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Nitric Oxide as an Upstream Signal of p38 Mediates Hypoxia/Reoxygenation-Induced Neuronal Death

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Key Words
Hypoxia · Hippocampal neuron · Cell death · Nitric oxide · Mitogen-activated protein kinase

Abstract
Nitric oxide (NO) and p38 have been shown to be involved in the ischemia/hypoxia-induced neuronal injury. In this study, we examined the activation patterns of mitogen-activated protein kinases and explored the relationship between NO and p38 in a model of hippocampal neuronal death induced by hypoxia/reoxygenation (H/R). p38 activity increased robustly during hypoxia and after reoxygenation, while the increase of c-Jun amino-terminal kinase and extracellular signal-related kinase activities showed mild tendency. Inhibition of p38 with SB203580 or SB202190 rescued neuronal death, whereas inhibition of extracellular signal-related kinases with PD98059 or c-Jun amino-terminal kinases with SP600125 offered no protection. p38 inhibitors also reduced neuronal death induced by the NO donor S-nitrosoglutathione. L-NAME, a nonspecific NO synthase inhibitor, blocked the p38 activation and rescued H/R-induced neuronal death. These results suggest that NO is an upstream signal of p38 that mediates the H/R-induced neuronal death.

Introduction

Hypoxia is an important pathophysiological feature of ischemic diseases such as stroke. Neurons are more vulnerable than other brain cells to reduced oxygen supply, and reoxygenation may further deteriorate the injury. Transient hypoxia/ischemia leads to neuronal death selectively in some brain areas, especially in the hippocampus. Evidence has accumulated that multitude of molecules such as glutamate, free radicals, protein kinases are involved in the hypoxic/ischemic injury. The intracellular signaling pathways, however, are poorly understood [1, 2]. Nitric oxide (NO) is an intra- and intercellular signaling molecule which plays important roles in regulating calcium influx, synaptic plasticity and cell survival in the adult nervous system in both physiological and pathological settings [3–6]. Upregulation of NO production during ischemia and reperfusion is thought to have either protective or deleterious effects depending on the NO synthase (NOS) isoform from which NO is produced [7]. The main source of NO in the brain, in absence of inflammation, is neuronal NOS [8]. Although the studies with pharmacological inhibitors and knockout mice implicate a neurotoxic effect of neuronal NOS in ischemic stroke [9, 10], the downstream signaling pathways by which NO mediates its neurotoxic effect remain unclear.

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Mitogen-activated protein kinases (MAPKs) play important roles in cellular response to different stimuli [11]. Among the MAPKs, extracellular signal-related kinases (ERK) are generally responsive to growth factors and have been widely associated with cell survival, whereas c-Jun amino-terminal kinases (JNK) and p38 are activated in response to cytokines or stress and are often implicated in cell death [12]. A growing body of evidence has shown that p38 pathway is activated following cerebral ischemia and contributes to ischemic/hypoxic neuronal death [13, 14], but its upstream signaling in mediating this kind of cell death is unknown. We report here that hypoxia/reoxygenation (H/R)-induced death of cultured hippocampal neurons was mediated by NO production and p38 activation. In addition, NOS inhibitors prevented the rapid activation of p38, and inhibition of p38 rescued NO-induced neuronal death. Taken together, these observations suggest that NO is an upstream molecule of p38 in the signaling pathway of hypoxic neuronal death.

Materials and Methods

Primary Cultures of Hippocampal Neurons

Primary hippocampal neuronal cultures were prepared from neonatal Sprague-Dawley rats (P1–2) as described previously [6, 15]. Hippocampi were dissected and digested by 0.125% trypsin at 37°C for 10 min. Cells were dispersed by gentle agitation and passed through a 200-mesh stainless steel sieve. Cells were plated with a density of 4×10^5 cells/cm^2 on poly-L-lysine-coated culture surface. Culture medium consisted of 90% DMEM (Gibco) and 10% fetal bovine serum. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Glial cell growth was controlled by treatment with 10^{-5} M cytosine arabinoside for 48 h at day 2 in vitro. The percentage of astrocytes is less than 4% when assessed by GFAP immunocytochemistry, and the percentage of GABAergic interneurons is no more than 5% when assessed by GAD65 immunocytochemistry. All experiments were performed on the cells cultured for 13–15 days.

