Hypoxic Preconditioning Induces Neuroprotective Stanniocalcin-1 in Brain via IL-6 Signaling

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Background and Purpose—Exposure of animals for a few hours to moderate hypoxia confers relative protection against subsequent ischemic brain damage. This phenomenon, known as hypoxic preconditioning, depends on new RNA and protein synthesis, but its molecular mechanisms are poorly understood. Increased expression of IL-6 is evident, particularly in the lungs of animals subjected to hypoxic preconditioning. Stanniocalcin-1 (STC-1) is a 56-kDa homodimeric glycoprotein originally discovered in bony fish, where it regulates calcium/phosphate homeostasis and protects against toxic hypercalcemia. We originally reported expression of mammalian STC-1 in brain neurons and showed that STC-1 guards neurons against hypercalcemic and hypoxic damage.

Methods—We treated neural Paju cells with IL-6 and measured the induction of STC-1 mRNA. In addition, we quantified the effect of hypoxic preconditioning on Stc-1 mRNA levels in brains of wild-type and IL-6 deficient mice. Furthermore, we monitored the Stc-1 response in brains of wild-type and transgenic mice, overexpressing IL-6 in the astroglia, before and after induced brain injury.

Results—Hypoxic preconditioning induced an upregulated expression of Stc-1 in brains of wild-type but not of IL-6–deficient mice. Induced brain injury elicited a stronger STC-1 response in brains of transgenic mice, with targeted astroglial IL-6 expression, than in brains of wild-type mice. Moreover, IL-6 induced STC-1 expression via MAPK signaling in neural Paju cells.

Conclusion—These findings indicate that IL-6–mediated expression of STC-1 is one molecular mechanism of hypoxic preconditioning-induced tolerance to brain ischemia. (Stroke. 2007;38:000-000.)

Key Words: hypoxia ■ IL-6 ■ ischemia ■ neuroprotection

Stanniocalcin-1 (STC-1) is a 56-kDa homodimeric glycoprotein hormone that was originally identified in bony fish,1 where it regulates calcium/phosphate homeostasis and protects against toxic hypercalcemia. STC-1 was considered unique to fish until the cloning of cDNA for human STC-1 in 1995 and mouse Stc-1 in 1996. STC-1 is conserved through evolution with human and salmon STC-1, sharing 60% identity and 80% similarity. Mammalian STC-1 is expressed in various organs,2 particularly in tissues containing highly specialized cells with absent or limited proliferative capacity, including neural cells, mature adipocytes, and megakaryocytes.3 We originally reported high expression of STC-1 in terminally differentiated mammalian brain neurons with the Purkinje cells, the large neurons of basal ganglia, and the pyramidal neurons in the neocortex being particularly rich in STC-1. In addition, we showed that STC-1 confers neuroprotection in neural Paju cells because expression of STC-1 cDNA increased their resistance to hypoxic and hypercalcemic stress. Furthermore, an upregulated expression of STC-1 was evident in neurons in the penumbra area of recent infarcts in human and rat brain.5

IL-6 is a multifunctional cytokine with major roles in the immune, hematopoietic, and nervous systems.6,7 IL-6 modulates bone metabolism, cell proliferation, differentiation, and apoptosis.8 Both in vivo and in vitro studies indicate that IL-6 mediates neuroprotective activity.8 Treatment with IL-6 increases the survival of retinal ganglion cells in vitro9 and protects cerebellar granule cells10 and neuroblastoma cells in culture11 against glutamate-induced toxicity and oxidative damage. Moreover, injection of IL-6 reduces the volume of induced brain infarcts in rats and protects against N-methyl-D-aspartate-induced toxicity in cortical, striatal, and retinal neurons.12 Inhibition of IL-6 signaling by treatment with monoclonal antibodies, however, aggravates ischemic cerebral injury in mice.13
The low level of IL-6 normally present in the central nervous system increases rapidly in response to mechanical, ischemic, or excitotoxic injury. The increased amounts of IL-6 in the injured brain originate mainly from local production in neuroglial and endothelial cells. We previously reported that mice with transgenic IL-6 overexpression, under the control of the glial fibrillary acidic protein (GFAP) gene promoter (GFAP–IL-6 mice), display an elevated resistance to neuronal damage and apoptotic cell death after brain injury and pellagra neurotoxicity. However, excitotoxic stress and brain cryoinjury increases degeneration and apoptotic cell death in brains of IL-6–deficient mice relative to wild-type (WT) controls. Although the molecular pathways by which IL-6 mediates neuroprotection have received much scientific attention, the molecular mechanisms still remain incompletely understood.

