Brain Research 1646 (2016) 410–417

Contents lists available at ScienceDirect

Brain Research

journal homepage: www.elsevier.com/locate/brainres

Research report

Hydrogen-rich saline mediates neuroprotection through the regulation of endoplasmic reticulum stress and autophagy under hypoxia-ischemia neonatal brain injury in mice

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ARTICLE INFO

Article history:
Received 13 April 2016
Received in revised form
2 June 2016
Accepted 12 June 2016
Available online 15 June 2016

Keywords:
Hydrogen-rich saline
Hypoxia-ischemia
Endoplasmic reticulum stress
Autophagy

ABSTRACT

Hydrogen as a new medical gas exerts organ-protective effects through regulating oxidative stress, inflammation and apoptosis. Multiple lines of evidence reveal the protective effects of hydrogen in various models of brain injury. However, the exact mechanism underlying this protective effect of hydrogen against hypoxic-ischemic brain damage (HIBD) is not fully understood. The present study was designed to investigate whether hydrogen-rich saline (HS) attenuates HIBD in neonatal mice and whether the observed protection is associated with reduced endoplasmic reticulum (ER) stress and regulated autophagy. The results showed that HS treatment significantly improved brain edema and decreased infarct volume. Furthermore, HS significantly attenuated HIBD-induced ER stress responses, including the decreased expression of glucose-regulated protein 78, C/EBP homologous protein, and down-regulated transcription factor. Additionally, we demonstrated that HS induced autophagy, including increased LC3B and Beclin-1 expression and decreased phosphorylation of mTOR and Stat3, as well as phosphorylation of ERK. Taken together, HS exerts neuroprotection against HIBD in neonatal mouse, mediated in part by reducing ER stress and increasing autophagy machinery.

1. Introduction

Neonatal hypoxia–ischemia (HI) brain injury remains a leading cause of mortality and severe long-term disabilities and neurological disorders (Lorenz et al., 1998). It has been known that the depletion of cellular energy production, decreased tissue glucose metabolism and the development of cell injury in the HI brain are closely related (Thornton et al., 2012). Consequently, there is an incontestable need to study the mechanisms underlying HI brain injury and search for additional possible therapeutic strategies.

The endoplasmatic reticulum (ER) regulates protein synthesis, protein folding and trafficking, cellular responses to stress, and intracellular calcium (Ca^{2+}) levels (Tajiri et al., 2004). Conditions that interfere with the ER functions cause accumulation of unfolded proteins in the ER lumen, referred to as ER stress, which activates a homeostatic signaling network known as unfolded protein response (UPR). Excessive and prolonged ER stress can trigger cell death (Tabas and Ron, 2011). Several studies have shown that ischemic injury causes a severe impairment of ER function, which in turn triggers shutdown of protein translation and apoptosis (Tajiri et al., 2004), suggesting that the ER plays an important role in cerebral ischemia. Thus, reducing ER stress may provide a therapeutic way to block the pathological process induced by cerebral ischemia.

Autophagy is a cellular catabolic process that contributes to quality control and maintenance of the cellular energetic balance through the turnover of protein and organelles in lysosomes. It has been suggested that autophagy is an adaptive mechanism that helps maintain cellular homeostasis during the early stage of disease in response to cellular stress. Autophagy occurs

Abbreviations: Chop, C/EBP homologous protein; CNS, central nervous system; ER, endoplasmatic reticulum; ERK, extracellular signal-regulated kinase; GRP78, glucose-regulated protein 78; HI, hypoxia–ischemia; HIBD, hypoxic-ischemic brain damage; HS, hydrogen-rich saline; MAPK, mitogen-activated protein kinase; PBS, phosphate buffered saline; PFA, paraformaldehyde; RT-PCR, reverse transcription–polymerase chain reaction; UPR, unfolded protein response

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http://dx.doi.org/10.1016/j.brainres.2016.06.020
0006-8993/

constitutively at a basal level, but can also be induced by both physiological and pathological stimuli. Given autophagy can be stimulated in both the adult- and neonatal-rat, it has been suggested that it may contribute to ischemic neuronal injury (Northington et al., 2011).

Hydrogen gas is a new medical gas that exerts organ-protective effects through regulating oxidative stress, inflammation, and apoptosis (Cai et al., 2008; Ohsawa et al., 2007; Ohta, 2012). Recently, accumulating evidence has suggested that hydrogen provides neuroprotection of oxidative stress-induced damage in neurological diseases, such as Alzheimer's disease, Parkison's disease, cerebral ischemia and spinal cord injury (Hong et al., 2010; Ohsawa et al., 2007). Hydrogen also affords neuroprotection against brain damage in a neonatal model of HI (Cai et al., 2008; Cai et al., 2009). Hydrogen-rich saline (HS) has biological benefits toward preventive and therapeutic applications; however, the molecular mechanisms underlying the marked effects of HS remain elusive.

In this study, we tested the hypothesis that whether ER stress and autophagy could be involved in the neuroprotective effect of HS administered after neonatal HI. We chose the Rice-Vannucci rat model to provoke hypoxic-ischemic brain damage (HIBD) (Hagberg et al., 1997).