Treatment of H/R

Before induction of hypoxia, the culture medium was replaced with DMEM without serum. Neurons were exposed to hypoxia for 4 h by transferring the culture plates to a humidified incubation chamber thermoregulated at 37°C and flushed by a gas mixture consisting of 95% N_2/5% CO_2. After the hypoxic treatment, the conditioned culture medium was changed back and the cultures were returned to standard normoxic atmosphere for up to 24 h, whereas control cells were constantly maintained under normoxic conditions [16].

Assessment of Neuronal Death

Neuronal death was assessed by both morphological observation and quantification. Dying neurons appeared as fragmented dendrites and condensed nuclear structures. Quantification was done by a spectrophotometric method using the tetrazolium salt MTT. Briefly, neurons were incubated for 3 h at 37°C with MTT (500 μg/ml), washed with ice-cold PBS, and lysed in DMSO. Optical density was measured at 570 nm, and data were compared with those obtained from sister control cells.

Western Blot Analysis

Activation of p38, JNK and ERK was assessed by Western blot analysis. Whole cell extracts were prepared following the manufacturer's protocol (Cell Signaling Technology). At the different time points after the beginning of hypoxia or reoxygenation, cells were washed twice with chilled (4°C) PBS and then homogenized on ice with lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue. The proteins were separated by electrophoresis on a 12% SDS-polyacrylamide gel and electrotransferred to a polyvinylidene difluoride membrane. After blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBS/TM), the membranes were incubated overnight at 4°C with anti-phospho-p38 antibody, anti-phospho-JNK antibody, anti-phospho-ERK antibody, anti-p38 antibody, anti-JNK antibody or anti-ERK antibody (Cell Signaling Technology) diluted 1:1,000 in TBS/TM. The membranes were then washed and incubated with HRP-conjugated secondary antibody (1:5,000) for 1 h. The immune complex was detected by chemiluminescence (Santa Cruz Biotechnology). The activations of p38, JNK and ERK were represented by the ratio of phosphorylated kinase bands normalized to the total kinase bands. Actin was used as a loading control.

Statistics

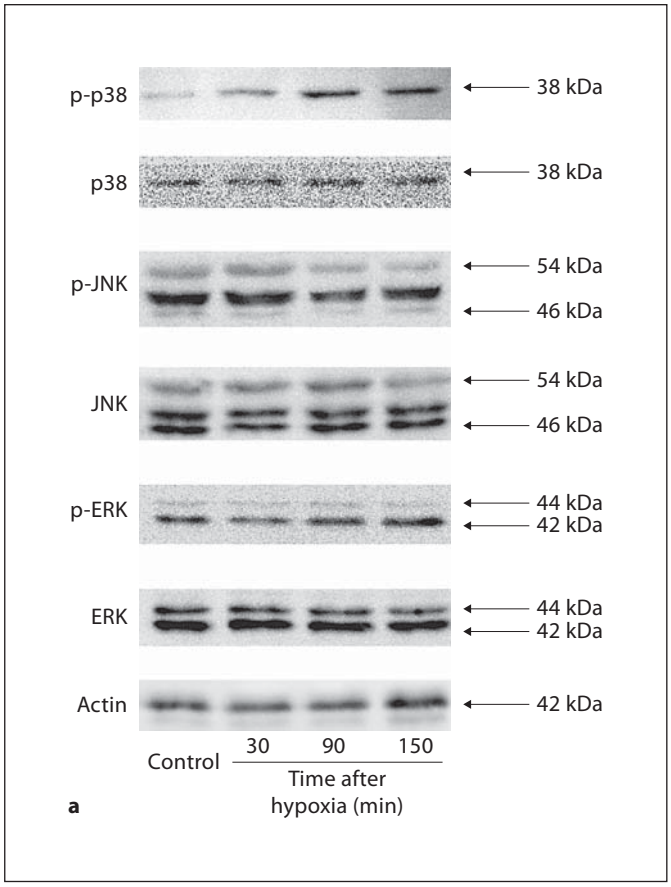
Data are expressed as the mean ± SEM for at least three independent experiments. Multiple comparisons were done using one-way ANOVA with LSD post-hoc tests. Changes were identified as significant if the p value was less than 0.05.

