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Temporal and regional patterns of Smad activation in the rat hippocampus following global ischemia

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ABSTRACT

In this study, we examined the temporal and regional patterns of Smad activation in the rat hippocampus following global ischemia. We also examined the association between Smad activation and ischemia-induced pathology in the hippocampus.
We found that 1) Smad1, -2, -3, and -5 proteins were detected in the rat hippocampus by means of western blot and immunohistochemistry; 2) after 5 min of ischemia, Smad2 and Smad3 proteins accumulated in the nuclei of pyramidal cells in the CA1 region, which is vulnerable to ischemia; 3) after 3 min of ischemia, which was non-lethal, there was no such nuclear accumulation of Smad2 and Smad3 in the CA1 region; 4) following injection of activin A, nuclear accumulation of Smad2 and Smad3 was induced not only in pyramidal cells of the CA1 region, but also in pyramidal cells of the CA3 region as well as in granule cells of the DG region; 5) activin A-induced nuclear accumulation of Smad2 and Smad3 neither caused degeneration of hippocampal neurons nor prevented degeneration induced by ischemia. These results suggest that in the hippocampus, ischemia-induced activation of Smad2 and Smad3 is associated with the response to stress but is not related to neuronal survival or death.

1. Introduction

Cardiac arrest causes global brain ischemia, often leading to cognitive or memory disturbances. The hippocampus plays an important role in cognition and memory formation, and is well known to be one of the brain regions most vulnerable to ischemia [1]. The pyramidal neurons of the CA1 region of the hippocampus are particularly vulnerable. In contrast, pyramidal neurons in the CA3 area of the hippocampus and granule cells of the dentate gyrus (DG) are both relatively resistant to ischemia. Previous studies using animal models of global ischemia have shown that pyramidal neurons in the CA1 region degenerate after 2 to 4 days of reperfusion [2,3]; this phenomenon is known as delayed neuronal death [2]. Delayed neuronal cell death has been the focus of a great deal of research in the hope of developing preventative interventions for patients with ischemia. Previous studies have demonstrated that the activation of a number of molecules is altered in neurons prior to the onset of ischemia-induced neuronal cell death. These molecules include calpain, a Ca^{2+}-dependent protease [4–6], MAPK/ERK kinase 1, a tyrosine/threonine kinase [7], Akt, a serine/threonine kinase [8,9], and CREB, a transcriptional factor [6,10,11]. Such findings suggest that ischemia disturbs the homeostasis in affected neurons. However, the precise mechanisms that mediate neuronal cell death have yet to be elucidated.

It is well known that the transforming growth factor-β (TGF-β) superfamily of proteins, including TGF-βs, activins, and bone morphogenetic proteins (BMPs), regulates multiple cellular functions in both fetal and adult tissues. TGF-βs inhibit the proliferation of epithelial cells and lymphocytes [12], activins regulate the release of follicle-stimulating hormone and the differentiation of erythrocytes [13–15], and BMPs contribute to the differentiation of osteoblasts [16]. Smad proteins are known to transduce the actions of the TGF-β family [17,18]. To date, 8 Smad proteins have been characterized: Smad1, -2, -3, -4, -5, -6, -7, and -8. These Smad proteins are divided into 3 subclasses on the basis of their function and structure: receptor-regulated Smads (R-Smads), the common partner Smad (Co-Smad), and inhibitory Smads (I-Smads). When TGF-βs, activins, and BMPs bind to their specific receptors on the cell surface, the receptors activate R-Smads via carboxy-terminal phosphorylation. R-Smads include Smad1, -2, -3, -5, and -8. Receptors for TGF-βs, activins and BMPs consist of 2 types of transmembrane serine/threonine kinase receptors; type I and type II. Seven type I receptors (activin receptor-like kinases (ALK) 1-7) and 5 type II receptors have been characterized. When type II receptors bind to their ligands, they activate type I receptors, which in turn phosphorylate R-Smads. ALK4, -5, and -7 form complexes with type II receptors that have affinity for TGF-βs and activins, and phosphorylate Smad2 and Smad3, while ALK1, -2, -3, and -6 form complexes with type II receptors with affinity for BMPs, and phosphorylate Smad1, -5, and -8. The only known mammalian Co-Smad is Smad4, which forms a complex with phosphorylated R-Smad before translocating to the nucleus. I-Smads include Smad6 and Smad7, and inhibit the activation

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of R-Smads by inducing degradation of the receptors or by competing with the R-Smads for type I receptor binding.

