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Subarachnoid Hemorrhage Induces Na^+/*myo*-Inositol Cotransporter in the Rat Brain

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Abstract

Neurons and glial cells respond to extracellular hyperosmolarity by accumulating small organic solutes, called ''osmolytes.'' Na^+/*myo*-inositol is one of the major organic osmolytes in the brain and Na^+/*myo*-inositol cotransporter (SMIT) regulates extracellular Na^+/*myo*-inositol content. Subarachnoid hemorrhage (SAH) is an osmotic stress-inducing event of the brain. The expression of SMIT messenger ribonucleic acid (mRNA) and protein was investigated with in situ hybridization and immunohistochemistry in rat brains with SAH induced by endovascular perforation. SMIT riboprobe was raised from a 490-bp rat SMIT complementary deoxyribonucleic acid. Anti-SMIT antibody was raised in rabbits. SMIT mRNA was expressed strongly in the cortex, hippocampus, and hypothalamus of the perforated side at 6 to 24 hours after SAH. Mild upregulation was noted in the contralateral cortex, hippocampus, and hypothalamus. The ventral aspect of the pons showed mild upregulation. Microautoradiography and immunostaining showed SMIT expression mainly in the neurons, but also in some non-neural cells in the hippocampus. The present results indicate that diffuse osmotic stress occurs in the host brain after SAH.

Key words: osmolytes, *myo*-inositol, osmolyte transporter, subarachnoid hemorrhage, Na^+/*myo*-inositol cotransporter

Introduction

Neuron and glial cells respond to the extracellular hyperosmolarity in many types of brain injury by accumulating small organic solutes, called ''osmolytes,'' that protect the cells from the perturbing effects of high intracellular concentrations of electrolytes. Na^+/*myo*-inositol is one of the major organic osmolytes in the brain. The intracellular uptake carrier of Na^+/*myo*-inositol is Na^+/*myo*-inositol cotransporter (SMIT),[6] which is expressed intensely and constitutively in the choroid plexus, pineal gland, and area postrema where the blood-brain barrier is absent. SMIT is also expressed in the hippocampus, locus ceruleus, suprachiasmatic nucleus, olfactory bulb, and Purkinje cells.[4] SMIT messenger ribonucleic acid (mRNA) expression is upregulated in focal cerebral ischemia,[10] hyperglycemia,[11] kainate-induced seizure,[7] and diffuse brain injury.[8]

Subarachnoid hemorrhage (SAH) is one such osmotic stress-inducing events of the brain. The subarachnoid space is packed with blood-borne materials that might induce osmotic stress to the neurons and glial cells of the adjacent brain tissue. Furthermore, ischemic stress is induced in the acute stage of SAH due to the transient increase in intracranial pressure and early spasm.[2,9] The ischemic stress might upregulate SMIT expression as found in focal ischemia.[10]

This study investigated the expression of SMIT mRNA and protein in the rat model of SAH.

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Materials and Methods

I. Animal preparation

Male Wistar rats (n = 28) weighing 300–350 g were used in this study. Anesthesia was induced with 3% halothane and maintained with 1–1.5% halothane during the operative procedure.

II. SAH model

The experimental SAH model was originally described by Veelken et al.[9] and modified in our laboratory. Details of the procedures are described elsewhere.[2,5] After induction of SAH, the animals

were returned to the cages for recovery from anesthesia and allowed free access to food and water. The animals were re-anesthetized deeply with nembutal and sacrificed at 1 hour, 6 hours, 12 hours, 24 hours, or 48 hours after SAH (n = 5 for each time point). Sham-operated rats (n = 3) were sacrificed one day after the procedure. The brains were removed immediately and fixed in 4% paraformaldehyde for 15 hours at 4°C. The fixed tissues were embedded in paraffin wax and serial 6-μm thick coronal sections were cut and mounted on glass slides.

III. In situ hybridization

In situ hybridization for SMIT mRNA was performed as described previously.[7,8,10,11] The antisense and sense (control) RNA probes for SMIT were synthesized from a 490-bp rat SMIT complementary deoxyribonucleic acid insert cloned into the vector pSPORT 1. In vitro riboprobe synthesis was carried out using the Promega kit (Promega, Madison, Wis., U.S.A.), and the probes were labeled with ^{35}S-uridine triphosphate. Efficacy of labeling was estimated by counting the radioactivity of the synthesized probe.