Results

Activation of MAPKs Induced by H/R Exposure

Since the MAPKs are proposed to be involved in the ischemic/hypoxic neuronal death and reoxygenation can further deteriorate the hypoxic injury, we first characterized the activation patterns of p38, JNK and ERK during hypoxia. Western blot analysis was used to examine the temporal changes in the phosphorylated kinase levels, which represent the activation of the kinases. As shown in figure 1, the activation of p38 was observed as early as 90 min after the beginning of hypoxia, while JNK (54 and 46 kDa) and ERK (44 and 42 kDa) showed no significant activation during hypoxia.

We then examined whether p38, JNK and ERK were activated after H/R. As seen in figure 2, robust activation of p38 was observed and the phosphorylation of p38 reached the highest level at 0 h, then decreased and recovered at 3 h after reoxygenation, while JNK and ERK only showed mild tendency of transient increase in H/R-treated cells.



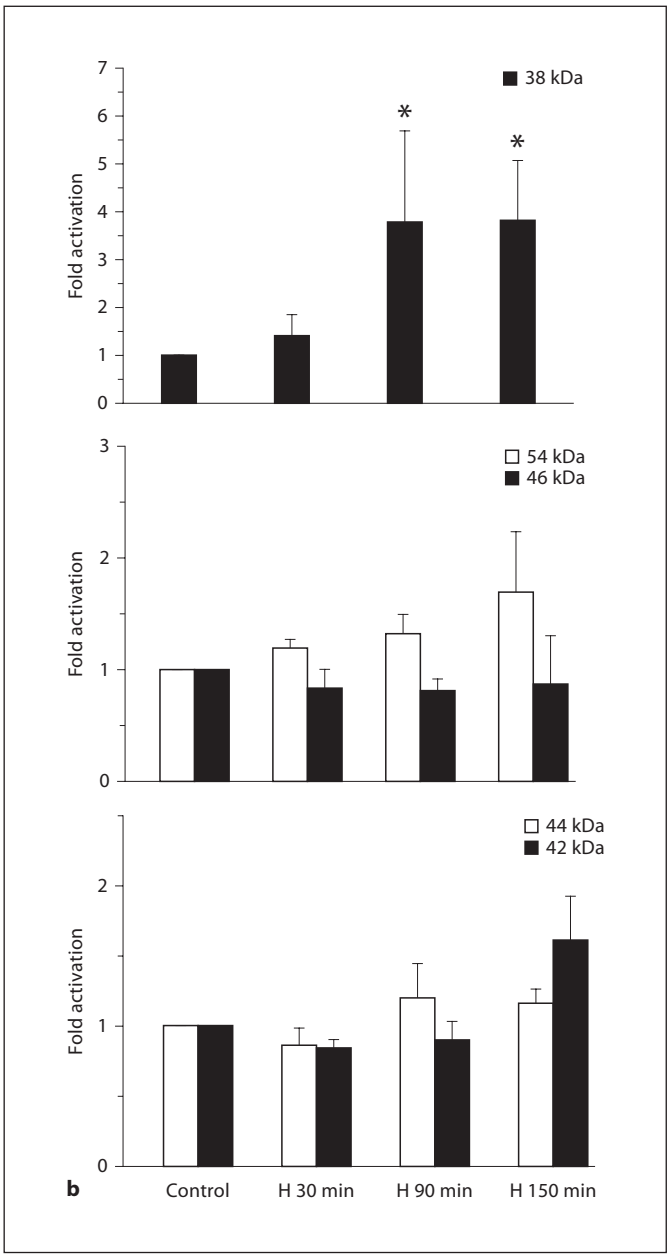


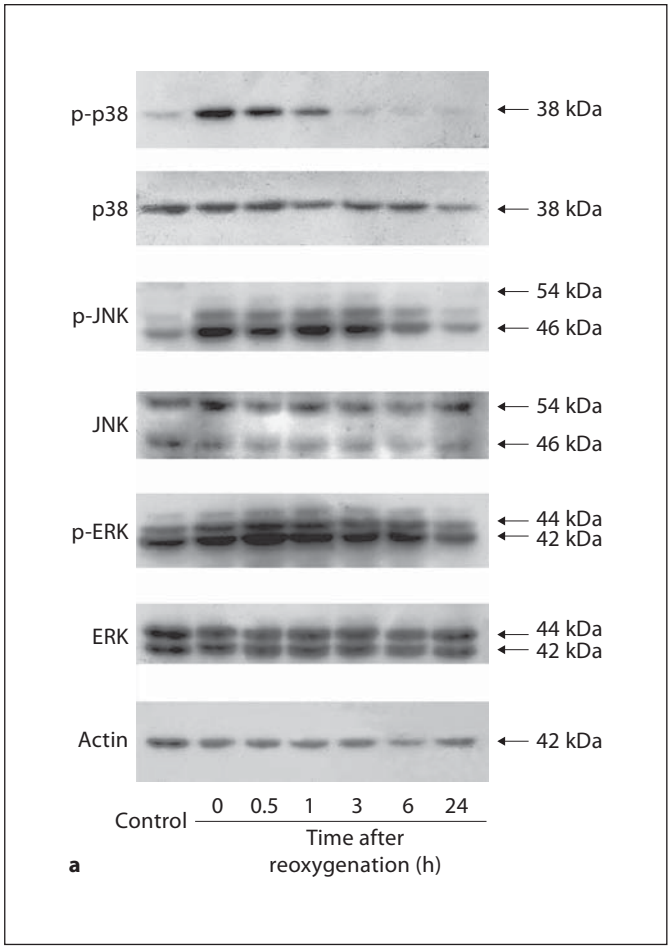
Fig. 1. Activation of p38, JNK and ERK during hypoxia. **a** Representative Western blots show the expression of phosphorylated and total kinases. **b** Quantification of the activations of p38, JNK and ERK. Data from three independent experiments * $p < 0.05$ compared with control.

Protection of H/R-Induced Neuronal Death by p38 Inhibitors

To evaluate the role of MAPKs activation in H/R-induced cell death, the effects of MAPK inhibition were tested. As shown in figure 3, H/R treatment caused about 30% neuronal death, as measured by the MTT method. Two specific p38 inhibitors, SB203580 (10 μM) or SB202190 (10 μM), rescued half of the neuronal death when applied before hypoxia, whereas the inactive analog SB202474 (10 μM), ERK inhibitor PD98059 (10 μM) or JNK inhibitor SP600125 (10 μM) afforded no protection, indicating that p38 acted as an important mediator of H/R-induced neuronal death.

NO Acts Upstream of p38 to Mediate H/R-Induced Neuronal Death

To clarify the role of NO in the H/R-induced neuronal death, a nonspecific NOS inhibitor, L-NAME, was used. L-NAME (0.1 mM) reduced cell death when applied before hypoxia but not after reoxygenation (fig. 4a). Then,