Here we report that IL-6, via signaling through the MAPK pathway, induces elevated expression of STC-1 in neural cells. We also compare the Stc-1 responses in the brains of WT mice, GFAP–IL-6 transgenic mice overexpressing IL-6, targeted to astroglia, and IL-6–deficient mice (II-6−/−) after hypoxic preconditioning (HOPC) and induced brain damage. The results indicate that one of the mechanisms by which HOPC leads to neuroprotection is through IL-6–mediated upregulation of STC-1 expression.

Materials and Methods

Reagents

IL-6 came from R&D Systems. The signaling pathway inhibitors AG490, PD98059, and Wortmannin came from Calbiochem. Primers for quantitative real-time polymerase chain reaction were from Proligo LLC.

Cell Culture and RNA Extraction

We cultured Paju cells, a human neural crest-derived tumor cell line, surface adherent in supplemented RPMI-1640. For signaling pathway experiments, we preincubated cells for 30 minutes with the respective inhibitor at 50 μmol/L (AG490), 25 μmol/L (PD98059), or 200 nmol/L (Wortmannin) before activation with 2.5 ng/mL IL-6. Cells were collected and RNA isolated by use of TRIZOL Reagent (Invitrogen) and performed quantitative real-time polymerase chain reaction with the Roche LightCycler instrument and the LightCycler FastStart DNA Master SYBR Green I kit (Roche). Human STC-1 primers were: 5′-CAAGCAGCTGAATGTCG-3′ and 5′-CAGGCTTCGAGAAGTCTGT-3′. Mouse primers were: Stc-1, 5′-ATGCTCCAAAACCTCAGCAGTGATTC-3′ and 5′-CAAGGCTTGGACAAGTCTGT-3′; Il-6, 5′-CTTCCCTACTTCACGAAGTCC-3′ and 5′-GCCACTCCTTCGATGTAC-3′; and Epo, 5′-ACCCTGCTGCTTTTACTCTC-3′ and 5′-ATGAAGCTGAAGGTCTCTG-3′. STC-1, Il-6, and Epo mRNAs were normalized against levels of mouse β-2-microglobulin (β2m) or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were: β2m, 5′-GCTATTCCAGAATCCCTCTA-3′ and 5′-ATGTCTCGATCCCTAGAC-3′; and GAPDH, 5′-GGTGAAGGTCCGAGTCAAC-3′ and 5′-CAATGAGCCCCAGCTTT-3′.

Experimental Animals

All procedures involving experimental animals were performed according to institutional and local guidelines. All efforts were made to minimize animal distress and to reduce the number of animals used.

WT mice (n=44) were of strain C57BL/6J. The construction and characterization of the GFAP–IL-6 mice, a generous gift from Dr Iain L. Campbell, have been previously described. The GFAP–IL-6 mice (n=8) have a mixed C57B6/J×SJL genetic background. Age and gender littermate WT mice (n=8) served as controls. II-6−/− mice (B6;129S2-I6−/−) (n=17) were from the Jackson Laboratory, Bar Harbor, ME (Stock 002254).

Hypoxia Treatment and Induction of Brain Lesions

We subjected mice to hypoxia in a controlled-environment chamber perfused with 8% N2 oxygen in nitrogen. The mice were euthanized at indicated time points and collected brain tissue was immediately snap-frozen in liquid nitrogen for extraction of total RNA as described.

For induction of brain lesions, the cryolesion method was used. Mice were lesioned under tribromethanol anesthesia and the skull was exposed over the right fronto-parietal cortex. Application of pellets of dry ice of consistent size during 60 seconds to the surface of the skull produced a focal cryoinjury. The mice were euthanized 24 hours later.

Quantitative Real-Time Polymerase Chain Reaction

We prepared cDNA with the cloned AMV first-strand synthesis kit (Invitrogen) and performed quantitative real-time polymerase chain reaction with the Roche LightCycler instrument and the LightCycler FastStart DNA Master SYBR Green I kit (Roche). Human STC-1 primers were: 5′-ACACAAAGCTGAATGTCG-3′ and 5′-CAGGCTTCGAGAAGTCTGT-3′. Mouse primers were: Stc-1, 5′-ATGCTCCAAAACCTCAGCAGTGATTC-3′ and 5′-CAAGGCTTGGACAAGTCTGT-3′; Il-6, 5′-CTTCCCTACTTCACGAAGTCC-3′ and 5′-GCCACTCCTTCGATGTAC-3′; and Epo, 5′-ACCCTGCTGCTTTTACTCTC-3′ and 5′-ATGAAGCTGAAGGTCTCTG-3′. STC-1, Il-6, and Epo mRNAs were normalized against levels of mouse β-2-microglobulin (β2m) or human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers were: β2m, 5′-GCTATTCCAGAATCCCTCTA-3′ and 5′-ATGTCTCGATCCCTAGAC-3′; and GAPDH, 5′-GGTGAAGGTCCGAGTCAAC-3′ and 5′-CAATGAGCCCCAGCTTT-3′.

