Neuron-specific phosphorylation of c-Jun N-terminal kinase increased in the brain of hypoxic preconditioned mice

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Abstract

Accumulated studies have suggested that mitogen-activated protein kinase (MAPK) play a pivotal role in the development of cerebral hypoxic preconditioning (HPC). By using our “auto-hypoxia”-induced HPC mouse model, we have reported increased phosphorylation level of p38 MAPK, and decreased phosphorylation and protein expression levels of extracellular signal regulated kinases 1/2 (ERK1/2) in the brain of HPC mice. In the current study, we investigated the involvement of c-Jun N-terminal kinase (JNK) in the brain of HPC mice. By using Western blot analysis, we found that the phosphorylation levels of JNK at Thr183 and Tyr185 sites (phospho-Thr183/Tyr185 JNK), but not its protein expression, increased significantly ($p < 0.05$, $n = 6$ for each group) both in the hippocampus and frontal cortex of early (H1–H4) and delayed (H5 and H6) HPC mice than that of the normoxic group (H0, $n = 6$). Similarly, enhanced phospho-Thr183/Tyr185 JNK was also observed by immunostaining in the hippocampus and frontal cortex of mice following series of hypoxic exposures (H3 and H6). In addition, we found that phospho-Thr183/Tyr185 JNK predominantly co-localized with a neuron-specific protein, neurogranin, in both the hippocampus and frontal cortex of HPC mice (H3) by using double-labeled immunofluorescence. These results suggest that the increased neuron-specific phosphorylation of JNK at Thr183/Tyr185, not protein expression, might be involved in the development of cerebral HPC of mice.

Ischemic/hypoxic preconditioning (I/HPC) is an endogenous strategy in which sublethal ischemic/hypoxic exposure protects the tissue from damage caused by a severe ischemic/hypoxic insult. This phenomenon can be induced in a variety of different organs including brain, heart, liver, kidney and so on [27]. Understanding the complex adaptive responses to a sublethal stress may lead to novel therapeutic strategies to prevent tissue injury and promote tissue recovery processes. However, the precise molecular mechanisms involved in the development of tolerance against hypoxic damage are unclear. Several studies have shown that hypoxic exposure results in altered gene expression and activation of various intracellular signaling pathways, some of which may contribute to the adaptive responses observed after hypoxia [1,29]. Signal transduction pathways leading to adaptation involve multiple kinases including protein kinase C (PKC) and mitogen-activated protein kinase (MAPK).

MAPK, which include extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK, has crucial roles in transducing signals from the cell surface to the nucleus and in regulating cell death and survival. JNK, also named stress-activated protein kinase (SAPK), is activated in response to environmental stress or membrane-bound receptor signaling through GTPase of Rho family through the MAPK kinase [2]. Activated JNK specially phosphorylates the N-terminal activation domain of transcription factor c-Jun at serine 73 and serine 63, thereby increasing transcriptional activity of c-Jun [20]. Some reports have provided important information in clarifying the roles of JNK in neuronal death and survival, but the results are still controversial [5,9,10,31].

To investigate the molecular mechanism of brain HPC, we have developed an “auto-hypoxia”-induced HPC mouse model that mimics clinical asphyxia [13,19]. Our previous results showed that the activation of several possible upstream and...
downstream mediators of the MAPK pathways involved in the development of cerebral HPC, including three isoforms of PKC: cPKCβII, cPKCγ and nPKCε [7,13,15,23]; mitogen and stress-activated protein kinase-1 (MSK1) [6]; neurogenin [16] and cyclic AMP response element binding protein (CREB) [4]. We also found that the other two members of MAPK family play a role in the development of cerebral HPC. ERK present a decreased phosphorylation following repeated hypoxic exposure [17], while p38 appeared an increased phosphorylation level in a cell-specific manner both in frontal cortex and hippocampus of HPC mice. In the present study, we investigated the involvement of JNK by examining its phosphorylation status and protein expression levels in the brain of mice following repetitive hypoxic exposures.

The following materials were obtained from the indicated sources: proteinase inhibitors (leupeptin, aprotinin, pepstatin A, and chymostatin); phosphatase inhibitors (okadaic acid, sodium pyrophosphate, potassium fluoride and orthovanadate); other reagents, such as ethylene diamine tetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-N,N',N''-tetraacetic acid (EGTA), sodium dodecyl sulfate (SDS), and Nonidet P-40 (NP-40), were purchased from Sigma–Aldrich Company (St. Louis, MO, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG were purchased from Bio-Rad Company (Hercules, CA, USA).