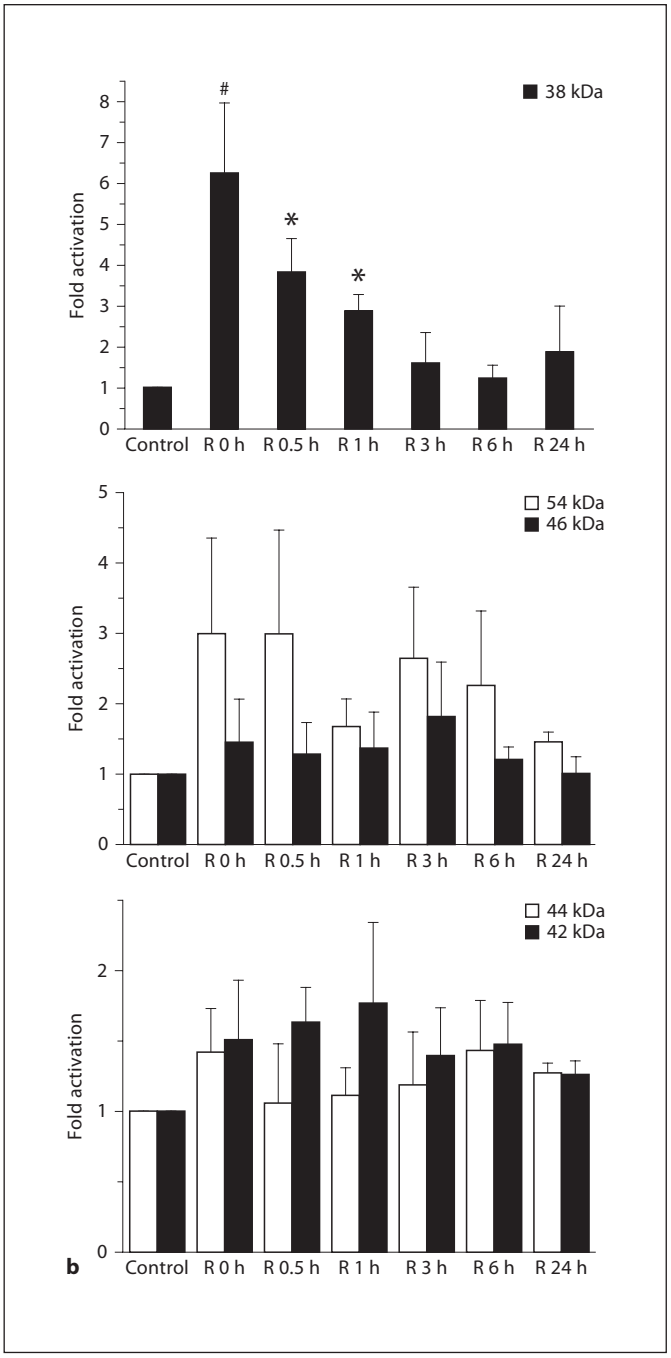
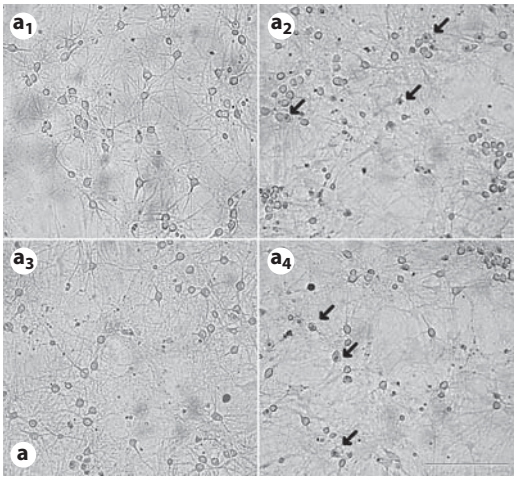


Fig. 2. Activation of p38, JNK and ERK after reoxygenation. **a** Representative Western blots show the expression of phosphorylated and total kinases. **b** Quantification of the activations of p38, JNK and ERK. Data from three independent experiments. # $p < 0.001$ and * $p < 0.05$ compared with control.

we further investigated the relationship between NO and p38 in the signaling pathway of neuronal death. S-nitrosoglutathione (GSNO), a NO donor that generates NO through mechanisms independent of NOS, alone induced the death of about 33% of normal cultured neurons at the concentration of 0.1 mM. SB203580 or SB202190 prevented GSNO-induced cell death, while SB202474 showed no effect (fig. 4b). This result suggested that the production of NO might stimulate the activation of p38. Moreover, in the presence of L-NAME, the activation of p38 by H/R was blocked (fig. 5), which confirmed that NO was an upstream signaling molecule of p38.



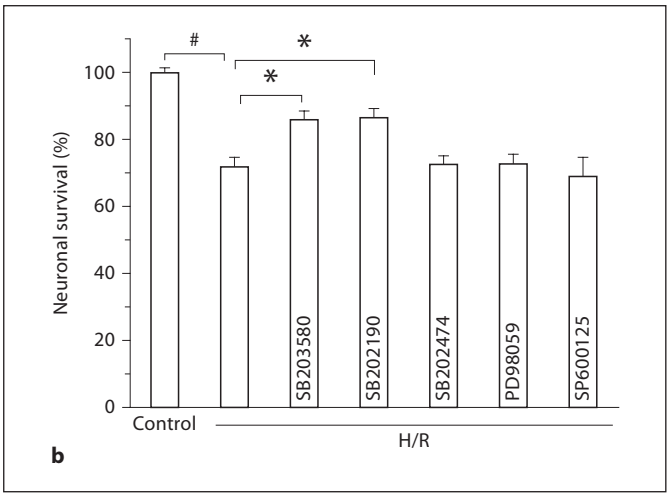
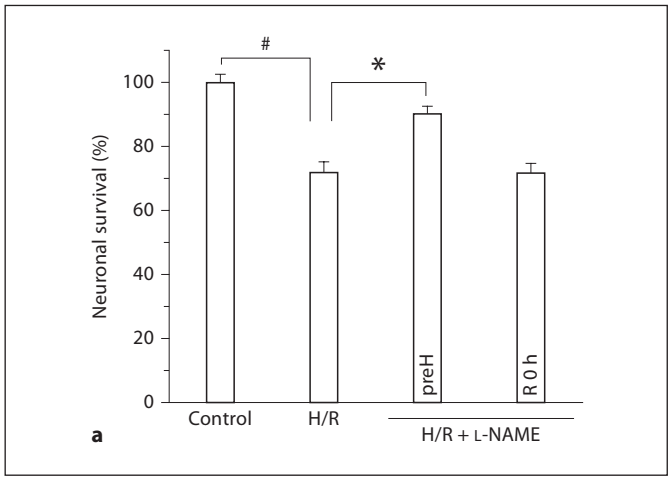


