that treatment with Sal in the rats caused a significant decrease in RME, WME and TE, compared to the exposure of IH [(RME: P = 0.001, 2Sal vs 2IH group; P = 0.003, 4Sal vs 4IH group); (WME: P = 0.048, 2Sal vs 2IH group; P = 0.043, 4Sal vs 4IH group); (TE: P = 0.001, 2Sal vs 2IH group; P = 0.001, 4Sal vs 4IH group)]. There were no significant differences between DMSO group and IH group [(RME: P = 0.281, 2DMSO vs 2IH group; P = 0.718, 4DMSO vs 4IH group); (WME: P = 0.687, 2DMSO vs 2IH group; P = 0.421, 4DMSO vs 4IH group); (TE: P = 0.533, 2DMSO vs 2IH group; P = 0.755, 4DMSO vs 4IH group)].
memory, we measured the number of TUNEL-positive cells in hippocampus and prefrontal cortex and calculated the index of apoptosis by TUNEL staining. As shown in Fig. 3, TUNEL-positive cells dying neurons with condensed nuclei in the hippocampus and in prefrontal cortex were evident in the CIH exposed groups (hippocampus: $F = 69.68$, $P = 0.001$; prefrontal cortex: $F = 16.32$, $P = 0.001$), especially in the $4\text{IH}$ group (2IH vs 4IH group, hippocampus: $P = 0.001$; prefrontal cortex: $P = 0.030$). Treatment with Sal significantly decreased the number of TUNEL-positive cells (hippocampus: $P = 0.001$, 2SAL vs 2IH group; $P = 0.001$, 4SAL vs 4IH group), (prefrontal cortex: $P = 0.048$, 2SAL vs 2IH group; $P = 0.018$, 4SAL vs 4IH group), while normal nuclear was seen in the control groups.

CIH induced cell apoptosis in hippocampus and prefrontal cortex

In order to determine that CIH induced cell apoptosis in the specific brain regions which are involved in brain functions related to spatial

![Fig. 1](image1.png)

Fig. 1. Mean ± SE changes in the fraction of arterial oxygen pressure. Arterial oxygen pressure ($\text{PaO}_2$), and arterial oxygen saturation ($\text{SaO}_2$) at 22.5 s intervals throughout a 90 s period of hypoxia/reoxygenation (A) and compressed air (B) respectively.

CIH induced ER stress

In this study, we treated the rats in CIH over 2 and 4 weeks then assessed the expression levels of immunoglobulin-binding protein (BiP), a central regulator of ER homeostasis because of its multiple roles in protein folding and activation of transmembrane ERS sensors. The data showed that BiP expression was upregulated in hippocampus and prefrontal cortex (hippocampus: $F = 153.01$, $P = 0.001$; prefrontal cortex: $F = 54.24$, $P = 0.001$) (Fig. 4A). These data indicated that exposure to CIH induced ER stress in hippocampus and prefrontal cortex.

Effect of CIH on UPR signaling pathway in hippocampus and prefrontal cortex

We determined whether the three sensor proteins were observed in the hippocampus and prefrontal cortex after CIH using the method of immunoblot. The data showed that although the total amount of PERK protein did not change, but the levels of phosphorylated PERK progressively increased in the hippocampus and prefrontal cortex after CIH in a time-dependent manner (hippocampus: $F = 636.616$, $P = 0.001$; prefrontal cortex: $F = 157.73$, $P = 0.001$) (Fig. 5B), and p-eIF2a, downstream target of PERK, was downregulated after CIH (hippocampus: $F = 124.08$, $P = 0.001$; prefrontal cortex: $F = 126.45$, $P = 0.001$) (Fig. 5F). The data demonstrated that the PERK signal pathway was sensitive to CIH-induced ER stress in the hippocampus and prefrontal cortex.

