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# Induction of suppressor of cytokine signaling-3 in astrocytes of the rat hippocampus following transient forebrain ischemia

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ABSTRACT

We investigated the spatiotemporal expression of suppressor of cytokine signaling-3 (SOCS-3) in the rat hippocampus following transient forebrain ischemia using *in situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Messenger RNA for SOCS-3 was constitutively expressed in neurons of the pyramidal cell and granule cell layers in control animals; however, significant induction was detected in reactive astrocytes preferentially located in the CA1 and the dentate hilar regions of the ischemic hippocampus. SOCS-3 mRNA was induced within 3 days of ischemia and maintained for more than 2 weeks. The *in situ* hybridization data agreed with the semiquantitative RT-PCR analysis. These results demonstrate SOCS-3 induction occurs in reactive astrocytes of the post-ischemic hippocampus, suggesting that SOCS-3 is involved in regulating the astroglial reaction to an ischemic insult.



The Janus kinase-signal transducer and activator of transcription (JAK-STAT) is critical in many biological responses to cytokines (for review, [2]). A number of studies have shown JAK-STAT signaling is involved in the activation of astrocytes in the lesioned brain, when following cerebral ischemia [4,8,23], excitotoxic brain injury [1,5], or axotomy lesions, for example [21].

Suppressors of cytokine signaling (SOCS) negatively regulate the JAK-STAT signaling pathway [6,22]. These proteins form a negative feedback loop by interfering with the receptor-mediated activation of signal transducers and activators of transcription (STATs). The SOCS family contains at least eight members, SOCS-1 to SOCS-7, and the cytokine-inducible Src homology 2-domain-containing protein. Several studies have shown SOCS-1 and SOCS-3 are negative regulators of JAK-STAT signaling in astrocytes *in vitro* [3,12], and that ischemic injury is associated with STAT3 activation in astrocytes [4,8,23], suggesting that alteration of SOCS-3 expression may occur in the ischemic brain. However, very little is known about the expression of SOCS-3 expression in the *in vivo* model of ischemic injury. Information regarding the level of expression of SOCS-3 in the ischemic brain, particularly the temporal pattern of expression and the specific cell types in which it is expressed during ischemic insults, should lead to a better understanding of the potential role of SOCS-3 in ischemic injury.

We have, therefore, examined the spatiotemporal expression of SOCS-3 in the rat hippocampus following transient forebrain ischemia using *in situ* hybridization and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Double labeling techniques were used to identify the phenotypes of cells expressing SOCS-3.

All experimental procedures performed on the animals were conducted with the approval of the Catholic Ethics Committee of the Catholic University of Korea, and in accordance with the US National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 80-23, revised 1996).

Adult male Sprague Dawley rats (250–300 g) were used in this study. Transient forebrain ischemia was induced by the four-vessel occlusion and reperfusion method described by Pulsinelli and Brierley [14], with minor modifications [9]. Briefly, the vertebral arteries were electrocauterized and cut to stop circulation in these vessels. After 24 h, both common carotid arteries were occluded for 10 min with miniature aneurismal clips. Only those animals showing completely flat electroencephalograms after vascular occlusion were classified as ischemic and used in the study. Body temperatures (measured rectally) were maintained at $37.5 \pm 0.3\,^{\circ}C$ with a heating lamp during and after ischemia. Sham-operated rats, with cauterized vertebral arteries and ligatures placed around the carotid arteries, were used as controls. No animal convulsed or died following reperfusion or sham operation.

Animals were allowed to live for 1, 3, 7, or 14 days after reperfusion. At each time point, three rats were sacrificed for use in RT-PCR analysis and seven were sacrificed for use in

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*in situ* hybridization histochemistry and immunohistochemistry. Sham-operated animals were treated using the same schedule as the ischemic/reperfused animals. At each time point following reperfusion, animals were deeply anesthetized with 16.9% urethane (10 ml/kg) and killed by transcardial perfusion with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), or by decapitation. This was followed by the removal of the hippocampi, which were quickly frozen in liquid nitrogen.

For semiquantitative RT-PCR, total RNA was extracted from microdissected pieces of the CA1 region of the hippocampus from each rat using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA samples were quantified by measuring optic absorbance at 260 nm with a spectrophotometer. First-strand cDNA was synthesized using Reverse Transcriptase M-MLV (Takara Korea Biomedical Inc., Korea) in accordance with the manufacturer's instructions. Equal amounts (1 μl) of the reverse transcription products were then PCR-amplified using Perfect Premix Version 2.1 (Ex Taq version; Takara Korea Biomedical Inc.). Amplification commenced with denaturation at 94 °C for 4 min followed by 25–30 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The final extension was made at 72 °C for 10 min. One picomole of each primer, using primers specific for SOCS-3 (sense, 5′-ACCAAGAACCTACGCATCCAGT-3′, nucleotides 285–306; antisense, 5′-CTCTGACCCTTTCTTTGCTCTTTA-3′, nucleotides 693–716) based on the rat SOCS-3 sequence (GenBank accession no. AF075383) was used in the amplification reaction. Ten microliters of each PCR reaction product were electrophoresed on 1.5% (w/v) agarose gels containing ethidium bromide (1 μg/μl). For semiquantitative measurements, we amplified the SOCS-3 mRNAs with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and optimized the number of PCR cycles to maintain amplification within a linear range. RT-PCR products were quantified by photographic densitometry of the ethidium bromide-stained agarose gel, and SOCS-3/GAPDH product ratios were calculated as indices of SOCS-3 mRNA expression. Three animals were used for PCR at each time point and three measurements were made per animal.