Fig. 3. Protection of H/R-induced neuronal death by p38 inhibitors. 10 μM of inhibitors specific to different kinases were added to the culture medium before hypoxia. **a** Representative phase-contrast microphotographs of control (**a1**), H/R (**a2**), H/R added with SB203580 (**a3**) and H/R added with SB202474 (**a4**). Photos were taken at 24 h after reoxygenation. Black arrows indicate the dying neurons. Bar = 200 μm. **b** Two p38 inhibitors, SB203580 or SB202190, rescued half of neuronal death, whereas its inactive analog SB202474, ERK inhibitor PD98059 or JNK inhibitor SP600125 afforded no protection. Neuronal survival was assessed using the MTT method and normalized with control as 100%. # $p < 0.001$ compared with control; * $p < 0.05$ compared with the H/R group (n = 12–16).



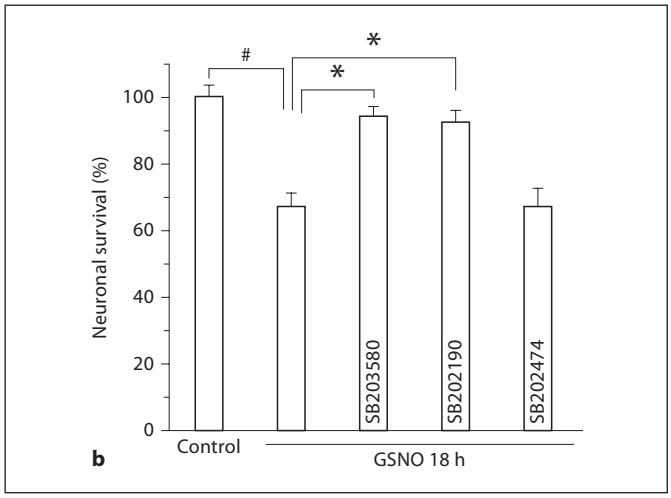


Fig. 4. Protection of H/R- and NO-induced neuronal death by NOS and p38 inhibitors, respectively. **a** Involvement of NO in H/R-induced neuronal death. Neuronal cultures were subjected to H/R. The NOS inhibitor L-NAME (0.1 mM) was applied either before hypoxia (preH) or at 0 h after reoxygenation (R0h). L-NAME reduced cell death when applied before hypoxia but not after reoxygenation. # $p < 0.001$ compared with control; * $p < 0.01$ compared with H/R group (n = 10–15). **b** Involvement of p38 in NO-induced neuronal death. Cultures were treated with 0.1 mM GSNO for 18 h, and 10 μM of MAPK inhibitors were added before GSNO treatment. p38 inhibitors prevented GSNO-induced cell death, while its inactive analog showed no effects. # $p < 0.001$ compared with control; * $p < 0.01$ compared with GSNO group (n = 10).

Discussion

p38 Is a Candidate Mediator of H/R-Induced Hippocampal Neuronal Death

The major finding in the present study is that NO acts as an upstream signaling molecule of p38 to mediate the H/R-induced cell death in the cultured hippocampal neurons. The result that p38, JNK and ERK were activated after H/R is consistent with previous reports in global or focal cerebral ischemia [13, 17]. The consequence of MAPK activation after insult is dependent on the immediate intracellular environment of the individual cell, the cell type, the number of kinase pathways activated at any given time, and the duration of kinase ac-