Tissue Processing and Immunohistochemistry

We transcardially perfused deeply anesthetized mice with heparinized 0.9% saline for 1 minute followed by Zamboni fixative for 8 to 10 minutes. Brains were removed and embedded in paraffin. For

Figure 1. Hypoxia induces Stc-1. Quantitative analysis of (A) Stc-1 mRNA and (B) Epo mRNA in brains of WT mice (n=4 per group) exposed to hypoxia. Brain tissue was harvested at indicated time points. Values are normalized against β2m mRNA levels and represent mean±SD.
immunohistochemistry, we pair-wise–mounted 4-μm sections from brains of control and GFAP–IL-6 mice on the same slide. Sections were further processed as described. Staining with pre-immune rabbit sera served as control.

In Situ Hybridization
We generated single-stranded antisense and sense RNA probes of a 356-bp mouse Stc-1 cDNA (position 147 to 502) fragment cloned into the pSPT18 plasmid (Invitrogen). Probes were labeled with digoxigenin-uridine triphosphate by in vitro transcription with SP6 and T7 RNA polymerases according to the manufacturer’s instructions (Roche). A sense probe was used as a negative control. Automated hybridization was performed with a Ventana Discovery Slide Stainer (Ventana Medical Systems). Sections were incubated with a monoclonal biotinylated anti-digoxin antibody (Jackson ImmunoResearch Laboratories) diluted 1:2000. The probe was detected with the Ventana Blue Map kit (Ventana Medical Systems).

Data Analysis
Data are expressed as means±SD. We examined normality of the data and performed all analyses with the Student t test or 2-way ANOVA. P<0.05 was regarded as statistically significant.

Results
Hypoxic Preconditioning Induces Stc-1 Expression in Brain
We exposed adult mice to 8% oxygen for 6 hours. After various times of re-oxygenation in normal air, we collected brain hemispheres and quantified Stc-1 mRNA expression. Upregulated expression of Stc-1 mRNA was evident already during the hypoxic period and declined gradually during 48 hours (Figure 1A). The kinetics of induced Stc-1 expression followed that of erythropoietin (Epo) (Figure 1B), a well-known HOPC-responsive gene. In situ hybridization confirmed the previously observed neuronal localization of Stc-1 expression in hypoxic brain (Figure 2).

IL-6–Treated Paju Cells Upregulate STC-1 Expression
Because IL-6 mediates neuroprotective activity, and because elevated levels of IL-6 are evident in animals exposed to HOPC, we investigated the role of IL-6 in induction of STC-1 expression. The human neural cell line Paju was treated with recombinant IL-6, which induced dose-dependent upregulation of STC-1 mRNA levels with a maximum at 9 hours, followed by a decline to background levels at 24 hours (Figure 3A,B). The concentration of IL-6 giving maximum stimulation was 2.5 ng/mL, ie, a concentration comparable to serum levels of IL-6 recorded in an in vivo experimental mouse central nervous system ischemia model. Notably, exposure of Paju cells to hypoxia overnight did not, however, directly induce increased STC-1 expression (data not shown).

IL-6 Activates STC-1 Through the MAPK Pathway
Cytokines of the IL-6 family bind to their subtype-specific soluble or membrane-associated α-receptor. The complex formed interacts with the common transmembrane β-receptor protein gp130, which signals through the STAT3, MAPK, or PI3K pathway, or via a combination of these. Treatment with the MAP kinase kinase inhibitor PD98059 completely blocked the IL-6–induced STC-1 response in Paju cells (Figure 3C). AG490, an inhibitor of JAK2 that blocks activation of STAT3, showed an intermediate inhibitory effect on the upregulation of STC-1 by IL-6. This may be explained by the suggested crosstalk occurring between the STAT3 and MAP kinase path-
The PI3K inhibitor Wortmannin failed to influence the increase of STC-1 mRNA levels by IL-6. These findings show that IL-6-induced STC-1 expression in Paju cells is dependent on the MAPK signaling pathway, but that some degree of regulation is also directed through the STAT3 pathway.

