Kainic acid induces expression of caveolin-1 in activated microglia in rat brain

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Abstract: Caveolin-1, a major constituent of caveolae, has been implicated in endocytosis, signal transduction and cholesterol transport in a wide variety of cells. In the present study, the expression of caveolin-1 was examined by immunohistochemistry in rat brain with or without systemic injection of kainic acid (KA). Caveolin-1 immunoreactivity was observed in capillary walls in brains of control rats. From one to seven days after KA injection, caveolin-1 immunoreactivity appeared in activated microglia in the cerebral cortex, hippocampus and other brain regions. The strongest immunoreactivity of microglia was seen after 3 days after KA administration. The expression of caveolin-1 was confirmed by RT-PCR and Western blot analysis, respectively. The induction of caveolin-1 expression in microglia activated in response to kainic acid administration suggests its possible role in a modulation of inflammation. (Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 1, 25–30)

Key words: caveolin 1 expression, brain, microglia, kainic acid, seizures, rat, immunohistochemistry

Introduction

Caveolin-1 is the major protein component of caveolae that are cholesterol- and sphingolipid-enriched microdomains of the plasma membrane [1]. Because caveolae are believed to be involved in two-way communication and exchange of molecules between the cell and extracellular environment, caveolin-1 has been implicated in multiple cellular functions including endocytosis, signal transduction and cholesterol transport [2]. In addition to caveolae, caveolin-1 was shown to be present in non-caveolar scaffold domains on the plasma membrane [3] and at many other intracellular locations [2]. Probably caveolin-1 plays important multiple biological roles in a heterogeneous cellular microenvironments.

In the brain, caveolin-1 has been reportedly found in endothelial cells and astrocytes [4]. The expression of caveolin-1 was also demonstrated in many types of cells in culture, including astrocytes [5], differentiating PC12 cells, dorsal root ganglion neurons [6], and oligodendrocytes [7]. Furthermore, injuries to the spinal cord caused an increase in phosphorylation of caveolin-1 in macrophages and microglia [8, 9]. These results suggest that virtually all types of cells in the nervous system are potentially capable of producing caveolin-1.

In rodents, injection of kainic acid (KA) results in recurrent seizures, behavioral changes, and subsequent degeneration of selective populations of neurons in the brain, showing characteristics similar to human temporal lobe epilepsy [10]. The hippocampus is particularly vulnerable to KA-induced neurotoxicity, where the pathological changes are characterized by pyramidal cell loss associated with glial activation, mossy fiber synaptic reorganization, and increased neurogenesis of dentate granule cells [10–12]. Thus, KA administration in rodents has widely been used as a model to elucidate the mechanisms of neurotoxicity of excitatory neurotransmitters. Bu et al. [13]
demonstrated that KA and other glutamate receptor agonists induce expression of caveolin-1 in a concentration-dependent manner in cultured primary hippocampal neurons of rats. This observation strongly suggests a role of caveolin-1 in KA-induced neuronal damage. To clarify the involvement of caveolin-1 in KA-induced excitotoxicity, we examined in the present study expression of caveolin-1 and its mRNA in rat brain after KA administration, by RT-PCR, Western blot analysis and immunohistochemistry.

**Material and methods**

**Animals.** Male Wistar rats (Clea Japan Inc., Tokyo, Japan), weighing 250–300 g, were used. The rats were handled in compliance with the principles of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1986). All efforts were made to minimize both the number of animals used and any suffering that they might experience. The rats received a single intraperitoneal (i.p.) injection of KA (Sigma, St. Louis, MO, USA; 12 mg/kg dissolved in physiological saline). Only the rats which showed generalized seizures (tonic-clonic convulsions) were used in this study.

**Immunohistochemistry.** Caveolin-1 immunohistochemistry was performed using the rats with survival periods of one, three and seven days after KA injection (n = 3 in each group). The rats injected with physiological saline served as the control group (1, 3, and 7 days post-injection; n = 2 in each group). Each animal was perfused through the ascending aorta with 10 mM phosphate-buffered saline (PBS, pH 7.4), followed by a fixative of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed from the skull and postfixed overnight in a fixative of 4% paraformaldehyde in 0.1 M PB at 4°C. After cryoprotection of the first strand cDNA using 500 pmol of oligo-dT12-18 (Amersham Biosciences Corp., Arlington Heights, IL) and 200 units of SuperScript II (Gibco BRL, Gaithersburg, MD). Single optical slice images were taken using 40 × LD Achromplan air interface objective lens at a z-axis step of 0.6 µm depth.

