

Hypertension-mediated enhancement of JNK activation in association with endoplasmic reticulum stress in rat model hippocampus with cerebral ischemia-reperfusion

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ABSTRACT. Acute brain ischemia can induce the activation of c-Jun N-terminal kinases (JNKs). Hypertension is a critical etiology for brain ischemia. We identified the effects of hypertension on the activation of JNK as well as its impact on SP600125, a JNK inhibitor, during endoplasmic reticulum stress (ERS) in the hippocampus using a rat model. Transient whole-brain ischemia was induced by 4-vessel occlusion (bilateral vertebral and bilateral common carotid arteries) in normal and spontaneous hypertensive rats. SP600125 (0.05 mg/kg, *iv*) was administered 30 min before ischemia. Morphological changes in hippocampal nerve cells were observed by cresyl violet staining. Phosphorylation of JNK, and expression levels of CHOP and GPR78, markers for ERS, were detected by western blot at 1, 6, 24, and 48 h, and

neurological outcomes were measured using an eight-arm radial maze 48 h after ischemia. Hypertension apparently aggravated impairment of memory function, decreased the density of surviving neurons, increased phosphorylation of JNK, and enhanced CHOP expression, but reduced GPR78 levels in hippocampal tissues following brain ischemia. SP600125 alleviated neurological dysfunction, improved neuron survival, decreased phosphorylation of JNK and levels of CHOP, but increased expression of GPR78 in rats with hypertension during cerebral ischemia by inhibition of ERS.

Key words: Hypertension; Ischemia/reperfusion; Endoplasmic reticulum stress; c-Jun N-terminal kinases; CAAT/enhanced I-binding protein

INTRODUCTION

Hypertension is an independent risk factor for ischemic cerebral stroke and may aggravate nerve injuries after ischemia, thus worsening prognosis (Duverger and MacKenzie, 1988; Nishimura et al., 2000). However, the exact underlying mechanisms remain unclear and require more research.

Mitogen-activated protein kinases (MAPKs) play a critical role in the survival or death of cells. c-Jun N-terminal kinase (JNK), a member of the MAPK family, is activated by a number of stimuli. Activated JNK can phosphorylate a number of downstream substrates, such as MEK and c-Jun, which regulate a number of genes such as *TP53* and *CASP3* involved in neuronal apoptosis (Park et al., 2000; Manning and Davis, 2003; Tripathi and Sodhi, 2009). The endoplasmic reticulum (ER) is an organelle where macromolecules are synthesized, and calcium ions are stored. The ER is very sensitive to external and internal stimuli and environmental changes inducing accumulation of unfolded proteins and imbalance of calcium homeostasis, a state known as ER stress (ERS). GRP78 and CHOP are proteins that are involved in ERS (Paschen et al., 1998; Rao et al., 2002). Increasing evidence suggests that GRP78 and CHOP may play either a protective or a harmful role after cerebral ischemia in hypertensive animals and humans. Aoki et al. (2001) and Hayashi et al. (2003) have proposed that focal cerebral ischemia-induced severe nerve damage is due to a reduction of GRP78 expression, and that preconditioning may protect the brain against focal cerebral ischemia/reperfusion (I/R) injuries by downregulating CHOP expression in the cerebral cortex. There is a relationship between ERS and the activation of the JNK signaling pathway, as evidenced by the fact that transient cerebral ischemia-induced nerve damage is associated with JNK activation and sustained c-Jun expression. Inhibition of ERS by curcumin leads to decreased JNK activity and c-Jun expression, decreasing nerve cell death (Hayashi et al., 2003).

However, there is a lack of data on whether JNK activation influences GRP78 and CHOP expression and whether SP600125, an inhibitor of JNK, can modulate cerebral I/R injuries in the hippocampus in cerebral ischemia combined with hypertension. The present study attempted to answer these questions by using normal and spontaneous hypertensive rats as a model, in an effort to understand the mechanisms involved and the biological consequences of JNK activation in cerebral ischemia under hypertension.

