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Delayed phosphorylation of p38 mitogen-activated protein kinase in the AT1a knock-out mouse striatal neurons during middle cerebral artery occlusion and reperfusion

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Abstract

To investigate whether the phosphorylation of p38 in cerebral ischemia occurs via angiotensin II receptor type 1a (AT1a), we examined the time course of phosphorylation of p38 and proline-rich tyrosine kinase 2 in AT1a knock-out mouse striatal neurons during middle cerebral artery occlusion (MCAO) and reperfusion. Phosphorylated-p38 was observed after 2 h and 5 h of reperfusion after 1 h of MCAO in C57/B6 mice and AT1a knock out mice, respectively. We demonstrated a delay of phosphorylation of p38 in the reperfusion model of the AT1a knock-out mouse, and detected microglia in the striatum on the ischemic side that were phosphorylated-p38-positive after 71 h of reperfusion in both animals. However, there was no association between AT1a and delayed neuronal cell death, or between AT1a and activation of caspase-9 in cerebral ischemia/reperfusion.

Keywords: Angiotensin; Angiotensin II receptor type 1a; Cerebral ischemia and reperfusion; Delayed neuronal death; Knock-out mouse; Microglia; p38 mitogen-activated protein kinase; Proline-rich tyrosine kinase 2

Petito et al. first reported that delayed neuronal death (DND) occurred after 48–72 h of transient cerebral ischemia in the rat hippocampus [9]. Many studies indicated that the mitogen-activated protein kinase (MAPK) family including extracellular signal-regulated kinase (ERK)-1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK (p38) were activated in neurons and glial cells after cerebral ischemia/reperfusion and contributed to DND [4,12]. Conversely, low-dose administration of 3-nitropropionic acid induced not only activation of JNK, but also ischemic tolerance to DND in CA1 neurons of gerbils [11]. Activation of ERK-1/2 was observed in only surviving neurons after ischemia/reperfusion [4]. These data suggest that ERK-1/2 and JNK contribute to protection against DND in reperfusion injury.

Meanwhile, a first generation p38 inhibitor, SB 203580, was reported to block mitochondria-dependent neuronal cell death in vitro [2]. It was also reported that a second generation p38 inhibitor, SB 239063, reduced infarct size in permanent MCAO animals [1]. However, there are no data on the relationship between the activation of p38 and resistance to DND in cerebral ischemia/reperfusion injury. Therefore, exploration of the p38 upstream signal pathway is important for clinical treatment of ischemic stroke.

The aim of this study was to investigate the activation of p38 in C57/B6 and AT1a knock-out mice to clarify whether AT1a participates in the phosphorylation of p38 and DND, because AT1a, one of the G protein-coupled receptors, phosphorylated p38 via Src in many cell types [5,14].

Seventeen adult male C57/B6 mice (body weight 15.4–21.6 g) were purchased from Charles River Laboratory (Kanagawa, Japan), and 16 adult male AT1a knock-out mice (body weight 18.4–25.0 g) were provided by Tanabe Seiyaku Co., Ltd. (Osaka, Japan). They were given food and water freely before and after surgery. All the experimental protocols relating to animals were given prior approval as meeting the Animal Experimentation Guidelines of the School of Medicine, Keio University, by the Laboratory Animals Care and Use Committee.

Sham-operated mice and mice subjected to 1 h of left MCAO/reperfusion for 2, 5, 11, 23, 47, and 71 h were

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produced by using a nylon thread as described by Hara et al. [3]. They were anesthetized with 1–2% isoflurane in a nitrous oxide/oxygen mixture (70%/30%) during operation and sacrificed by injection of excess sodium pentobarbital (over 100 mg/kg, i.p.) before removing the brain tissue. Cerebral blood flow in the left MCA territory was continuously measured through the exposed skull with a Doppler flowmeter (ALF21R; Advance, Tokyo, Japan), and right-sided hemiparesis was confirmed in all animals except the sham-operated animals.