**Stronger STC-1 Response After Induced Brain Injury in GFAP–IL-6 Mice**

To study the interplay between IL-6 and STC-1 expression in vivo, we used mice with transgenic overexpression of IL-6 targeted to the central nervous system astroglia under the control of the GFAP gene promoter (GFAP–IL-6 mice). We extracted mRNA from brains of GFAP–IL-6 mice and WT controls before and 24 hours after induction of a focal brain injury. Brains of GFAP–IL-6 mice showed a higher constitutive expression of Stc-1 mRNA than did the brains of WT mice. Both GFAP–IL-6 and WT mice upregulated brain Stc-1 mRNA 24 hours after induced focal brain injury, but this response was strongly enhanced in the GFAP–IL-6 mice (Figure 4A). When sections from a GFAP–IL-6 mouse brain and from a control (WT) mouse brain, mounted on the same slide, were immunohistochemically stained for STC-1, the GFAP–IL-6 mice revealed a stronger staining for STC-1, particularly in the large pyramidal neurons of the parafrontal cortex (Figure 4B, 4C).

**Lack of Enhanced Stc-1 Induction in IL-6–Deficient Mice**

Our results show a transient Il-6 mRNA increase in WT mouse brains after exposure to hypoxia (Figure 5A). To further substantiate the regulatory influence of IL-6 on Stc-1 expression, we exposed Il-6/H11002/H11002/H11002 mice to HOPC and quantified the induction of Stc-1 mRNA in the brain. HOPC that induced elevated Stc-1 mRNA in the brains of WT mice did not elicit a measurable Stc-1 response in the brains of the Il-6/H11002/H11002/H11002 mice (Figure 5B). However, brains from both WT mice and Il-6/H11002/H11002/H11002 mice, not exposed to hypoxia, showed similar levels of Stc-1 mRNA (control animals in Figure 1A and Figure 5B, respectively), indicating that IL-6 is not required for the constitutive expression of STC-1 in brain neurons. The Epo response in Il-6/H11002/H11002/H11002 mice was similar to that measured in WT mice (Figure 1B and Figure 5C), confirming that the 2 groups experienced similar hypoxic stress.
Discussion

Activation of the hypoxia-inducible factor-1 (HIF-1) is a well-established response to HOPC. This transcription factor induces Stc-1 expression in nasopharyngeal cancer cells subjected to oxygen deprivation. Other hypoxia-inducible transcription factors include NF-IL6, the transcription factor for IL-6. Enhanced activity of NF-IL6, in response to hypoxia, occurs in heart, lung, and kidney, but not in the liver, suggesting tissue specific regulation. Our present data show that induction of Stc-1 expression in the brain, after HOPC, is under the control of IL-6 because hypoxia fails to upregulate Stc-1 in brains of Il-6−/− mice under conditions in which transcription of the HIF-1 target gene Epo is fully activated.

Astrocytes produce IL-6 under hypoxic conditions in vitro. We show here that a transient increase in IL-6 mRNA is evident in brains of WT mice after exposure to hypoxia (Figure 5A). Moreover, Yan et al reported an accumulation of IL-6 in the lungs of mice kept in low oxygen. Because IL-6 is known to penetrate the blood–brain barrier, the lungs may represent an additional source of IL-6 that upregulates the Stc-1 expression in the brain and also in other organs sensitive to ischemic damage.

The ultimate mechanism(s) by which STC-1 exerts cytoprotection is still unclear. STC-1 acts as an “emergency hormone” in fish, where elevated levels of environmental calcium trigger its synthesis and secretion from kidney-associated specialized glands, the corpuscles of Stannius.

Human neural Paju cells transfected with STC-1 cDNA maintain an increased ATP synthesis in the presence of CoCl2 and show increased survival as compared with mock-transfected cells. McCudden et al reported that mammalian STC-1 localizes to the inner mitochondrial membrane and that addition of recombinant STC-1 enhances the electron transport in submitochondrial particles. Mitochondrial calcium metabolism is a main regulator of cell survival. Given that STC-1 blocks the influx of environmental calcium in fish, it is tempting to speculate that mammalian STC-1 regulates mitochondrial calcium fluxes and thus modifies the respiratory rate. Alterations of the oxidative phosphorylation is intimately linked to hypoxic stress including uncoupling of mitochondrial respiration occurring during HOPC. It is conceivable that STC-1 contributes to the maintenance of the integrity of mitochondrial function, ie, cellular energy metabolism, under stressful conditions.

Our findings may have therapeutic implications for management of stroke. It is also noteworthy that physical exercise, inducing elevated serum levels of IL-6, also confers protection against neurodegenerative disorders like Alzheimer disease. Regular exercise also has a beneficial effect on disease progression in a mouse model of amyotrophic lateral sclerosis. Induction of elevated expression of STC-1 in neurons may be a mediator in these events.

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