TGF-βs include 3 mammalian isoforms: TGF-β1, -β2, and -β3 [17]. In the adult rat hippocampus, the distributions of TGF-β1, -β2, and -β3 proteins and their mRNAs have been characterized using immunohistochemistry and in situ hybridization. TGF-β1, -β2, and -β3 proteins and their mRNAs were detected in pyramidal cells in the CA1 and CA3 regions, as well as in granule cells in the DG region [19–21]. Following ischemia, the immunoreactivities of TGF-β1, -β2, and -β3 in the hippocampus were found to be altered. The immunoreactivities were decreased in CA1 pyramidal neurons, although they did not change in the CA3 pyramidal neusons or in DG granule cells [19]. In contrast, the immunoreactivities of TGF-β1, -β2, and -β3 in astrocytes were increased after ischemia [19]. There are 3 types of activin isoforms known, including activin A, activin B, and activin AB [18]. Activin A and activin B are homodimers of inhibin βA and inhibin βB subunits, respectively. Activin AB is a heterodimer formed by inhibin βA and inhibin βB subunits. In situ hybridization revealed that inhibin βA subunit mRNA was expressed in the adult CA1, CA3, and DG regions, and that inhibin βB subunit mRNA was expressed in the CA1 and CA3 regions [22]. In addition, studies using a global ischemia model in adult rats and a hypoxic–ischemic brain injury model in infant rats demonstrated that treatment with either TGF-β1 or activin A decreased neuronal death in the CA1 region after ischemia [23,24]. These findings suggest that TGF-β1 and activin A may act as endogenous neuroprotective factors following ischemia. However, it has also been reported that TGF-β and activin induce cell death in an oligodendroglial cell line [25,26]. Furthermore, very little is known regarding the association between Smad activation and the pathology of the ischemic hippocampus.

In this study, we examined the temporal and regional patterns of Smad activation in the rat hippocampus following global ischemia, and investigated the association between Smad activation and ischemia-induced pathology in the hippocampus.

2. Materials and methods

2.1. Animal surgery and experimental groups

Male Sprague–Dawley rats, each weighing 250–350 g, were subjected to global ischemia or to intracerebroventricular injection of activin A. Rats were divided into 8 groups: (a) 5-min and (b) 3-min ischemia group, rats subjected to 5 min and 3 min of ischemia, respectively; (c) sham group, rats subjected to the same operation without ischemia; (d) intracerebroventricular injection of activin A group, rats subjected to injection of activin A into the bilateral cerebral ventricles; (e) intracerebroventricular injection of vehicle group, rats subjected to injection of vehicle control into the bilateral cerebral ventricles; (f) intracerebroventricular injection of activin A + 5-min ischemia group, rats subjected to injection of activin A into the bilateral cerebral ventricles followed by 5 min of ischemia; (g) intracerebroventricular injection of vehicle + 5-min ischemia group, rats subjected to injection of vehicle into bilateral cerebral ventricles followed by 5 min of ischemia; and (h) intracerebroventricular injection of vehicle + sham group, rats subjected to injection of vehicle into bilateral cerebral ventricles followed by sham operation. The experimental design for each group is summarized in Fig. 1. The number of animals used is shown in Table 1.