We also determined whether the other ER sensor pathways were activated in the hippocampus and prefrontal cortex with CIH exposure. Under ER stress, full-length ATF6 (90 kDa) is cleaved in the Golgi apparatus by SP1 and SP2 proteases to yield a cytosolic fragment (50 kDa) that migrates to nucleus and activates transcription of genes containing ER stress response elements (ERSE) sequence on their promoters (Lee et al., 2003). Following the analysis of PCR revealed that there was no significant difference in c-ATF6 (cleaved ATF6, 50 kDa) (hippocampus: $F = 1.46$, $P = 0.296$; prefrontal cortex: $F = 0.71$, $P = 0.572$) and ERSE expression (hippocampus: $F = 3.73$, $P = 0.060$; prefrontal cortex: $F = 3.34$, $P = 0.077$) in CIH groups in comparison with the control groups (Figs. 4E, C). IRE-1 activation was mediated by the increase in the processed form of xbp1, which would be responsible for the observed EDEM (Badiola et al., 2011). We found an upregulation of p-IRE-1 (hippocampus: $F = 298.87$, $P = 0.001$; prefrontal cortex: $F = 239.44$, $P = 0.001$) (Fig. 5A), but no increase of spliced xbp-1 (Xbp-1s) (hippocampus: $F = 3.19$, $P = 0.084$; prefrontal cortex: $F = 0.24$, $P = 0.865$) and EDEM (hippocampus: $F = 2.30$, $P = 0.154$; prefrontal cortex: $F = 3.48$, $P = 0.070$) that is downstream of xbp-1 after CIH (Figs. 4C, F), and demonstrated that splicing of xbp-1 mRNA, activated via the IRE-1 pathway did not occur significantly after CIH.

Intermittent hypoxia activates ER proapoptotic proteins

CHOP and JNK are important signal molecules linking the endoplasmic reticulum stress to cell apoptosis. In endoplasmic reticulum stress, ATF4 can be transferred to the cell nucleus, and induced the expression
of CHOP gene. Our data showed that ATF4 and CHOP were significantly upregulated after CIH over time (Figs. 4B, D, 5G). In stress state, the phosphorylation of IRE-1 combined with TRAF2, recruited apoptotic signaling kinase 1 (ASK1), and formed a complex, when p-IRE-1/TRAF2/ASK1 complex formation, c-Jun amino-terminal kinase JNK in downstream area was activated and phosphorylated (Nishitoh et al., 2002). Therefore, we have evaluated CIH induced changes in TRAF2, p-ASK1, JNK and p-JNK processing in the hippocampus and prefrontal cortex. There was a significant increase in p-JNK/JNK (hippocampus: $F = 123.03, P = 0.001$; prefrontal cortex: $F = 142.97, P = 0.001$), p-ASK1 (hippocampus: $F = 110.71, P = 0.001$; prefrontal cortex: $F = 197.51, P = 0.001$) and TRAF2 (hippocampus: $F = 280.80, P = 0.001$; prefrontal cortex: $F = 245.64, P = 0.001$) (Figs. 5C–E).

**Sal inhibits CIH-induced neuronal apoptosis**

It is suggested that Salubrinal, a small molecule counteracts ER stress-induced cell degeneration by selectively inhibiting eIF2a dephosphorylation both in vitro and in vivo and Salubrinal is able to penetrate into the brain tissue and thereby affords protection against excitotoxic...
(Sokka et al., 2007). Western blot analysis was revealed that Sal induced the phosphorylation of eIF2a significantly after CIH in the hippocampus and prefrontal cortex (hippocampus: $F = 124.08$, $P = 0.001$; prefrontal cortex: $F = 126.45$, $P = 0.001$) (Fig. 5F), indicating Sal in line with eIF2a for its action. CHOP was significantly downregulated with treatment of Sal, compared to the CIH groups [(hippocampus: $P = 0.009$, 2SAL vs 2IH group); (prefrontal cortex: $P = 0.017$, 2SAL vs 2IH group; $P = 0.013$, 4SAL vs 4IH group)] (Fig. 5G). Further, Sal reduced TUNEL-positive cells significantly after CIH injury as revealed by TUNEL staining (Fig. 3), and improved the memory errors (Fig. 2). All the results revealed that Sal inhibits CIH-induced neuronal apoptosis in hippocampus and prefrontal cortex.

Discussion

Obstructive sleep apnea hypopnea syndrome (OSAHS) is characterized by chronic intermittent hypoxia (CIH). Increasing evidence demonstrates that CIH plays an important part in the pathophysiology of neurocognitive dysfunction of OSAHS patients. In this study we modified a computer controlled CIH model and examined the effect of CIH on the spatial memory in growing rats by using an 8-Arm radial maze test and investigated the possible mechanisms of CIH-induced apoptosis. We suggested that CIH exposure during a critical period of neuronal development can lead to substantial deficits in spatial memory by ER stress mediated cell apoptosis.