MATERIAL AND METHODS

Materials

Mouse monoclonal antibodies against phosphorylated JNK, CHOP, and GPR78 were obtained from Cell Signaling Biotechnology (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The secondary antibodies used in our experiments were goat anti-mouse IgG obtained from Sigma-Aldrich (St. Louis, MO, USA).

Experimental design

Normotensive adult male Wistar-Kyoto and spontaneous hypertension male rats (SHR) weighing 250-300 g (Beijing Experimental Animal Center, Chinese Academy of Science, Beijing, China) were selected as a model in this study. Rats were given free access to food and water before surgery. The Wistar-Kyoto rats were divided into two groups: 1) normal rat sham operation (NSO); and 2) normal rat I/R (NI/R). The SHR rats were divided into four groups: 1) hypertensive rat sham operation (SHRSO); 2) hypertensive rat cerebral I/R (SHR+I/R); 3) SHR+I/R+SP600125; and 4) SHR+I/R+dimethyl sulfoxide (DMSO). Normal and hypertensive rats were decapitated at 1, 6, 24, and 48 h after subjection to I/R for 30 min. The experimental procedures were approved by our local Ethical Committee for animal experiments.

Ischemia induction

Transient brain ischemia (30 min) was induced by the four-vessel occlusion method, as previously described (Zhang et al., 2003). Under anesthesia using chloral hydrate (300-350 mg/kg, *ip*), vertebral arteries were electrocauterized and common carotid arteries were exposed. Rats were allowed to recover for 24 h and fasted overnight. Ischemia was then induced by occluding the common carotid arteries using aneurysm clips. Animals were selected for the present experiment during ischemia as indicated by the following conditions: 1) a completely flat bitemporal electroencephalogram; 2) maintenance of dilated pupils; and 3) absence of corneal reflex. Carotid artery blood flow was restored by releasing the clips. Rectal temperature was maintained at approximately 37°C during, and for 2 h after, ischemia. The sham operation was performed using the same surgical exposure procedures except that the carotid arteries were not occluded.

Drug treatment

SP600125 was dissolved in 1% DMSO (200 nmol in 5 μ L DMSO) (Cell Signaling Technology, La Jolla, CA, USA). Drug infusion was performed using a micro-injector in both cerebral ventricles (from the bregma: anteroposterior, 0.8 mm; lateral, 1.5 mm; depth, 3.5 mm) as previously described (Wang et al., 2011). A volume of 5 μ L each was infused over 5 min. SP600125 was given to the SHR rats 30 min before ischemia and then once every 24 h for 2 days. Controls received the vehicle DMSO only.

Histology

Rats were perfusion-fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) under anesthesia 1, 6, 24, or 48 h after I/R. Brains were quickly removed and further fixed with the same fixative at 4°C overnight. Post-fixed brains were embedded in paraffin, and cut into 5 µm coronal sections using a microtome. Paraffin-embedded brain sections were deparaffinized with xylene and rehydrated using an ethanol gradient (100% to 70% v/v), followed by washing with water. Sections were stained with 0.1% w/v cresyl violet and examined by light microscopy. The number of surviving hippocampal CA1 pyramidal cells with obvious cell membranes, nucleus, and nucleolus was counted as the neuronal density (Wang et al., 2011).