Specific sequences for SOCS-3 were prepared using RT-PCR, and antisense and sense riboprobes were labeled with digoxigenin (DIG) by *in vitro* transcription using a DIG RNA Labeling Kit (Roche, Germany). Coronal cryostat sections (25 μm thick) were hybridized with antisense or sense probes diluted in hybridization solution (150 ng/ml) at 52 °C for 18 h. Hybridization was visualized using an alkaline phosphatase-conjugated sheep anti-DIG antibody (Roche; dilution 1:2000) with 4-nitroblue tetrazolium chloride (0.35 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.18 mg/ml) as substrates. Tissue sections were visualized using a microscope and photographed using a digital camera (Jenoptik, Germany). Images were converted to TIFF format and contrast levels adjusted using Adobe Photoshop v. 7.0 (Adobe Systems, San Jose, CA, USA).

Some hybridized sections were double or triple labeled for glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor molecule 1 (Iba1), or neuronal nuclear antigen (NeuN) immunohistochemistry; these are established markers for astrocytes, microglia, and neurons, respectively. After hybridization, as described above, the sections were incubated for 2 h with biotin-conjugated mouse monoclonal anti-DIG antibody (Jackson ImmunoResearch, West Grove, PA, USA; dilution 1:200) at room temperature. For double- or triple-immunofluorescence histochemistry, sections were incubated overnight at 4 °C with following antibodies; monoclonal mouse anti-GFAP (Chemicon International Inc., Temecula, CA, USA; dilution 1:500), polyclonal rabbit anti-GFAP (Chemicon International Inc.; dilution 1:1500), monoclonal mouse anti-NeuN (Chemicon International Inc.; dilution 1:500) and polyclonal rabbit anti-Iba1 (Wako Pure Chemical Industries, Ltd., Osaka, Japan; dilution 1:500). Antibody staining was visualized with the following secondary antibodies; Cy3-conjugated streptavidin (Jackson ImmunoResearch; dilution 1:1500), FITC-conjugated anti-mouse antibody (Jackson ImmunoResearch; dilution 1:50), FITC-conjugated anti-rabbit antibody (Jackson ImmunoResearch; dilution 1:50) and Cy5-conjugated anti-mouse antibody (Jackson ImmunoResearch; dilution 1:500). Slides were viewed with a confocal microscope (LSM 510 Meta; Carl Zeiss Co., Ltd., Germany).

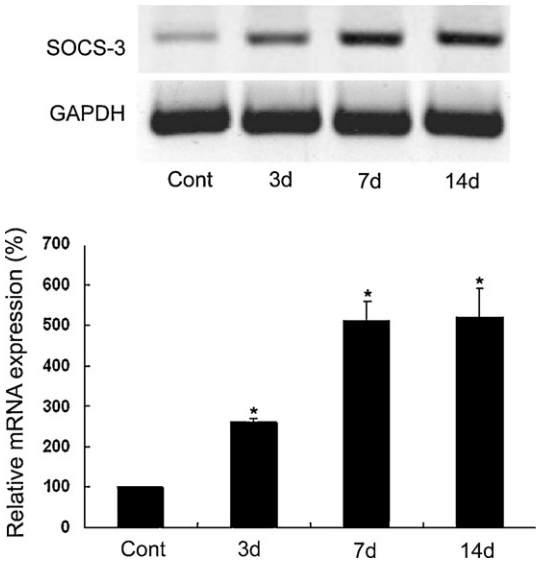


**Fig. 1.** RT-PCR analysis of the gene for SOCS-3 in the microdissected CA1 region of the hippocampus of control (Cont) and experimental rats 3, 7, and 14 days after transient forebrain ischemia. As an internal standard, GAPDH mRNA was measured. PCR products were run on an agarose gel and relative optical densities of the bands were obtained from three independent experiments, each performed in triplicate. The density of the SOCS-3 band was divided by the density of the band for GAPDH mRNA, representing the normalization factor. SOCS-3 signals increased 3 days after ischemia, reached a peak at 7 days, and remained elevated at 14 days. The data are expressed as the mean ± S.E.M. $^{*}P < 0.05$ compared with sham-operated controls.

The semiquantitative RT-PCR analysis showed that SOCS-3 expression was upregulated in the hippocampal CA1 region following transient forebrain ischemia. The level of SOCS-3 mRNA significantly increased 3 days after ischemia, reached a maximum 7 days after ischemia, and enhanced expression was maintained until at least day 14, which was the latest time point examined (Fig. 1).

The distribution and cellular localization of SOCS-3 mRNA in the hippocampus following a 10 min period of ischemia was examined using *in situ* hybridization histochemistry. Constitutive expression of SOCS-3 mRNA was localized to neurons of the pyramidal cell and granule cell layers in the hippocampi in control rats at all time points (Fig. 2A, E). The signals were also evident at a weaker intensity in some scattered cells located outside of the pyramidal cell layer and in the dentate hilar region. Adjacent sections in these animals were routinely processed for *in situ* hybridization with the SOCS-3 sense probe and no specific cellular labeling was observed (data not shown). Three days after the 10 min period of ischemia, the signals for SOCS-3 mRNA were observed in small cells predominately in the strata radiatum and oriens of the CA1 region, and the dentate hilar region (Fig. 2B, F). The distribution pattern and morphology of these cells suggested they might represent glial cells. At 7 (Fig. 2C, G) and 14 (Fig. 2D, H) days after reperfusion, labeling profiles were similar in number and intensity to those seen at 3 days after ischemia, with prominent enrichment in the strata radiatum and oriens of the CA1 region. Double or triple label-