Previous study suggested that OSAHS can lead to brain injury, especially in cognitive impairment (Fung et al., 2012). More and more structural alterations in brain regions implicated in cognitive functions have been revealed by sensitive and quantitative MRI techniques. Torelli et al. (2011) showed regions of decreased GM volume in hippocampus and within more lateral temporal areas is related to cognitive impairment in patients with OSAHS. While Morrell et al. (2010) suggested that OSAHS was associated with loss of gray matter in the right middle temporal gyrus and the cerebellum in a large group of patients with OSAHS. These results above suggested that OSAHS was associated with brain areas vulnerable to hypoxia involved in different cognitive deficits. In our study, we choose the 8-Arm radial maze to test the effects of CIH on spatial memory in growing rats and showed that CIH exposure can cause substantial deficits in spatial memory in growing rats, which is in agreement with the above. The investigators identified that the main anatomical location of learning and memory was in the hippocampus and prefrontal cortex, and the function of hippocampus CA1 region was associated with the spatial memory (Siesjo et al., 1980), furthermore, we examined the apoptosis cells in the brain hippocampus CA1 region and prefrontal cortex by TUNEL assay to determine the effect of CIH on neuronal cell apoptosis. This study revealed that CIH caused the neuron damage in the hippocampus and prefrontal cortex, resulting in the impairment of spatial memory, which is consistent with the findings of previous studies (Archbold et al., 2009; Yauhi et al., 2009). However, the specifics of how spatial memory is impaired remain elusive.

ER stress can be activated by a variety of factors, including intermittent hypoxia in cells. The latter is also the cause of OSAHS. Our previous research has showed that ER stress plays an important role in the impairment of learning and memory during the exposure of CIH (Zhou et al., 2012), but the definitive mechanisms remain unclear. In the current study, we investigated the effects of CIH on the induction of ER stress and apoptosis in the hippocampus and prefrontal cortex and then determined the underlying molecular mechanism.

In physiological state, Bip binds to the effectors of UPR, PERK, IRE-1 and ATF6, to suppress their activity. Under conditions of ER stress, when unfolded or misfolded proteins accumulate in the ER lumen, Bip dissociates from these effectors allowing their activation (Lee do et al., 2010). Increased expression of Bip serves as a marker for ER stress and protects against various types of cell death induced by ER stress (Schorer and Kaufman, 2005). An obvious increase in the expression of Bip mRNA levels was found both in the hippocampus and prefrontal cortex in our study. It determined that Bip can induce the ER stress. Our results also showed that Bip mRNA levels had no increase in time-independent manner, but the expression of CHOP, a marker of cell apoptosis, was upregulated and the TUNEL positive cells were significantly increased, so we suggested that the temporal increase of Bip mRNA expression after CIH might relax the ER stress and prevent the cell apoptosis. However, prolonged ER stress accompanied by failure of adaptive response may eventually result in cell death.

Activated PERK phosphorylates eIF2a, which leads to inhibition of global protein synthesis. Moreover, phosphorylated eIF2a also leads to the paradoxical increased translation of ATF4 and CHOP (Greenberg et al., 2006). Our data showed a number of ER stress products, namely PERK, ATF4, and CHOP were upregulated after exposure of CIH in time-independent manner, and treatment with Sal reduced CHOP mRNA and protein. In addition, knockdown of PERK gene expression suppressed the PERK/ATF4/CHOP signaling pathway during sodium palmitate-induced ER stress and significantly inhibited sodium palmitate-induced apoptosis in L02 and HepG2 cells (Cao et al., 2012). Our study demonstrates that the PERK/CHOP signaling pathway may...
play an important role in mediating CIH-induced ER stress and apoptosis.