Western blotting analysis

Rats were decapitated under anesthesia at specific time points from reperfusion after 30-min ischemia. The hippocampal tissue was separated into CA1 and CA3/DG regions and CA1 cells were rapidly frozen in liquid nitrogen. Hippocampus tissues were homogenized in ice-cold homogenization buffer [50 mM 3-(N-morpholino)propanesulfonic acid, pH 7.4, 100 mM KCl, 50 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-phosphoglycerol, 320 mM sucrose, 0.2 mM dithiothreitol, 1 mM edetic acid, 1 mM egtazic acid, 0.5 mM MgCl₂, 1 mM Na₃VO₄, 1 mM p-nitrophenyl phosphate, 5 µg/mL each of aprotinin, leupeptin, and pepstatin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine]. Homogenates were then centrifuged at 800 g for 15 min at 4°C. Supernatants were collected and protein concentrations were determined by the Lowry method. Samples were stored at -80°C until required. Samples (20 µg protein each) were separated by 10 or 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose membranes (Hayashi et al., 2003). After blocking with 3% bovine serum albumin for 3 h, membranes were probed with the proper primary antibody at 4°C overnight. Bound antibodies were detected using alkaline phosphatase-conjugated goat anti-mouse IgG for 2 h. Immunoreactivities were detected using an NBT/BCIP assay kit, according to the manufacturer instructions (Sigma-Aldrich, St. Louis). Protein bands were scanned and analyzed with an image analyzer (LabWorks Software, UVP Inc., Upland, CA, USA).

Spatial memory in the radial arm maze apparatus

The radial maze was made from gray polyvinylchloride and consisted of eight arms (length: 32 cm, width: 8 cm, height: 19 cm) extending radially from an octagonal central area (20 cm across). Rats were trained to the radial maze for two consecutive days with two trials per day. Each rat was placed in the central area of the maze with all arm entries closed. After 10 s, the doors were opened and the rat was permitted to enter any of the eight arms. Only three of the eight arms contained water. The rationale for using three instead of four baited arms was to increase the sensitivity of the task in measuring reference memory errors (RME) by decreasing the probability of making a correct choice by chance. The three arms containing water were randomly determined for each rat and were unchanged over the 2-day learning period. A trial was terminated after either all baits were consumed or after 10 min, whichever occurred first.

Forty-eight hours after injury, the memory function was assessed using a previously described method (Hayashi et al., 2003). An experimenter who was blinded to the experimental groups scored each rat based on: 1) RME: entering into an arm that was never baited; and 2) working memory errors (WME): re-entering into an arm already visited.

Statistical analysis

Values are reported as means \pm SD, from five independent rats. Statistical analysis was performed by one-way analysis of variance, followed by Duncan's new multiple range method, using the SPSS statistical package for social science (SPSS Inc., Chicago, IL, USA). P values < 0.05 were considered to be significant.

RESULTS

Neuronal cell viabilities in hippocampal CA1 regions

Cresyl violet staining was used to determine the survival of CA1 pyramidal neurons. Normal cells showed round and weakly stained nuclei. Shrunken cells with pyknotic nuclei after I/R were counted as dead cells. Transient brain ischemia followed by reperfusion induced severe cell death at 1, 6, 24, and 48 h after reperfusion. Compared with brain ischemia in normal rats, hypertension-aggravated cell death and neuron survival were markedly decreased in the SHR+I/R group. Pretreatment with SP600125 before ischemia significantly decreased neuronal degeneration and increased neuron survival in the SHR+I/R+SP600125 group compared with the SHR+I/R group. DMSO had no effect on neuronal survival (Figure 1 and Table 1).