Hydrated sections were treated with 10 mg/ml of proteinase K in 50 mM Tris-HCl and 5 mM ethylenediaminetetra-acetic acid for 10 minutes at room temperature and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB). After washing in PB, the sections were acetylated, dehydrated, and air-dried. The ^{35}S-labeled RNA probes diluted in hybridization buffer were denatured and hybridized on 6-μm thick coronal sections for 12–17 hours at 55°C. The slides were then subjected to high-stringency washing, treated with ribonuclease A, counterstained with thionine, and dried. The slides were laid on contact x-ray film. After 5 days of exposure, the films were developed and the images were checked. The sections were then covered with photographic emulsion (Kodak NTB-2; Eastman-Kodak, Rochester, N.Y., U.S.A.), exposed for 28 days, developed in Kodak D-19 (Eastman-Kodak), and fixed with photographic fixer.

IV. Immunohistochemistry

Anti-SMIT antibody was raised in rabbits by injecting a synthetic peptide, CTPPPTKEQ, which corresponds to the amino acids 533–540 of SMIT. Purity of the antibody was confirmed by affinity column chromatography and Western blot analysis.[1] The antibody was used for immunohistochemistry. Dewaxed and hydrated tissue sections were immersed in 3% hydrogen peroxide in methanol for 30 minutes to suppress the activity of endogenous peroxidase, rinsed in 0.01 M phosphate-buffered saline (PBS) (pH 7.4), treated with 0.5% bovine serum albumin (BSA)-PBS for 15 minutes, and reacted with the primary antibody diluted 1:500 with BSA-PBS for 1 hour at room temperature. The slides were washed with PBS, then incubated with biotinylated anti-rabbit immunoglobulin G (DAKO, Carpinteria, Calif., U.S.A.) for 30 minutes at room temperature, followed by streptavidine-peroxidase (DAKO) for 30 minutes at room temperature before diaminobenzidine substrate color development. Sections were counterstained with hematoxylin. Normal rabbit serum was used as primary antibody to confirm the specificity of the antibody.

Results

I. Extent of SAH

Most rats subjected to endovascular perforation developed massive SAH at the base of brain adjacent to the area of arterial perforation. SAH was also demonstrated on the dorsal aspect of the cerebral hemisphere. None of the sham-operated animals exhibited hemorrhage in the subarachnoid space.

II. Expression of SMIT mRNA after SAH

The antisense probe for SMIT mRNA showed constitutive expression in the hippocampus, choroid plexus, and cerebral and cerebellar cortices of the sham-operated rat (Figs. 1A and 2A). The sense probe for SMIT showed no hybridization signal (data not shown) suggesting the high sensitivity of the antisense probe.

In animals with SAH, expression of SMIT mRNA started to increase at 6 hours, peaked at 12 hours after surgery, and lasted till 24 hours after SAH, then subsided at 48 hours after SAH, and returned to the level seen in the sham-operated animals (Fig. 1B–F). Expression was observed in the cortex, hippocampus, and hypothalamus of the perforated side. There was mild upregulation of the SMIT mRNA expression in the contralateral hippocampus, hypothalamus, and temporal cortex. The distribution of SMIT mRNA expression at 12 hours showed diffuse cortical SMIT mRNA upregulation on the side of the perforation (Fig. 2B). Moderate expression was observed in the periaqueduct area, pyramidal tract of the midbrain, and pontine nucleus. Microautoradiography of the hippocampal CA1 at 12 hours showed dense signals in some neurons and non-neural cells (Fig. 3).

III. Expression of SMIT protein

Expression of SMIT protein showed dense staining in the some of the hippocampal CA1 neurons at 12 hours after SAH (Fig. 4A). Some of the cortical