ATF6 and IRE-1 are the other two UPR transducers. Both factors are known to work, at least in part, through XBP-1. Following the analysis of PCR products and western-blot revealed that there was no significant difference in XBP-1 expression in CIH groups in comparison with the control. These data demonstrated that CIH exposure did not have the spliced version of XBP-1. Previous study also showed that IRE-1 activates ASK1, directly interacts with TRAF2 and constitutes an IRE-1–TRAF2–ASK1 complex that led to JNK activation. The JNK pathway

**Fig. 5.** Intermittent hypoxia activates ER proapoptotic proteins CHOP and JNK. Salubrinal inhibits CIH-induced neuronal apoptosis. A, B, p-IRE1 and p-PERK were significantly upregulated after CIH over time. C–E, There was a significant increase in p-JNK, p-ASK1 and TRAF2 in hippocampus and prefrontal cortex treated with CIH using immunoblotting, but no change in JNK. F, Immunoblots of eIF2α treated with CIH and Sal. Note the inhibition of eIF2α phosphorylation in CIH groups. Sal induced the phosphorylation of eIF2α significantly after CIH in hippocampus and prefrontal cortex. G, CHOP expression was significantly upregulated after CIH over time, and downregulated with treatment of Sal. n = 3 in each group. Statistically significant differences are indicated by *P* < 0.05 vs 2C group; **P** < 0.01 vs 2C group; ***P*** < 0.01 vs 4C group; ****P*** < 0.01 vs 2IH group; *****P*** < 0.01 vs 4IH group; and ■■*P* < 0.05 vs 4IH group.
was involved in cell-death and JNK-3 was revealed to be essential for excitotoxic damage in the brain (Yang et al., 1997). We observed a significantly higher increase in TRAF2 levels in CIH-treated hippocampal neurons, compared to the control group. JNK, p-ASK1 and p-JNK expressions were also increased time-independently. We suggested that chronic intermittent hypoxia can induce neuronal apoptosis through IRE-1/TRAF2/ASK1-JNK pathway but not IRE-1/XBP-1.

Sal was shown to inhibit dephosphorylation of eIF2α and to counteract ER stress in cultured cells (Kessel, 2006). We have observed that intraperitoneal administration of Sal significantly induced the phosphorylation of eIF2α after CIH in rats. Interestingly, Sal made CHOP expression downregulated and reduced significantly the apoptosis in the hippocampus and prefrontal cortex after CIH as revealed by TUNEL staining. Therefore, it appeared that Sal may act via eIF2α for its neuroprotective effect in agreement with Zhu et al.’s (2008) study. However, Zhu et al. (2008) observed that long-term intermittent hypoxia (LTIH) exposure was clearly injurious to select brainstem motoneurons, and suggested a differential susceptibility across motoneuronal groups was different in response to LTIH.

As shown by our data, the protective effect of Sal was robust, although not complete. This reveals that other pathways not blocked by Sal are also involved in neuronal apoptosis after the brain injury. It is known that ER interacts with other cell organelles, including mitochondria. Dysregulated mitochondrial functions have been considered to underlie excitotoxic and brain insult (Ankarcrona et al., 1995). In the previous study, we have observed the ultrastructural changes in the hippocampus with electron microscope and found the swollen mitochondria vacuoles were evident in the CIH exposed groups (Cai et al., 2010), so in our future research, the role of mitochondria in CIH-induced apoptosis will be studied.

Conclusions

In summary, this study demonstrates an involvement of ER stress responses in neuronal damage induced by CIH. The effect of on ER occurred rapidly and was associated with the induction of different signaling pathways from the ER after CIH. The data with Sal showed that the inhibition of ER stress is neuroprotective. Sal preventing ER stress may be valuable in novel therapies for brain disorders. It will be important to discern whether the injury we observe in the rat model occurs in humans with OSAHS and whether this injury can be reduced with therapies increasing ER homeostatic efforts.
Acknowledgment

This work is supported by Zhejiang Province Science and Technology Grant (2008C33011, 2013C33174), Natural Science Foundation of Zhejiang Province (Y2110277), Wenzhou City Science and Technology Bureau Grant (Y20080083, H20130001), Scientific Research Foundation of Health Bureau of Zhejiang Province (2011RCB027, 2014ZDA014), and Project of National Key Clinical Subspecialty from the Ministry of Health of the People's Republic of China. We appreciate the statistical help from Prof. Guang-Yun Mao, the School of Environmental Science & Public Health of Wenzhou Medical University. We thank Prof. Jianguo Shao, the College of Medicine of Boston University School of Medicine for the critical review and comments. We sincerely thank the staff of the Department of Science and Research Center at the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University for their constant and unselfish help.

References