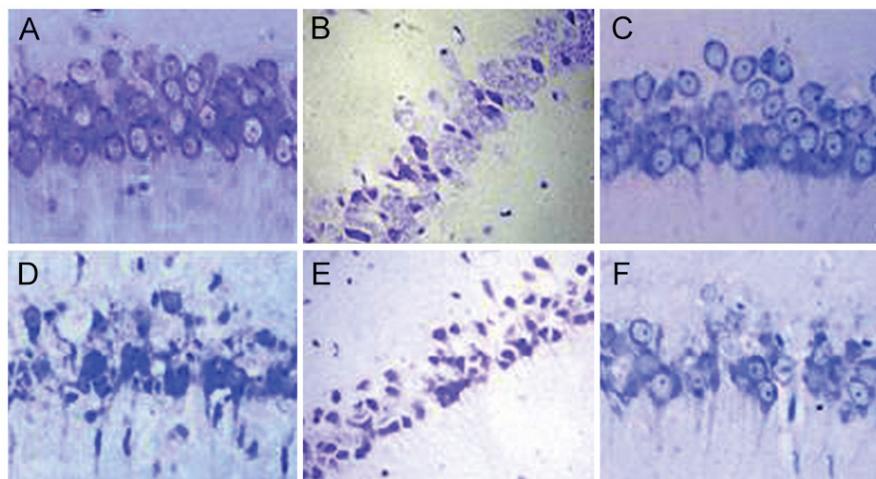


Figure 1. Representative photomicrographs of cresyl violet-stained sections of the hippocampus in rats at 24 h after subjection to ischemia/reperfusion (I/R). **A.** Normal rats subjected to sham operation. **B.** Normal rats subjected to I/R. **C.** Spontaneous hypertension rats subjected to sham operation. **D.** Spontaneous hypertension rats subjected to I/R. **E.** Spontaneous hypertension rats subjected to I/R plus dimethyl sulfoxide vehicle; and **F.** spontaneous hypertension rats with I/R plus SP600125. Magnification 400X.

Table 1. Quantitative analysis of neuronal survival in the hippocampus in normal and hypertensive rats subjected to ischemia/reperfusion (I/R).

Groups	Neuronal density in CA1 region			
	1 h	6 h	24 h	48 h
NSO	188.65 ± 20.90	189.8 ± 19.85	190.10 ± 20.05	189.80 ± 19.90
NI/R	120.80 ± 16.75	100.75 ± 12.50	65.90 ± 10.75	40.80 ± 9.75
SHRSO	170.10 ± 19.15	171.24 ± 18.80	172.10 ± 18.05	172.50 ± 18.40
SHR+I/R	80.50 ± 12.48 ^a	60.75 ± 10.50 ^a	38.75 ± 8.70 ^a	26.70 ± 6.10 ^a
SHR+I/R+DMSO	81.45 ± 12.45	59.80 ± 10.45	39.25 ± 8.60	27.20 ± 6.25
SHR+I/R+SP600125	112.65 ± 18.50 ^b	96.80 ± 11.65 ^b	80.60 ± 11.80 ^b	78.75 ± 10.80 ^b

NSO = normal rats subjected to sham operation; NI/R = normal rats subjected to I/R; SHRSO = spontaneous hypertensive rats subjected to sham operation; SHR+I/R = spontaneous hypertensive rats subjected to I/R; SHR+I/R+DMSO = spontaneous hypertensive rats subjected to I/R plus dimethyl sulfoxide (DMSO) vehicle; SHR+I/R+SP600125 = spontaneous hypertensive rats subjected to I/R plus SP600125. Data are reported as means ± SD from five rats. ^aP < 0.05 vs NI/R; ^bP < 0.05 vs SHR+I/R+DMSO.

Activation of JNK after I/R

To assess the JNK activation after brain ischemia under hypertension or treatment with SP600125, we examined levels of phospho-JNK at 1, 6, 24, and 48 h after I/R. The level of phospho-JNK increased rapidly in the NI/R group compared with the NSO group after 1h, peaked at 6 h, and decreased after 24 h before returning to the control level after 48 h. JNK activation was strong at all time points in the SHR+I/R group; it steadily increased to a maximum level at 24 h and slightly decreased 48 h after reperfusion in comparison with the SHRSO group. Therefore, hypertension enhanced ischemia-induced JNK activation. The use of SP600125 blocked JNK phosphorylation induced by brain ischemia under hypertension in comparison with the DMSO-treated control group (Figure 2).