For Western blot analysis, the left cerebral hemisphere was removed after sacrifice and homogenized in ice-cold lysate buffer (200 mmol/l HEPES [pH 7.5], 250 mmol/l sucrose, 1 mmol/l dithiothreitol, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 1 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l (ethylenebis(oxethylenenitrilo))tetraacetic acid, 0.1 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 2 μg/ml aprotinin) in a Teflon homogenizer. The homogenate was spun at 54 000 × *g* for 60 min, and the resulting supernatant was used as the cytosolic fraction. Each 40 μg of protein was electrophoresed in 4–12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotted using primary antibodies: a rabbit polyclonal anti-p38 antibody (1:500, Cat# 9210, New England Biolabs, MA, USA), a rabbit polyclonal anti-phosphorylated-p38 antibody (1:500, Cat# 9210, New England Biolabs), a rabbit polyclonal anti-phosphorylated-proline-rich tyrosine kinase 2 (pyk2) antibody (1:1000, Cat# 44-618, BioSource International, CA, USA), and a rabbit polyclonal anti-caspase-9 antibody (1:500, Product# AAP-109, StressGen Biotechnologies, BC, Canada), and a secondary antibody: an alkaline phosphatase-conjugated anti-rabbit IgG antibody (1:1000, Code Number 111-055-144, Jackson ImmunoResearch Laboratories, PA, USA).

For immunohistological analysis, the brain tissues were fixed by 4% paraformaldehyde/0.1% PBS perfusion, sliced into 10–12 μm-thick frozen coronal sections, and stored at −80 °C until use. The primary antibodies used were: a rabbit polyclonal anti-p38 antibody (1:100, Cat# 9210, New England Biolabs) and a mouse monoclonal anti-bromodeoxyuridine (Brd-U) antibody (1:200, Product# B2531, SIGMA, MO, USA), and the secondary antibodies were: a biotinylated anti-rabbit IgG antibody (1:200, Cat# BA-1000, Vector Laboratories, CA, USA) and a Texas-Red dye-conjugated anti-mouse IgG antibody (Code Number 715-075-150, Jackson ImmunoResearch). Fluorescein isothiocyanate-labeled lectin (Product# L 2895, SIGMA) was used as a microglial marker.

To investigate whether AT1a activates p38 via src, we performed Western blot analysis of phosphorylated-pyk2, phosphorylated-p38, and p38 in cytosolic fractions prepared from sham-operated mice and mice subjected to 1 h of ischemia before reperfusion in both C57/B6 and AT1a knock-out mice. There were significant amounts of phosphorylated-p38 after 2 h of reperfusion in the C57/B6 mouse and after 5 h in the AT1a knock-out mouse (Fig. 1A). Meanwhile, the same amounts of phosphorylated-pyk2 and p38 were detected at all time points in the ischemia/reperfusion mice and sham-operated mice in both C57/B6 and AT1a knock-out mice (Fig. 1A). It was shown that phosphorylation of p38 was delayed in the cerebral ischemia/reperfusion model in AT1a knock-out mice.

Immunohistochemical analysis on the ischemic side of the striatum showed phosphorylated-p38-positive neurons after 2 h of reperfusion in the C57/B6 mouse (Fig. 1B). On one hand, there were few phosphorylated-p38-positive neurons at the same time point (Fig. 1B) and a significant increase in the number of phosphorylated-p38-positive neurons between 5 and 11 h of reperfusion (data not shown) in the AT1a knock-out mice. Conversely, we found phosphorylated-p38-positive glial cells after 71 h of reperfusion in both C57/B6 and AT1a knock-out mice (Fig. 1B).

To clarify the characteristics of glial cells after 71 h of reperfusion in both animals, we performed fluorescence immunohistochemical analysis of glial cells after 71 h of reperfusion on the ischemic side of the striatum. The same glial cells were positive for both phosphorylated-p38 (Fig. 2A) and lectin which is a microglial marker (Fig. 2B) in the AT1a knock-out mouse (Fig. 2C). The same microglial cells were positive for Brd-U (Fig. 2D), which means that these cells underwent cell division after the Brd-U administration at 1 h before sacrifice. These data imply that these glial cells were microglia that had proliferated, at least after reperfusion.

To evaluate the delayed neuronal cell death induced by ischemia/reperfusion, we performed Western blot analysis of pro- and cleaved-caspase-9 in the cytosolic fraction and TUNEL staining at all time points in ischemia/reperfusion and sham-operated animals in both C57/B6 and AT1a knock-out mice. We observed a gradual increase of pro-caspase-9 above the level in sham-operated animals up to 23 h of reperfusion and time-dependent augmentation of cleaved-caspase-9 between 2 and 23 h of reperfusion in both animals (data not shown). There was no statistically significant difference between the number of TUNEL-positive cells in the striatum on the ischemic side in sham-operative mice and ischemia/reperfusion mice after 23 h and 71 h of reperfusion in C57/B6 mice and AT1a knock-out mice using Mann–Whitney's *U*-test (0 vs. 0/mm^2 in sham; 275.0 ± 222.2 vs. 452.0 ± 163.8/mm^2, $P = 0.17$, at 23 h of reperfusion; 895.0 ± 311.4 vs. 1025 ± 188.7/mm^2, $P = 0.40$, at 71 h of reperfusion; taking five random windows at each time point).