**RT-PCR.** After perfusion with 10 mM PBS, brains were removed from a saline-injected control rat and a KA-injected rat 3 days post-injection. Total RNA was extracted from fresh tissues of right cerebral cortex and hippocampus using the acid guanidium thiocyanate-phenol method. Five µg of each total RNA was reversely transcribed for the synthesis of the first strand cDNA using 500 pmol of oligo-dT12-18 and 200 units of SuperScript II. For a control, PCR for β-actin gene was done in a separate tube. The sequences of the primers were: 5'-CTCAAGCCCAACCAAGGGC-3' (5'-sense primer), and 5'-AGGAAGCTTCTTGATGCAGCT-3' (3'-antisense primer) (GenBank no. AF439778). For the control, PCR for β-actin gene was done in a separate tube. The sequences of the primers for rat β-actin were: 5'-TG-CTGGGTATGGGCTAGAGTGT-3' (5'-sense primer), and 5'-CATGGGCTGGGTGGTTCAGCTCA-3' (3'-antisense primer). The expected sizes of the PCR products were 342 base pair (bp) for caveolin-1 and 265 bp for β-actin, respectively.

Each 25 µl reaction mixture consisted of 2 ng/µl of the template cDNA, 0.8 µM of each of the primers, 0.2 mM of...
each of 4 deoxynucleotide triphosphates and 0.25 units of AmpliTaq Gold polymerase (Perkin Elmer) in 1xPCR buffer (Perkin Elmer). After preincubation for 10 min at 95°C, the amplification program was performed for 34 cycles with denaturation for 0.5 min at 95°C, annealing for 0.5 min at 64°C, and extension for 1.5 min at 72°C. The PCR products were electrophoresed on a 3% agarose gel and stained with ethidium bromide. After dissecting out the expected bands from the gel, the target PCR product was eluted, and DNA was cloned using a TA cloning system (Invitrogen Corp., Carlsbad, CA), followed by sequencing using the ABI-PRISM cycle sequencing kit and 310 DNA sequencer (Perkin Elmer).

Western blot analysis. After perfusion with 10 mM PBS, tissues of right cerebral cortex and hippocampus were obtained from both a saline-injected control rat and a rat 3 days after KA injection. They were homogenized in 10 volumes of ice-cold 20 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, and 60 mM octyl-glucoside, under the presence of protease inhibitor cocktail tablets, Complete Mini (Roche Diagnostics, Mannheim, Germany; one tablet/10 mL). The homogenates were centrifuged at 14000 g for 20 min at 4°C. About 25 µg of the crude extracted protein and Prestained Precision Protein Standards (Bio-Rad, Hercules, CA, USA) were electrophoresed on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel under a reducing condition, and then transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Tokyo, Japan). The membrane was incubated overnight with the rabbit antibody against caveolin-1 (N20; Santa Cruz Biotechnology; diluted 1:2 000) in 25 mM TBS containing 0.1% Tween 20 (TBST) at 4°C, and then for 2 h with a peroxidase-coupled anti-rabbit IgG Fab’ fragment (Histofine; Nichirei Corporation, Tokyo, Japan; diluted 1:100). The peroxidase labeling was detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Results
In saline-injected control rats, capillary walls were positively stained for caveolin-1 in many brain regions including the cerebral cortex (Figure 1A) and hippocampus (Figure 1B). One day after KA administration, caveolin-1-positive cells appeared in the brain including the cerebral cortex and hippocampus. Three days after KA injection, the number of positive cells increased in the cerebral cortex (Figure 1C). Many
immunoreactive (ir) positive cells had small, round to ovoid cell bodies with several processes, while some positive cells showed pyramidal profiles (Figure 1C). In the hippocampus, particularly in the CA3 area, a dense accumulation of ir-positive cells was observed (Figure 1D). Similar aggregations of immunoreactive cells were also detected in the thalamus and entorhinal cortex.

To better characterize ir-positive cells, brain sections of the rats 3 days after KA injection were doubly stained for caveolin-1 and one of the selected three markers of various brain cell populations. As shown in Figure 2, caveolin-1 immunoreactivity was observed in virtually all CD11b-positive (OX42-labelled) microglia and only a few GFAP-positive astrocytes, but not in NeuN-positive neurons. Thus, the major population of caveolin-1-positive cells was found to consist of activated microglia.

Seven days after KA-injection, the number and staining intensity of positive microglia decreased in the cerebral cortex (Figure 1E). Only a few positive cells showed morphology of activated microglia. Positive microglial aggregates were found in the regions vulnerable to the toxic effects of KA, such as the CA3 area of the hippocampus (Figure 1F), some thalamic nuclei, amygdala and entorhinal cortex.