GPR78 and CHOP expressions during ERS following I/R

To explore ERS in the hippocampus after brain ischemia under hypertension in the rat model, tissue expression levels of CHOP and GPR78, markers for the ERS, were identified at various time points after I/R. The levels of both GPR78 and CHOP increased rapidly after 1 h, peaked at 24 h, and then decreased after 48 h following I/R in the normal rat group (NI/R compared with NSO). Although hypertensive rats seemed to have high basal levels of GPR78 and CHOP (SHRSO compared with NSO), they were able to rapidly respond to I/R by increasing these proteins to maximum levels at 6 h for GPR78 and 24 h for CHOP, then reducing their expression levels to below the control level (SHR+I/R compared with SHRSO) suggesting strong ERS occurring in the hippocampus. Pretreatment with SP600125 apparently resulted in different responses for GPR78 and CHOP in hypertensive rats under I/R conditions: GPR78 was upregulated while CHOP was downregulated (SHR+I/R+SP600125 compared with SHR+I/R). Note that the DMSO vehicle had no effect on GPR78 and CHOP expression levels (SHR+I/R+DMSO compared with SHR+I/R) (Figure 3A and B).

Memory function after I/R

The results for RME and WME obtained from the memory trials are shown in Table 2. Memory damage was induced in normal rats 48 h after I/R (NI/R compared with NSO).

Marked increases in the number of RME and WME in the SHR+I/R group suggest that hypertension apparently aggravated memory impairment induced by I/R (SHR+I/R compared with NR+I/R). Marked decreases in the number of RME and WME in the SHR+I/R+SP600125 group suggest that pretreatment with SP600125 before ischemia significantly improved recovery of memory functions in hypertensive rats in response to I/R. DMSO had no effect on the RME or WME in the memory trial.