Recent studies suggest that activated *N*-methyl-D-aspartate receptor subunit 2A interacts with pyk2 and Src, and phosphorylated pyk2 in neurons and microglial cells directly induced phosphorylation of p38 after cerebral ischemia/reperfusion in rats [6,13]. However, there was a discrepancy between these data and our western blot analysis, the latter of which showed that the amount of

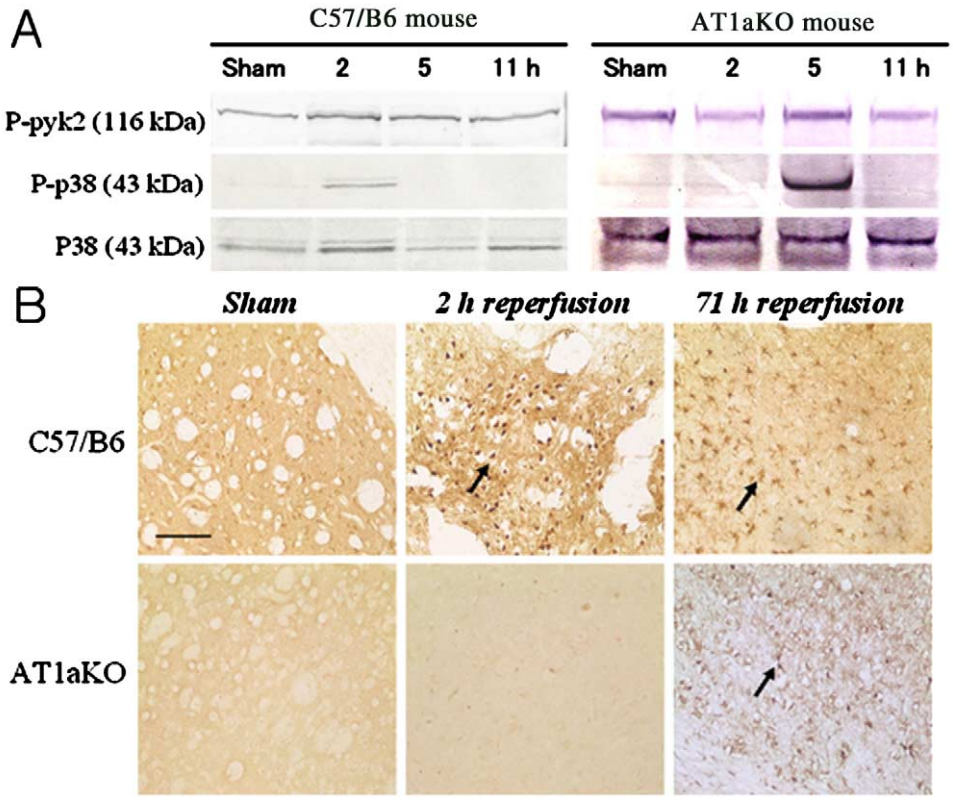

Fig. 1. Phosphorylation of p38 induced by MCAO/reperfusion in the mouse. (A) Each Western blot analysis of cytosomal samples shows phosphorylated-pyk2, phosphorylated-p38 and p38. After 2 h of reperfusion in the C57/B6 mouse and after 5 h in the AT1a knock-out mouse, there was a significant increase in the amount of phophorylated-p38. On the other hand, phosphorylated-pyk2 and p38 showed the same amounts in all the samples. (B) Immunohistochemical analysis of the ischemic side of the striatum. Phosphorylated-p38-positive neurons (arrow) were found after 2 h of reperfusion in the C57/B6 mouse, and phosphorylated-p38-positive microglia (arrows) after 71 h of reperfusion in both C57/B6 and AT1a knock-out mice. Scale bar in (B) = 200 μm.