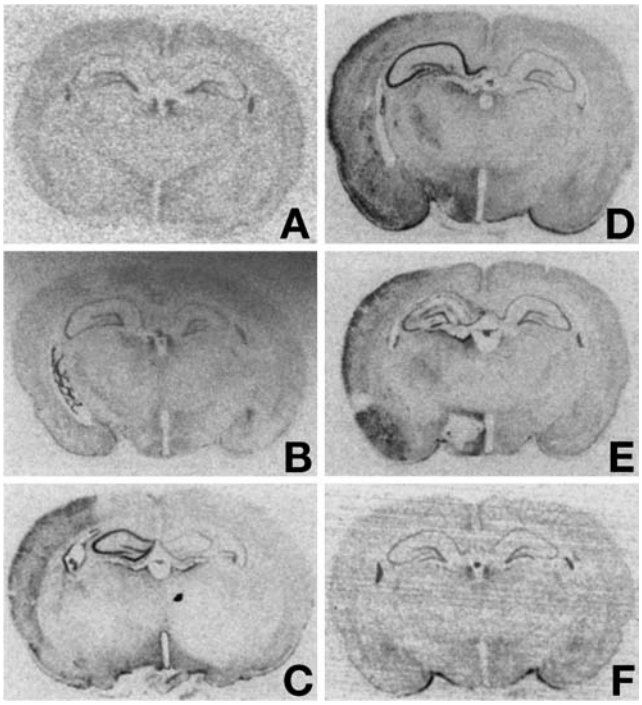

Fig. 1 **Autoradiographs of in situ hybridization for Na^+/*myo*-inositol cotransporter (SMIT) messenger ribonucleic acid (mRNA) in rats with Subarachnoid hemorrhage (SAH). Riboprobe for SMIT was applied to tissue obtained at 1 (B), 6 (C), 12 (D), 24 (E), and 48 hours (F) after SAH. Tissue from sham-operated rats (A) was processed as well. Upregulation of SMIT mRNA expression was noted at 6, 12, and 24 hours after SAH.**

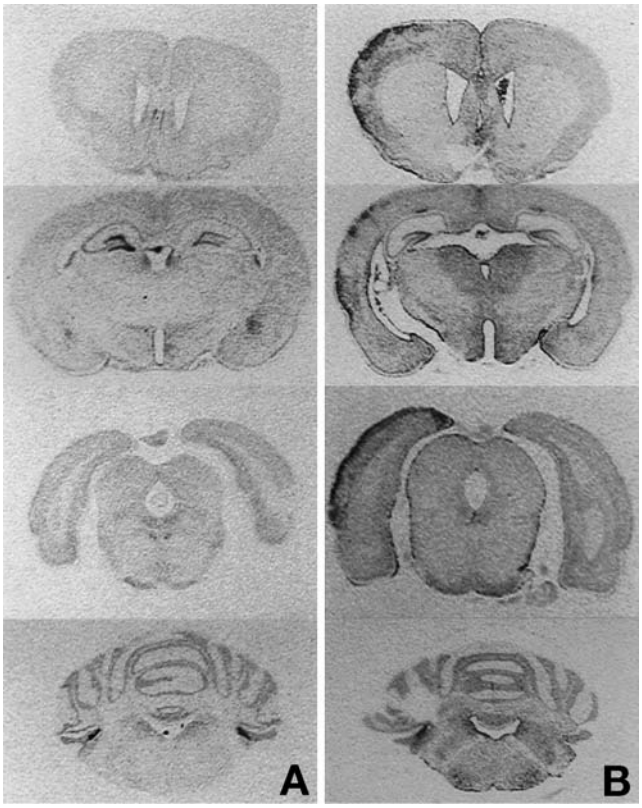

Fig. 2 **Autoradiographs showing the distribution of Na^+/*myo*-inositol cotransporter (SMIT) messenger ribonucleic acid expression in rats with sham operation (A) and subarachnoid hemorrhage (SAH) at 12 hours after perforation (B). Constitutive SMIT expression was noted in the hippocampus, choroid plexus, and cerebral and cerebellar cortices (A). SAH induced SMIT expression in the cortex, hypothalamus, and ventral aspect of pons (B).**

neurons were stained as well (Fig. 4B). Neurons in the amygdala facing to the basal subarachnoid cistern were also stained (Fig. 4C).

Discussion

The present study clearly demonstrated upregulation of SMIT mRNA and protein in the rat SAH model. The mechanism of SMIT overexpression in SAH may vary from site to site. The hypothalamus, amygdala, and temporal cortex may reflect the direct effect of SAH, because these areas face the subarachnoid space packed with blood-borne materials. SMIT mRNA expression in those area is highly upregulated at 6 to 24 hours after SAH. The time course agrees with local accumulation of SAH clot and hemolysates, which may diffuse into adjacent brain tissue and cause local hyperosmolarity.

SMIT is also upregulated by transient ischemia of the brain. The rat model of SAH causes ischemia, mainly in the perforation-side hemisphere and partly in the contralateral hemisphere.[9] The ischemia is rather mild and does not result in focal necrosis, but does induce expression of 70-kDa heat-shock protein (hsp70) and immediate early genes.[2,5] This focal ischemia may also cause focal osmolarity change. Increases in focal osmolarity were measured by vapor pressure osmometer 3 to 6 hours after rat middle cerebral artery (MCA) occlusion.[3] The present study found that SMIT expression started to upregulate at 6 hours. A different pattern of SMIT expression was found in the rat MCA-clip occlusion model,[10] compared to the SAH model.