Figure 3A shows expression of caveolin-1 mRNA analyzed by RT-PCR. As shown, the amplification

Figure 2. Confocal fluorescent images of the cerebral cortex doubly stained for caveolin-1 and one of the cell marker antigens. (A–C) Caveolin-1-positive cells (green; A), NeuN-positive neurons (red; B), and a composite image of A and B (C), showing no overlap of these two types of cell. (D–F) Caveolin-1-positive cells (green; D), GFAP-positive astrocytes (red; E), and a composite image of D and E (F), showing only a partial overlap of these two types of cell. (G–I) Caveolin-1-positive cells (green; G), OX42-labelled microglia (red; H), and a composite image of G and H (I), showing an overlap of caveolin-1-positive cells and OX42-labelled microglia (yellow). Bar = 50 mm
product for the caveolin-1 gene was detected with the expected size (342 bp) in the cerebral cortex and hippocampus of both saline-injected and KA-treated rats. The nucleotide sequence analysis confirmed the identity of the PCR product with the caveolin-1 gene fragment. No mutation was found within the nucleotide sequence of analyzed fragments. In Western blot analysis, the caveolin-1 antibody revealed a positive band of about 22 kDa in the cerebral cortex and hippocampus of both saline-injected and KA-treated rats (Figure 3B).

Discussion

In the present study, two major types of cells are recognized to express caveolin-1 in rat brain. First, caveolin-1 immunoreactivity was present in capillary endothelial cells in vehicle-injected control rats. Second, caveolin-1-positive microglia were widely distributed in the brain areas including the cerebral cortex and hippocampus from 1 day to 3 days after injection. Seven days post-injection, positive microglia were confined to such vulnerable areas as the hippocampus, thalamus and entorhinal cortex. The present study demonstrates for the first time that the expression of caveolin-1 is induced in activated microglia in rat brain after systemic treatment with KA. The expression of caveolin-1 and its mRNA in the brain of KA-treated rats were confirmed by Western blot analysis and RT-PCR, respectively, though the changes in their expression levels should be determined by future quantitative analyses.

Microglia and macrophages are the cells of the mononuclear phagocyte lineage originating from monocytes. Although the expression of caveolin-1 in immune cells has long been debated [14], a body of evidence indicates that macrophages actually express caveolin-1 in pathological conditions [15]. In addition, it is reported that the expression of caveolin-1 gene is up-regulated in monocytes after activation by adhesion to endothelial cells [16]. The present study provides additional evidence indicating that caveolin-1 plays roles in tissue injuries through cells of the mononuclear phagocytic system. Because CD11b antigen is expressed by two groups of morphologically indistinguishable phagocytic cells, resident brain microglia and infiltrating peripheral macrophages [17], the exact origin of the caveolin-1-positive brain cells remains to be determined. Although a previous in vitro study has shown that cultured primary hippocampal neurons from rats express caveolin-1 after excitotoxic treatment [13], the present study failed to show any caveolin-1-positive neurons in KA-treated rat brains.

The precise functional role of caveolin-1 in activated microglia is unclear. It is probable that caveolin-1 is involved in lipid metabolism in microglia [14]. More importantly, it is suggested that caveolin-1 is a potent modulator of inflammation. Bucci et al. [18] showed a role of caveolin-1 in suppression of inflammation, by using the scaffolding domain of caveolin-1 to inhibit edema formation and vascular leakage in rats. Such anti-inflammatory effects of caveolin-1 have partly been ascribed more directly to the action of caveolin-1 in macrophages. For example, caveolin-1 deficient macrophages display impaired phagocytosis [19]. Wang et al. [20] demonstrated that caveolin-1 acts as a potent immunomodulatory effecter molecule in murine alveolar and peritoneal macrophages. They showed that caveolin-1 has a protective role for inflammation by suppression of pro-inflammatory cytokine production (TNF-α and IL-6) and augmentation of anti-inflammatory cytokine production (IL-10) [20]. Thus, it is tempting to speculate that caveolin-1 in activated microglia participates in modulation of inflammation in the brain in response to excitotoxic insults.

In summary, we have demonstrated for the first time that KA administration induces the expression of caveolin-1 in activated microglia in rat brain. The results suggest an immunomodulatory role of caveolin-1 in KA-induced brain damage. Because activat-
ed microglia are known to act both neurotoxic and neuroprotective effects [21], it is unclear whether the action of microglial caveolin-1 is toxic or protective to neurons. Another question is whether caveolin-1 induced in activated microglia by KA is phosphorylated or not, because an increase in phosphorylation of caveolin-1 has been reported in microglia in rat spinal cord in some experimental conditions [8, 9, 22]. In addition, the apparent alterations in the expression level of caveolin-1 should be clarified by further quantitative analysis. Future studies are needed to address these issues.

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References


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