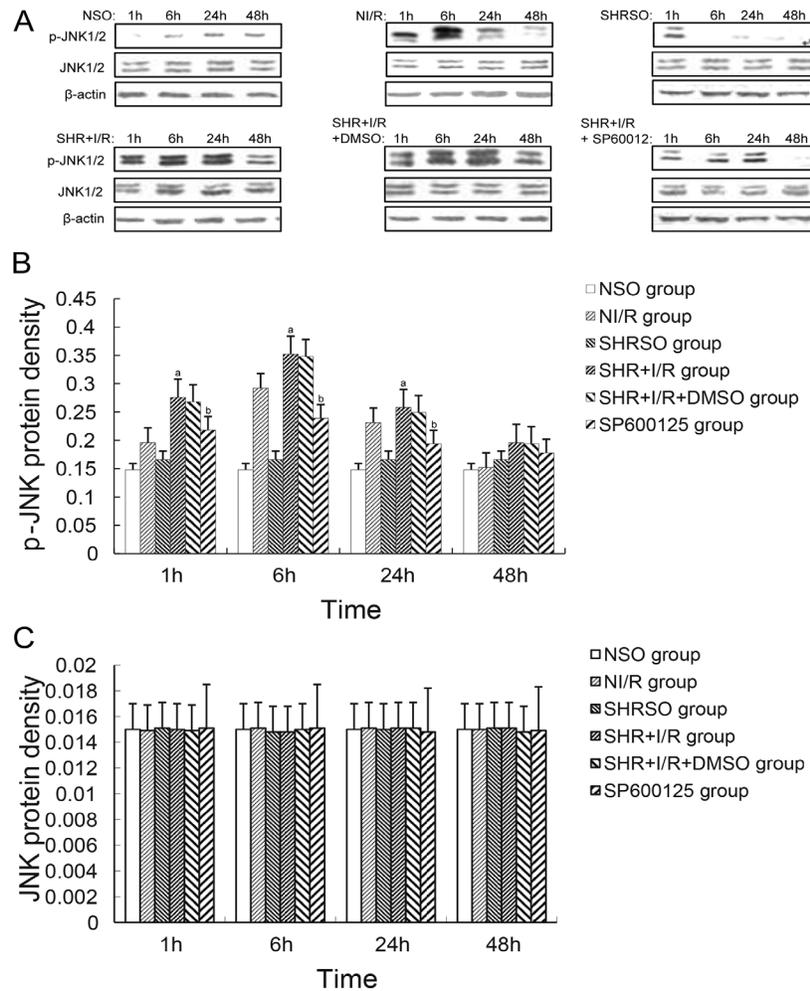


Figure 2. c-Jun N-terminal kinase (JNK) activation in the hippocampus in normal and spontaneous hypertension rats subjected to ischemia/reperfusion (I/R). NSO = normal rats subjected to sham operation; NI/R = normal rats subjected to I/R; SHRSO = spontaneous hypertension rats subjected to sham operation; SHR+I/R = spontaneous hypertension rats subjected to I/R; SHR+I/R+DMSO = spontaneous hypertension rats subjected to I/R plus dimethyl sulfoxide (DMSO) vehicle; SHR+I/R+SP600125 = spontaneous hypertension rats subjected to I/R plus SP600125. **A.-C.** p-JNK (phospho-JNK), JNK, and β -actin expression levels were examined by immunoblotting analysis. **B.** and **C.** p-JNK, JNK, and β -actin were scanned and the intensities were normalized to β -actin. Data are reported as means \pm SD from five rats. ^aP < 0.05 vs NI/R; ^bP < 0.05 vs SHR+I/R+DMSO.