Experiments were conducted on male BALB/c mice at the age of 8–10 weeks (weighing 18–22 g). The animal protocol was approved by the University Institution Animal Care and Use Committee of Capital Medical University and is consistent with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The “Auto-hypoxia”-induced HPC mouse model that could mimic the clinical conditions of asphyxia was prepared as our previously reported [13,19]. Briefly, mice were placed individually in a 125 ml airtight jar with fresh air and sealed with a rubber plug to duplicate a progressive auto-hypoxia environment. Mice were removed from the sealed jar as soon as the first gasping appeared, and the hypoxic tolerant time was recorded. After recovery from the previous hypoxic exposure (at least 30 min under normoxic conditions), the mice were switched into another hermetically sealed jar with fresh air of the same volume. This procedure was repeated one to four times. The mice were then designated into groups as follows: the normoxic control group (H0), which was kept in an open jar; hypoxic group (H1) was exposed to hypoxia once; and the repetitive hypoxic exposures became groups H2–H4. Some mice of the H4 group were kept under the normoxic condition 24 h for recovery before the fifth and sixth hypoxic exposure on the following day. Then, the delayed HPC groups were generated: the H5 and H6 group with additional one-time and two-time hypoxic exposures, respectively. At the end of the experiments, the mice were sacrificed and the brain was quickly removed, and then placed into ice-cold artificial cerebral spinal fluid (ACSF, in mM: NaCl 125, KCl 2.5, CaCl2 2.0, NaHCO3 26, NaH2PO4 1.25, MgCl2 1.0, glucose 10, pH 7.4) bubbling with air flow mixed with 95% O2 and 5% CO2. The hippocampus and frontal cortex regions were collected, frozen in liquid nitrogen, and kept frozen at −70 °C for later analysis.

To determine the phosphorylation and protein expression levels of JNK, protein extracts were generated from whole hippocampus and frontal cortex as our previous reports [6]. The frozen samples were thawed and homogenized at 4 °C in 150 μl homogenized buffer [50 mM Tris–Cl, pH 7.5, containing 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 5 mg/ml each of leupeptin, aprotinin, pepstatin A, and chymostatin, 50 mM KF, 50 mM okadaic acid, 5 mM sodium pyrophosphate, 1 mM orthovanadate, and 2% SDS] and sonicated to dissolve the tissue completely. Then, the protein amounts of samples were determined by BCA kit (Pierce Company, USA).

The procedure of SDS-PAGE and Western blot analysis were carried out as previously reported [13]. Briefly, 50 μg of protein from each sample was loaded per lane for SDS-PAGE (10% SDS gel). At the end of electrophoresis, the proteins were transferred onto the nitrocellulose (NC) membrane (Schleicher and Schell, USA) at 4 °C, 400 mA for 3 h. The transferred membrane was washed for 10 min in TTBS (20 mM Tris, pH 7.5, containing 0.15 M NaCl, and 0.05% Tween-20) and then blocked with 10% nonfat milk for 1 h. After washed for three times (each for 10 min), the blocked NC membrane was incubated with different primary rabbit polyclonal antibodies against phosphorylated JNK at Thr183 and Tyr185 sites (phospho-Thr183/Tyr185 JNK, Cell Signaling Technology, USA), and total JNK (T-JNK, Cell Signaling Technology, USA), and total JNK (T-JNK, Cell Signaling Technology, USA) at a 1:1000 dilution for 3 h to determine JNK phosphorylation and protein expression levels, respectively. Next, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG as second antibodies at 1:4000 dilutions for 2 h. Finally, immunoblotting signals were visualized by using ECL-plus Kit (Perkin-Elmer Life Science Inc., USA).

To detect the changes in phosphorylation levels of JNK, those membranes blotted with antibodies against phospho-Thr183/Tyr185 JNK were re-probed with primary antibody against T-JNK. The ratio of phospho-Thr183/Tyr185 JNK to T-JNK of group H0 was normalized to 100%, and the data in the H1–H6 groups were expressed as the percentage of the value from the group H0. For JNK protein expression levels, the expression of β-actin was determined at the same time. Those membranes blotted with antibodies against the T-JNK were stripped using buffer containing 62.5 mM Tris–Cl, pH 6.7, 100 mM 2-mercaptoethanol and 2% SDS, for 50 min at 55 °C. Next, they were re-probed with primary mouse monoclonal antibody against β-actin (Sigma–Aldrich Company, USA) at a 1:1000 dilution for 3 h. The value of the relative optical density of each band corresponding to T-JNK was normalized to the value of β-actin to demonstrate JNK protein expression level. The ratio of the values of group H0 was regarded as 100%, and the data from H1 to H6 groups were expressed as a percentage of the value from the group H0.