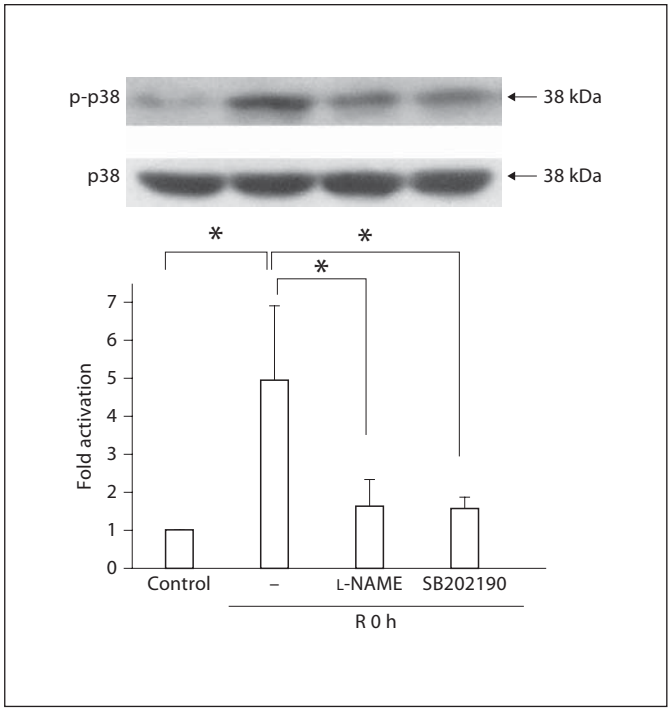


Fig. 5. Blockade of H/R-induced p38 activation by NOS inhibitor. L-NAME (0.1 mM) and SB202190 (10 μM) were added before hypoxia. SB202190 was used as a positive control. Activation of p38 at 0 h after reoxygenation was assessed by Western blot analysis. Results are from three experiments. * $p < 0.05$.

tivation [18]. Although the inhibition of p38 was demonstrated to provide protective effects on glutamate- or NO donor-induced neuronal death [19, 20], the role of p38 in neurons subjected to hypoxic insult was inconsistent [21, 22]. In cortical neurons, inhibition of p38 reduced hypoxic neuronal death [21]. However, in spinal cord neurons, no change in p38 kinase was detected after H/R, and inhibition of p38 did not provide protection [22]. Our data from hippocampal neurons suggest that p38 is a candidate mediator of neuronal death induced by H/R. The activation of p38 was transient in the neuronal cultures subjected to H/R, and the protective effects appeared only when the specific p38 inhibitors were applied before hypoxia, which indicates that p38 may be an early mediator in the starting up of neuronal death signaling induced by H/R. As shown in figure 2, although there is a tendency of greater activation fold of p38 at the later stage of reoxygenation, H/R-induced global suppression of protein translation due to activation of the unfolded protein response in the ER may lead to less phopho-p38 than in control conditions.

It was reported that reduction in ERK or JNK activations by treatment of specific inhibitors offered neuroprotection against ischemia [18, 23]. Although H/R evoked mild activation of ERK and JNK in the present study, their inhibitors failed to protect the cells. This discrepancy may reflect the differences in severity of injury to neurons by the experimental ischemia model applied. In addition, changes that happened in multiple types of cells in ischemic animal make the explanation of results more complex than that in culture systems.

NO Acts Upstream of p38 in H/R-Induced Hippocampal Neuronal Death

It has been shown that exogenous NO induces p38 activation in cultured neurons [19, 24]. In a recent study, NO was found as an upstream regulator of p38 in glutamate-induced cerebellar granule neuronal death [20]. Consistent with this, our present results show that inhibition of NOS by L-NAME when applied before the induction of hypoxia blocks the activation of p38 and rescues the hypoxic neuronal death, indicating that endogenous NO production precedes p38 activation to mediate the H/R-induced neuronal injury. NO mediates the cellular effects mainly by two signaling mechanisms, cGMP pathway and S-nitrosylation. The cGMP pathway has been extensively documented for the effect on proteins by phosphorylation, whereas S-nitrosylation of proteins by chemical reactions with NO can modify protein functions [4]. Either increased cGMP levels or nitrosative stress have been reported to promote p38 activation in nonneuronal cells [25, 26]. The mechanisms underlying NO modulation of p38 activity in ischemic/hypoxic neuronal death need further elucidation.

Acknowledgements

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