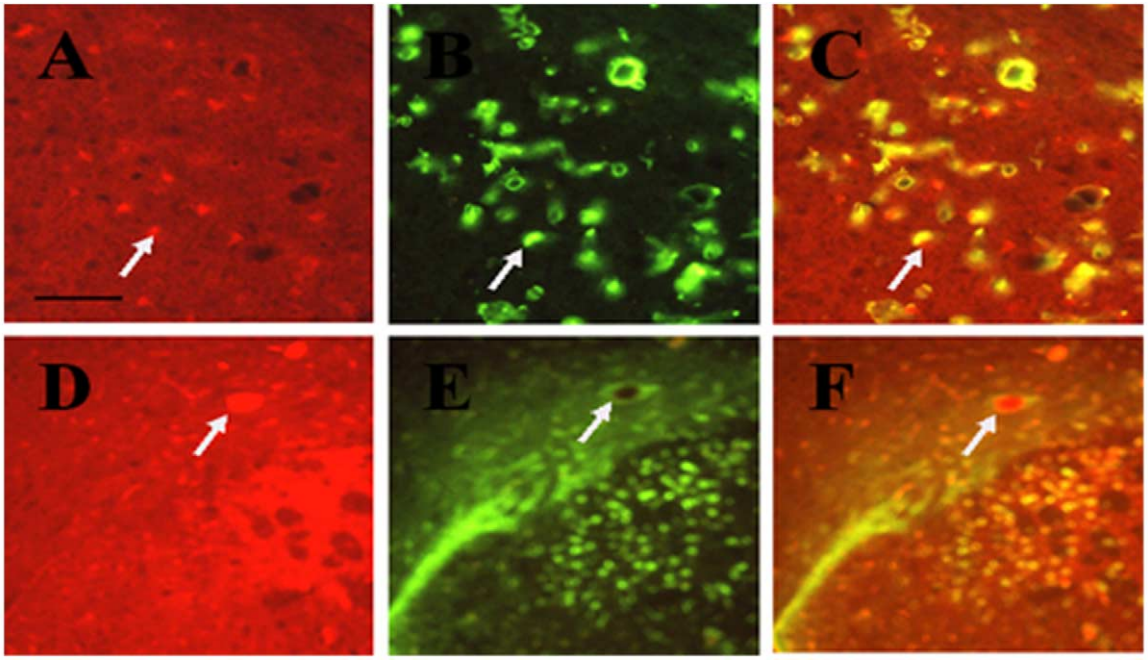

Fig. 2. Fluorescence immunohistochemical analysis of microglia after 71 h of reperfusion in the ischemic side of the striatum. (A–C) Glial cells are double-stained for phosphorylated-p38 (A,C) and lectin which is a microglial marker (B,C). (D–F) Glial cells are double-stained for Brd-U which is a maker of cell division (D,F) and lection (E,F). The same cells are indicated by white arrows. Scale bar in (A) = 100 μm.

phosphorylated-pyk2 was the same at all the time points examined.

Physiologically, AT1 receptors were seen in dopamine-synthesizing neurons of the substantia nigra, the caudate nucleus and putamen of the human and rat brain [15]. AT1a and AT1b receptor mRNA levels were not altered, but AT2 mRNA level was about twice as high in the striatum of the ischemic side compared with the contralateral side after 3 h of reperfusion after 10 minutes of whole brain ischemia in the rat [7]. Excess AT2 receptor in vitro had ligand-independent ability to induce apoptosis via phosphorylation of p38 [8]. The delayed phosphorylation of p38 in the striatum on the ischemic side in the AT1a knock-out mouse might mean that AT1a receptors contribute to p38 phosphorylation. However, there was no difference in the activation of caspase-9 and the number of TUNEL-positive cells at all time points between the two animals. We speculated that the AT1a receptor first induced the activation of p38, and then the induced AT2 receptor contributed to DND by activating p38 secondarily. The AT1a knock-out mice we used had hypotension and hyperreninemia [10]. We consider that these factors could affect the severity of ischemia and the size of the ischemic region induced by MCAO, but cerebral blood flow detected by flowmeter was decreased by more than 80% during occlusion and increased more than 3-fold during reperfusion in all cases in both animals.

In conclusion, we detected a delay of phosphorylation of p38 in cerebral ischemia/reperfusion in AT1a knock-out mice, and found phosphorylated-p38-positive microglia in the striatum on the ischemic side after 71 h of reperfusion in the AT1a knock-out mouse and C57/B6 mouse. However, there was no difference in delayed neuronal death and activation of caspase-9 between the two animals in cerebral ischemia/reperfusion.

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