For immunostaining, mice were anesthetized with chloral hydrate (240 mg/kg, i.p.) and perfused transcardially with 0.9% NaCl, followed by 4% paraformaldehyde as our previous reports [6]. The brains were removed quickly, postfixied in 4% paraformaldehyde for 24 h, and dehydrated in 20% sucrose solution. The tissue was frozen and sectioned on a sliding microtome at 30 μm thickness. Then the slices were placed in ice-cold
10 mM phosphate buffered saline (PBS). To block the activity of endogenous peroxidase, the slices were incubated with 3% H$_2$O$_2$ for 10 min and washed in PBST (10 mM PBS, pH 7.4, containing 0.5% Triton X-100). To reduce non-specific staining, sections were blocked with 10% bovine serum for 1 h. The blocked slices were incubated with primary rabbit polyclonal antibodies against phospho-Thr183/Tyr185 JNK at 1:25 dilution at 4 °C overnight. Alternate sections from each brain sample were incubated without the primary antibody as negative controls. The specimens were incubated in secondary antibody of streptavidin-conjugated horseradish peroxidase solution (Sigma–Aldrich Company, USA) at a 1:200 dilution for 2 h and then incubated with 3,3′-diaminobenzidine (DAB, 60%) was added to visualize the slices. The images were captured by Leica microscope imaging system (Leica Company, Germany).

Additionally, a double-labeled immunofluorescent staining was performed to determine the cell type and co-localization of phospho-Thr183/Tyr185 JNK and neurogranin, a neuron-specific protein [12,14]. After incubation with primary antibody against phospho-Thr183/Tyr185 JNK overnight at 4 °C, secondary fluorescein isothiocyanate-labeled anti-rabbit IgG (green, Sigma–Aldrich Company) was added at a 1:200 dilution for 30 min in the dark at 37 °C. The slices were extensively washed in PBS and blocked with 10% bovine serum for 30 min and then incubated with primary rabbit polyclonal antibody against neurogranin (Upstate Company, NY, USA) at a 1:400 dilution overnight at 4 °C and secondary rhodamine-labeled anti-rabbit IgG (red, Sigma–Aldrich Company, USA) at a 1:200 dilution for 30 min at 37 °C in the dark. Negative controls included alternate sections incubated without one or both primary antibodies. The images were visualized and captured by a fluorescent microscope imaging system (Leica Company, Germany).
Quantitative analysis for Western blot results was performed after scanning of the X-ray film with Quantitative-One software (GelDoc-2000, Bio-Rad Company, USA). The values were presented as mean ± S.E. from six independent experiments. Statistical analysis was conducted by one-way analysis of variance, followed by all pairwise multiple comparison procedures using Bonferroni test. Significance was regarded as at least $p < 0.05$.

As shown in Fig. 1, the phosphorylation levels of JNK at Thr183/Tyr185 sites increased in the hippocampus and frontal cortex of HPC mice. Immunolabeled bands corresponding to the two forms of phosphorylated (p-p46 JNK and p-p54 JNK) or total JNK (T-p46 JNK and T-p54 JNK) were detected at the molecular weight of 46 and 54 kDa, respectively, while β-actin at 42 kDa. The representative results of Western blot are depicted in Fig. 1A, and quantitative analysis in Fig. 1B. A significant increase of JNK phosphorylations could be observed as follows: p-p54 JNK both in the hippocampus and frontal cortex from H1 to H6 groups, and p-p46 JNK in the hippocampus from H1 to H6 and frontal cortex from H3 to H6 groups. Unlike the increased phospho-Thr183/Tyr185 JNK, there were no significant changes in JNK protein expression both in the hippocampus and frontal cortex of early (H1–H4) and delayed (H5–H6) HPC mice (Fig. 1A, and the negative results of quantitative analysis did not show).

According to the findings from the Western blot study, the mice from H3 to H6 groups, which represent the early and delayed HPC, respectively, were selected for the immunostaining. We found that the numbers of phospho-Thr183/Tyr185 JNK positive cells increased in the CA3 region of hippocampus (Fig. 2B and C) and layer IV of frontal cortex (Fig. 2E and F) of HPC mice from H3 to H6 when compared to that of H0 group (Fig. 2A and D).