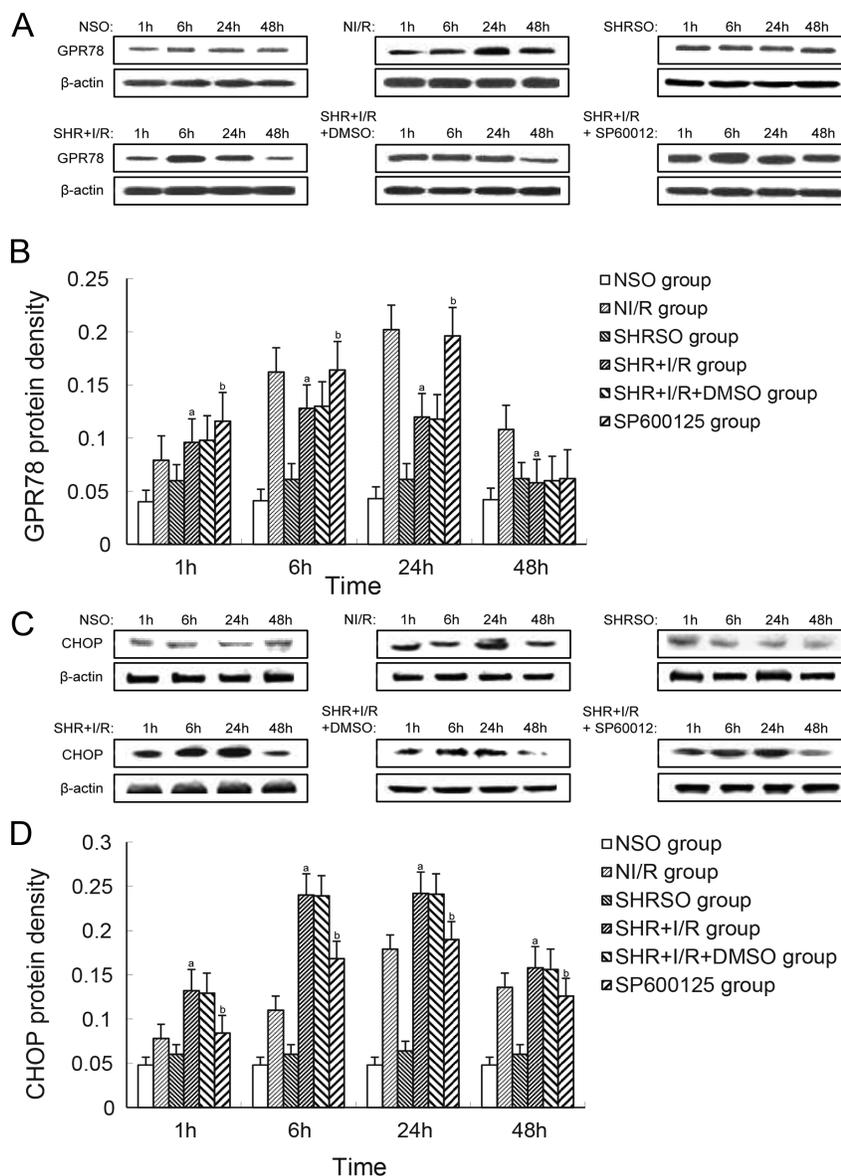


Figure 3. CHOP and GPR78 expression levels in the hippocampus in normal and spontaneous hypertension rats subjected to ischemia/reperfusion (I/R). NSO = normal rats subjected to sham operation; NI/R: normal rats subjected to I/R; SHRSO = spontaneous hypertension rats subjected to sham operation; SHR+I/R = spontaneous hypertension rats subjected to I/R; SHR+I/R+DMSO = spontaneous hypertension rats subjected to I/R plus dimethyl sulfoxide (DMSO) vehicle; SHR+I/R+SP600125 = spontaneous hypertension rats subjected to I/R plus SP600125. **A.-D.** GPR78 (A and B) and CHOP (C and D) expression levels were examined by immunoblotting analysis. **B.** and **D.** GPR78, CHOP, and β-actin were scanned and the intensities were normalized to β-actin. Data are reported as means ± SD from five rats. ^aP < 0.05 vs NI/R; ^bP < 0.05 vs SHR+I/R+DMSO.

Table 2. Quantitative analysis of memory injuries in normal and hypertensive rats at 48 h after subsection to ischemia/reperfusion (I/R).

Group	RME	WME (error times)
NSO	2.03 ± 1.26	1.28 ± 0.45
NI/R	5.37 ± 1.64	3.01 ± 1.40
SHRSO	3.26 ± 1.38	1.85 ± 0.80
SHR+I/R	7.35 ± 2.80 ^a	5.85 ± 2.10 ^a
SHR+I/R+DMSO	7.40 ± 2.75	5.90 ± 2.12
SHR+I/R+SP600125	5.12 ± 1.56 ^b	2.86 ± 1.24 ^b

RME = reference memory errors; WME = working memory errors; NSO = normal rats subjected to sham operation; NI/R = normal rats subjected to I/R; SHRSO = spontaneous hypertension rats subjected to sham operation; SHR+I/R = spontaneous hypertension rats subjected to I/R; SHR+I/R+DMSO = spontaneous hypertension rats subjected to I/R plus dimethyl sulfoxide (DMSO) vehicle; SHR+I/R+SP600125 = spontaneous hypertension rats subjected to I/R plus SP600125. Data are reported as means ± SD from six rats. ^aP < 0.05 vs NI/R; ^bP < 0.05 vs SHR+I/R+DMSO.

DISCUSSION

As reported, activation of JNK is involved in brain damage induced by hypertension and cerebral I/R (Sugino et al., 2000; Li et al., 2009; Hu et al., 2012; Ormiston et al., 2013). Activation of JNK enhances phosphorylation of c-Fos and c-Jun and facilitates AP-1 formation, which further triggers caspase-3-dependent or -independent signals, implicated in neuron death and memory loss (Tang et al., 2002; Guan et al., 2006). Thus, JNK is an early target and effector for ischemia-induced neuronal damage. In this study, we observed the JNK status in normal and hypertensive rats in response to I/R and explored the modulation effects of SP600125, an inhibitor of JNK, on JNK-mediated phenotype changes. Our results indicate that hypertension exacerbates I/R-induced structural and memory damage to the hippocampus, as proved by the activation of JNK. SP600125 displayed neuro-protective effects mediated by inhibition of ERS.