2. Results

2.1. HS protected against brain injury

Representative whole brain morphology from mouse pups in each group, at 3 d after HI insult is shown in Fig. 1A. The HIBD brain after 3 d insult appeared edematous and apparent sign of liquefaction area in the ipsilateral side of the whole brain (shown by arrows) as compared to the sham group. However, HS treatment could alleviate edema and this morphological damage, especially in the higher dose of HS group. Moreover, quantification of brain water content in the ipsilateral and contralateral brain hemisphere was measured 3 days after HI. The results showed that the water content of the ipsilateral hemispheres in the HI group was significantly increased compared with that in the sham group. Brain water content was significantly reduced by HS treatment

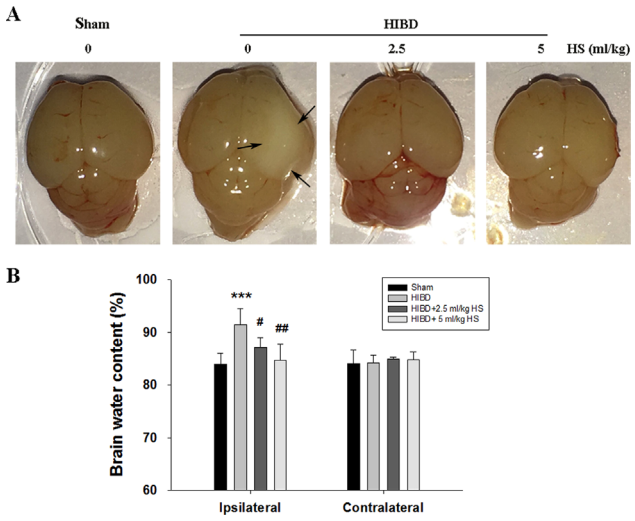

Fig. 1. Effects of the HS on HI-induced brain injury (A) Representative whole brain morphology of animals shown at 3 days after HI. The ipsilateral hemisphere to the ligation appeared edematous and showed apparent sign of liquefaction area (black arrows) in HIBD brain. (B) Brain water content of ipsilateral and contralateral brain hemisphere was measured at 3 days after HI, n=6. Values represent the mean ± SD, $^{***}p < 0.001$ HIBD VS Sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ HIBD+HS VS HIBD.

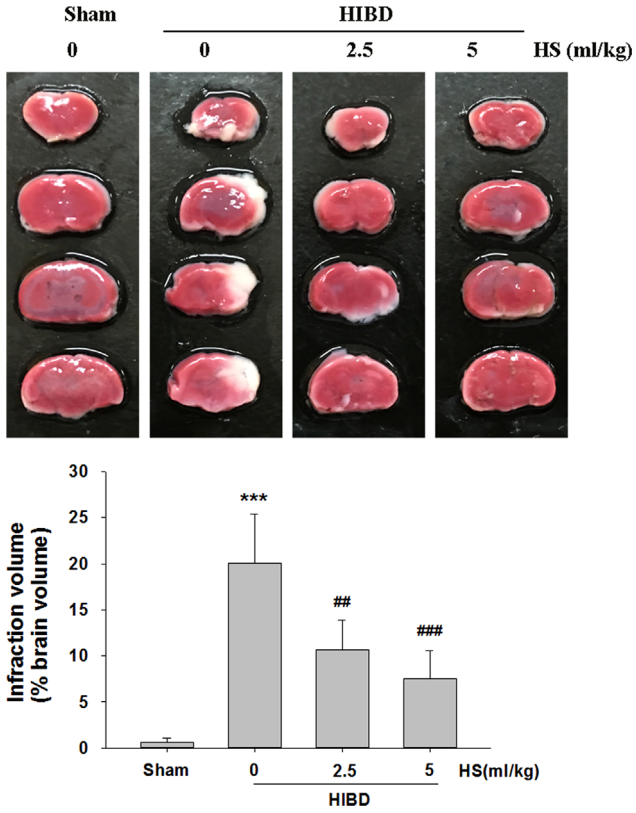

Fig. 2. HS alleviates SAH-induced infarct volume (A) Representative TTC stained brain sections from Sham, HI and treatment groups with different dosages of HS were shown. (B) Quantitative analysis of infarct volume, n=6. Values represent the mean ± SD,$^{***}p < 0.001$ HIBD VS Sham; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ HIBD+HS VS HIBD.

(Fig. 1B).

Representative photographs of TTC-stained sections from mouse pups in each group, at 3 d after HI insult were shown in Fig. 2. The infarct ratio in HI group was markedly higher than that in the sham group. However, HS treatment at two doses after HI therapy dramatically decreased the volume of infarction, especially in the higher dose of HS group.

2.2. HS decreased apoptotic markers

Apoptotic markers in the lesioned hemisphere, Bcl-2 and Bax, were quantified by Western blot and RT-PCR analysis. The Bcl-2/Bax ratio was decreased significantly after HI injury at protein and mRNA levels, which was reversed by HS treatment (Fig. 3). To further investigate the potential protective mechanism of HS, we performed Western blot to assess cleaved-caspase 3 protein levels after HI insult. As shown in Fig. 3, HI insult significantly increased cleaved-caspase 3 protein levels compared with the corresponding controls. However, HS treatment significantly suppressed this HI induced-cleaved-caspase 3 increase (Fig. 3).

2.3. HS reduced ER stress after HI

To study whether the reduction of ER stress was involved in the neuroprotective effect of HS, we assessed the expression and activation of several proteins involved in the UPR. Glucose-regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP) are molecular markers of ER stress (Tabas and Ron, 2011). In agreement with previous findings, immunohistochemical analysis revealed that many GRP78/Chop-positive cells were observed in the lesioned hemisphere, whereas the GRP78/Chop-positive cells were