To determine the neuronal co-expression of phospho-Thr183/Tyr185 JNK with neurogranin, a neuron-specific marker, expressed predominantly in neurons of hippocampus and cortex [12,14,16], a double-labeled immunofluorescent study was performed in the brain of HPC mice from H3 group. Double-labeled immunofluorescent images as shown in Fig. 3 indicated that the immunostaining of phospho-Thr183/Tyr185 JNK (green, Fig. 3Band E) predominantly co-localized with neurogranin (red, Fig. 3A and D) both in the hippocampus (merged image in Fig. 3C) and frontal cortex (merged image in Fig. 3F) of HPC mice.

Treatments that induced tolerance to brain injury include hypoxia [19], ischemia [30], hypothermic/hyperthermic preconditioning [22,24], hyperbaric oxygenation [26], spreading depression [8], metabolic inhibitors [32] and cytokine stimulation [21]. In this study, we used an “auto-hypoxia”-induced HPC mouse model that mimics clinical asphyxia [13,19]. There are two phases of HPC: the first one is an early protective phase, which appears rapidly after sublethal ischemia or hypoxia loading; the second one is a delayed phase (second window of protection), which develops 24–72 h later. The two phases of HPC differed in duration and induction mechanisms [20]. Huge progress has been made in understanding the signal transduction pathways that convey the extracellular signal, initiated by the preconditioning stimuli, to the intracellular targets, and include the activation of the MAPK cascades.

As a member of MAPK family, JNK is encoding by three genes, jnk1, 2 and 3. The genes of jnk1 and jnk2 are ubiquitously expressed, whereas jnk3 expression is predominantly restricted to the brain and testes [33]. Alternative splicing of three jnk genes yields proteins of p46 JNK and p54 JNK, with the molecular weight 46 and 54 kDa, respectively. JNK activation requires dual phosphorylation of Thr183 and Tyr185 sites [11]. The activated JNK has been found in the heart of ischemia/reperfusion and stretch preconditioned rodents [3,25,28], and the brain of thrombin or hypothermic preconditioned rats [5,18]. In this study, we demonstrated that repetitive hypoxic exposures could increase the phospho-Thr183/Tyr185 JNK levels both in the hippocampus and frontal cortex of HPC mice.
It has been reported that JNK phosphorylation correlates with the development of preconditioning in the heart. In an in vivo rat model of ischemia-reperfusion, ischemic preconditioning and pharmacologic preconditioning with the opioid receptor agonist, TAN-67 significantly increased JNK phosphorylation, which may be an important component of cardioprotection [3]. By using isolated rat hearts subjected to four cyclic episodes of 5-min ischemia and 10-min reperfusion (PC) followed by 30-min ischemia and 2-h reperfusion (I/R), Sato et al. found that JNK phosphorylation was involved in the cardioprotection provided by ischemic preconditioning [28]. In the brain, intraperitoneal administration of 3-nitropropionic acid, a mitochondria toxin, induced JNK activation, tolerance to subsequent ischemia, and prevented delayed neuronal death in hippocampus CA1 subfield of gerbil [31]. In our “auto-hypoxia”-induced HPC model, we found that the phospho-Thr183/Tyr185 JNK levels, not total protein expression levels, increased significantly both in the hippocampus and frontal cortex neurons of HPC mice as phospho-Thr183/Tyr185 JNK mainly occurred in neurogranin (neuron-specific marker) positive cells by using double-labeled immunofluorescence. Neurogranin is a neuron-specific protein composed of 78 amino acid residues, mainly located in the cerebral cortex, hippocampus, and olfactory bulb in mammals [12,14,16]. It has been described that both damaging events and defense mechanisms took place in the brain following ischemic/hypoxic stimulation. However, based on the results from earlier reports and our current findings, it is still unclear what role activated JNK played in the development of cerebral HPC of mice. Further studies regarding the relationship between the differential signaling proteins which we found in the brain of HPC mice, need to be carried on.

In conclusion, our study is the first to demonstrate the changes in JNK phosphorylation and protein expression levels in the brain of HPC mice, which indicates that the increased neuron-specific phosphorylation of p54/p46 JNK at Thr183/Tyr185 sites, not protein expression, might be involved in the development of cerebral HPC of mice.

Acknowledgments

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References