Intense SMIT expression in the hippocampus of the SAH model is mainly due to focal ischemia, because hsp70, an indicator of ischemic stress, is also expressed in the pyramidal layer of hippocam-

pus.[2,5] SMIT protein was also detected in the pyramidal CA1 neurons (Fig. 4A) suggesting that ischemic stress might cause local osmolarity change and induce SMIT mRNA and protein.

Expression of SMIT in cortical layer 2 (Figs. 1C–E and 2B) is rather specific for the direct effect of SAH, because the MCA clip-occlusion model showed strong expression of SMIT in layer 5 but not in layer 2. SMIT protein was also expressed in the layer 2 neurons in the SAH model (Fig. 4B). Macroscopic observation of the SAH model showed rather diffuse spread of SAH over the cortical surface,[2] where SMIT was expressed. Expression in the hypothalamus, amygdala, and temporal cortex is the result of dense SAH, because those areas face the basal subarachnoid space. Immunohistochemistry for SMIT showed neuronal expression in the amygdala (Fig. 4C).

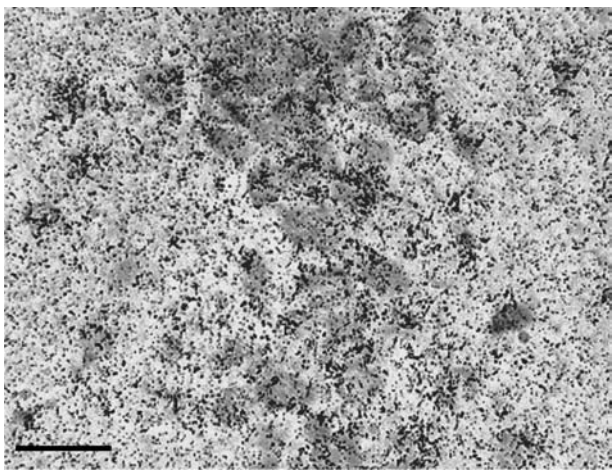

Fig. 3 Microautoradiograph of Na^+/*myo*-inositol cotransporter messenger ribonucleic acid expression in the hippocampal CA1 at 12 hours after subarachnoid hemorrhage. Hybridization signals appear on the large neurons, but some signals are also seen on the small non-neural cells. Bar = 50 μm.

SMIT mRNA expression in the ventral aspect of the pons may be due to basal SAH, but increased intracranial pressure and diffuse ischemia may also be the cause (Fig. 2B). Our previous study with the diffuse brain injury model found SMIT mRNA is upregulated in the glia of this area, which increased signal transduction of p44/42 mitogen-activated protein kinase.[8] SMIT expression in the diffuse brain injury model persisted for 6 to 24 hours after injury, which accords with the present SAH model. We did not perform microautoradiography in this area, but SMIT expression in the ventral aspect of pons might be caused by activated glia. Non-neural expression of SMIT also occurs in the hippocampus and cortex of the hyperglycemic animals.[11] Our SMIT mRNA microautoradiography showed accumulation of sig-