2.2. Global ischemia

Global ischemia was induced using the four-vessel occlusion method. The surgical procedure and induction of ischemia were performed as has been described previously [5]. Briefly, the bilateral vertebral arteries were permanently occluded by electrocauterization under chloral hydrate anesthesia (i.p., 400 mg/kg). After allowing a 24 h recovery, rats were anesthetized with 1.5% halothane in 30% oxygen and 70% nitrous oxide, and ischemia was induced by occluding the bilateral common carotid arteries with aneurysm clips. Sham-operated animals were treated similarly to those subjected to ischemia, except for the occlusion of the common carotid arteries. Body temperature was maintained at 37.0 ± 0.5 °C with a rectal thermistor and heat lamp until rats fully recovered from the anesthesia. Variability of results was minimized by excluding rats that failed to show complete loss of the righting reflex and bilateral pupil dilation during ischemia. Rats that stopped breathing during ischemia were also excluded. All procedures were approved by the Animal Experiment Committee of the Osaka Prefecture University. All surgeries and subsequent experiments including histology, immunohistochemistry, western blot, and RT-PCR were performed by operators blinded to the treatment group.

2.3. Intracerebroventricular injection

One microgram of recombinant human activin A (Peprotech, Rocky Hill, NJ) was dissolved in 5 μl of vehicle (0.1% bovine serum albumin diluted in 0.01 M PBS (pH 7.2)). Rats were anesthetized with 1.5% halothane in 30% oxygen and 70% nitrous oxide, and placed into a stereotactic apparatus (Muromachi, Tokyo, Japan). Recombinant human activin A was delivered into the bilateral cerebral ventricle using a microinjector (Muromachi, Tokyo, Japan) (0.5 mm posterior to bregma, 1.5 mm lateral to the midline, 5 mm ventral from the cranium). A total volume of 5 μl was administered at a rate of 1 μl/min. An equal volume of vehicle infusion served as the vehicle control.

2.4. Histological assessment

Animals were deeply anesthetized with chloral hydrate and perfused transcardially with physiological saline containing 10 U/ml heparin sulfate, followed by 4% paraformaldehyde-0.1 M phosphate buffer (PB) (pH 7.4). Brains were quickly removed from the skull and post-fixed for 20 h at 4 °C, then dehydrated and embedded in paraffin. Coronal sections (3.5 μm thick) containing areas of the hippocampus were cut between −3.3 mm and −3.8 mm from bregma and stained with hematoxylin/eosin. The number of neurons considered to have survived ischemia in the bilateral CA1 regions was counted. Counting of neurons was performed as described previously [5]. Briefly, neurons were considered to have survived if they had an intact round or oval nucleus. Four sections from each animal were analyzed, with the sections separated from one another by at least 20 μm.

2.5. RT-PCR

Brains were removed, rinsed in ice-cold physiological saline, and sliced at 2-mm intervals using Brain Matrices (BrainScience-Idea, Osaka, Japan). The brain sections were placed onto chilled plates, and the hippocampus was removed from the slices under a dissection microscope for mRNA expression analysis of Smad1, -2, -3, -5, and -8. For the analysis of inhibin βA, -βB, TGF-β1, -β2, -β3, Smad6, and Smad7 mRNA expression, the sliced hippocampi were further divided into 2 regions; the CA1 region and CA3/DG regions. These tissues were then snap-frozen in liquid nitrogen, and stored at −80 °C until further processing. Total RNA was isolated from the frozen tissue samples using TRIZOL reagent (Invitrogen Japan K.K., Tokyo) according to the manufacturer's instructions. Reverse transcription of 1 μg of the total RNA was performed using M-MLV reverse transcriptase (Promega, Madison, WI) and an oligo (dT)18 primer at 42 °C for 50 min. cDNA was amplified using HybriPol™ DNA Polymerase (Bioline Ltd. London, UK), with the annealing temperatures, and PCR cycles dependent on the target genes. Primers, annealing temperatures and PCR cycles used are listed in Table 2. The identities of the RT-PCR products were confirmed by direct sequencing of PCR products using an automated ABI 3130 Genetic Analyzer (Life Technologies, Tokyo, Japan).