Ischemia-elicited ERS may be a critical mechanism for JNK signal activation (Liu et al., 2008; Li et al., 2009). Our results showed that in response to I/R, hypertensive rats exhibited an increase in GRP78 from 1 to 6 h followed by a marked decrease from 6 to 48 h, and increases in CHOP and phospho-JNK from 1 to 24 h followed by decreases from 24 to 48 h. Thus, a decrease in GRP78 was accompanied by an increase in CHOP in association with an increase in the level of phospho-JNK 24 h after I/R in hypertensive rats. Decreased GRP78 in extensive ERS increases the accumulation of free inositol-requiring enzyme 1 (IRE1), activates PKR-like endoplasmic reticulum kinase and apoptosis signal regulation kinase IRE1/TRAF-2/ASK-1, and enhances phosphorylation of JNK and upregulation of CHOP, leading to the loss of nerve cells (Urano et al., 2000). Alleviation of ERS decreased phosphorylation of JNK and death of myocardial cells in a myocardial I/R model (Liu et al., 2008). Preconditioning to ERS inhibited the expression of phosphorylated JNK and decreased neural cell death in the hippocampus in a focal brain ischemia model (Xu et al., 2012). SP600125 reversibly competes for ATP, suppressing JNK, which is followed by downstream inhibition of the generation of phosphorylated c-Jun, inflammatory factor epoxidase-2, interleukin-2, immunoreactivity fiber, tumor necrosis factor, and oxygen-based free radicals (Bennett et al., 2001; Kim et al., 2008). In addition, a “network communication” exists between JNK and a variety of signal pathways, such as the ERK and AKT pathways (Hu et al., 2004; Hong and Kim, 2007). Thus, in hypertensive rats subjected to cerebral I/R, SP600125 inhibition of JNK activation, promo-

tion of neuron survival, and improvement of memory functions by stabilization of cellular ER may be collectively achieved by reducing inflammatory responses, antagonizing oxidative stress, and balancing “network communication”.

ERS is an adaptive response. Moderate ERS is marked by an increase in the levels of GRP78 and calreticulin, which enhance a cell's ability to activate the unfolded protein response, and promote ERS recovery. However, severe or continuous ERS can stimulate the activation of CHOP and caspase-12, and may trigger ERS-related apoptotic pathways, leading to cell death and tissue injury (Aoki et al., 2001; Nieto-Miguel et al., 2007). Tajiri et al. (2004) detected the CHOP protein in the hippocampus and striatum of wild-type and CHOP^{-/-} mice after bilateral common carotid artery ligation for 15 min. The CHOP levels were related to the number of apoptotic cells at 48 h after reperfusion. The number of dead nerve cells in the hippocampus and striatum was markedly decreased in homozygous *Chop* knockout mice. Lehotský et al. (2009) reported that the apoptosis rate and CHOP and caspase-12 expression levels decreased while GRP78 increased at each time point in rats after a 10-min ischemia. In our experiments, GRP78 and CHOP expression levels exhibited a different time course to reach maximum levels in hypertensive rats in response to I/R. As indicated, two peak time points were differentiated at about 18 h. More interesting, GRP78 and CHOP also displayed different responses to SP600125, a JNK inhibitor. GRP78 expression was enhanced but CHOP expression inhibited in hypertensive rats treated with I/R. The possible explanation is that under hypertension I/R may further increase cytotoxic factors, such as glutamic acid, calcium overload, and oxygen radicals (Hirata et al., 1995; Srinivasan and Sharma, 2012) inducing severe ERS, as evinced by increased CHOP and decreased GRP78 levels, resulting in nerve cell death and a severe memory loss.

In summary, during cerebral ischemia hypertension enhanced activation of JNK and upregulation of CHOP, but inhibited expression of GPR78 in association with severe neuronal cell death in the hippocampus and memory loss. SP600125 protects against I/R injuries in hypertensive rats by inhibition of ERS. These findings provide a new insight into the role of JNK and yield promising therapeutic options for hypertensive patients with stroke.

Conflicts of interest

The authors declare no conflict of interest.

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