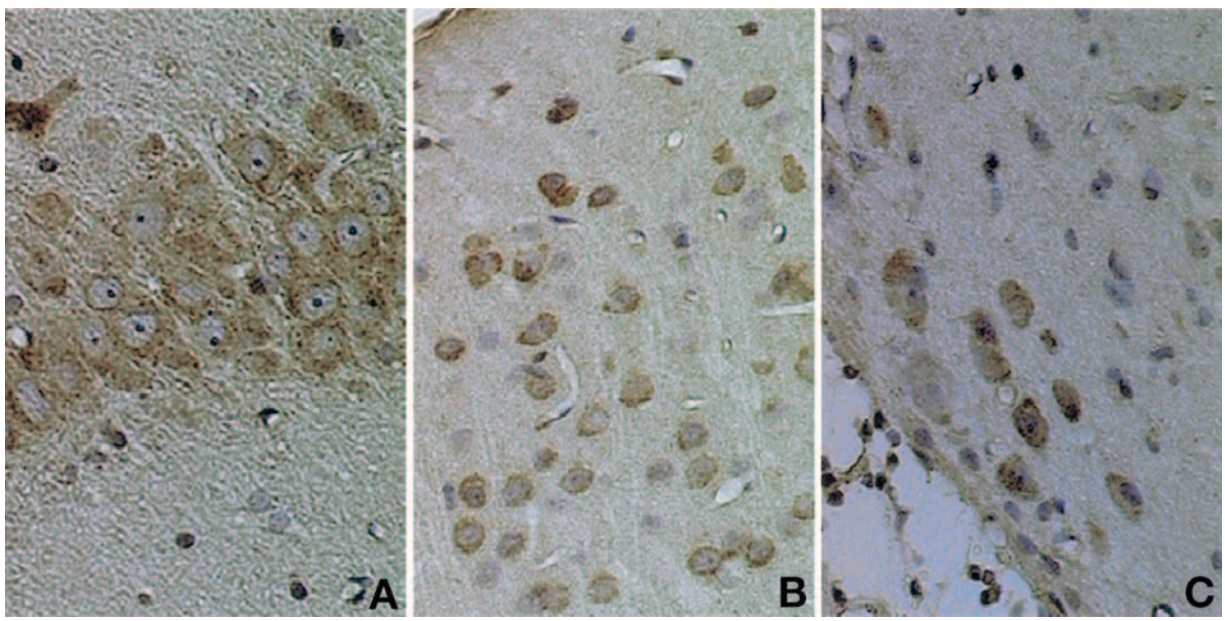

Fig. 4 Photomicrographs showing immunohistochemistry for Na^+/*myo*-inositol cotransporter (SMIT) protein in hippocampal CA1 of the perforation side (A), parietal cortex of the perforation side (B), and amygdala of the perforation side (C). Neurons in these areas were mainly stained for anti-SMIT antibody. Counterstained with hematoxylin, ×200.

nals in some non-neural cells of the hippocampal CA1 area (Fig. 3), and immunocytochemistry also demonstrated non-neural cells positive for SMIT protein (Fig. 4A). Therefore, SMIT expression is not neuron-specific but rather an ubiquitous response of the brain cells.

The present findings clearly indicated that SAH caused diffuse osmotic stress to the host brain. Expression of SMIT mRNA and protein was observed in the host neurons and glial cells, and might reflect the stress-related response of the brain to SAH, because the response is similar to those found in cerebral ischemia,[10] hyperglycemia,[11] and diffuse brain injury.[8] Overexpression of SMIT might reverse local extracellular hyperosmolarity. Therefore, expression of SMIT is neuroprotective, but this response is transient and self-limiting. The observations that subarachnoid clot causes focal osmolarity change and SAH causes transient ischemia should be considered when treating patients with SAH.

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Commentary on this paper appears on the next page.

Commentary

The authors have used highly technical, elegant methods in a rat subarachnoid hemorrhage model to examine the question of intracellular osmolyte accumulation as a protective mechanism against extracellular hyperosmolarity after SAH. The specific osmolyte studied was sodium/myo-inositol, along with its co-transporter SMIT; upregulation of this pathway has been shown in several different forms of brain injury or insult. The study has shown similar effects after experimental SAH. Two possible mechanisms are apparent, the osmotic effect of the subarachnoid space being packed with blood and its products, affecting areas close to a surface; and a probable secondary effect due to raised intracranial pressure and ischemia, mostly in more remote, deeper parts of the brain. This protective mechanism against high intracellular osmolarity has not received much attention, especially after SAH. Could this be a further argument for early surgery followed by cisternal perfusion or drainage, to decrease subarachnoid osmolarity as well as remove spasmogenic blood products?

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In this article, the authors demonstrated the increase of a neuronal osmolyte, Na^+/myo-inositol, in the rat brain after subarachnoid hemorrhage (SAH). At 6 to 24 hours after endovascular perforation of the rat cerebral artery to induce SAH, up-regulation of Na^+/myo-inositol cotransporter (SMIT) messenger ribonucleic acid (mRNA) was found in the cortex, the hippocampus, and the hypothalamus of the bleeding hemisphere. Mild up-regulation of SMIT-mRNA was also noticed in the contralateral hemisphere and non-neural cells. Thus, the authors concluded that SAH causes diffuse osmotic stress to the brain. It is well documented that up-regulation of SMIT-mRNA occurs in focal cerebral ischemia. The observation in the present study probably only reflects the occurrence of cerebral ischemia as a consequence of vasospasm after SAH. Different degrees of up-regulation of SMIT-mRNA expression in the different sites of the brain should relate to various degrees of clot thickness and consequent vasospasm after SAH. For the further elucidation of cerebral osmotic stress after SAH, studies to correlate the degree of vasospasm after SAH and up-regulation of SMIT-mRNA expression should be conducted. It is also advisable to study the SMIT expression at different time intervals and up to a week after SAH when the degree of vasospasm is at its maximum.

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