Neuroprotection by Interleukin-6 Is Mediated by Signal Transducer and Activator of Transcription 3 and Antioxidative Signaling in Ischemic Stroke

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Background and Purpose—Interleukin-6 (IL-6) has been shown to have a neuroprotective effect in brain ischemic injury. However, its molecular mechanisms are still poorly understood. In this study, we investigated the neuroprotective role of the IL-6 receptor (IL-6R) by IL-6 in the reactive oxygen species defense system after transient focal cerebral ischemia (tFCI).

Methods—IL-6 was injected in mice before and after middle cerebral artery occlusion. Coimmunoprecipitation assays were performed for analysis of an IL-6R association after tFCI. Primary mouse cerebral cortical neurons were transfected with small interfering RNA probes targeted to IL-6Ra or gp130 and were used for chromatin-immunoprecipitation assay, luciferase promoter assay, and cell viability assay. Reduction in infarct volumes by IL-6 was measured after tFCI.

Results—IL-6R was disrupted through a disassembly between IL-6R and gp130 associated by protein oxidation after reperfusion after tFCI. This suppressed phosphorylation of signal transducer and activator of transcription 3 (STAT3) and finally induced neuronal cell death through a decrease in manganese-superoxide dismutase. However, IL-6 injections prevented disruption of IL-6R against reperfusion after tFCI, consequently restoring activity of STAT3 through recovery of the binding of STAT3 to gp130. Moreover, IL-6 injections restored the transcriptional activity of the manganese–superoxide dismutase promoter through recovery of the recruitment of STAT3 to the manganese–superoxide dismutase promoter and reduced infarct volume after tFCI.

Conclusions—This study demonstrates that IL-6 has a neuroprotective effect against cerebral ischemic injury through IL-6R-mediated STAT3 activation and manganese–superoxide dismutase expression. (Stroke. 2011;42:3574-3579.)

Key Words: cerebral ischemia ■ IL-6 ■ Mn-SOD ■ neuroprotection ■ STAT3

Many studies have demonstrated the neuroprotective effects of interleukin-6 (IL-6) in ischemic brain injuries, although a few studies suggest that IL-6 has an injurious effect. Continuous injection of IL-6 reduced neuronal loss after forebrain ischemia, and injection of the IL-6 receptor (IL-6R) antibody after middle cerebral artery occlusion increased cell death and infarct volume. However, the mechanism of neuroprotection by IL-6 in relation to IL-6R in cerebral ischemia has not been fully elucidated.

The action of IL-6 begins at the cell surface through its binding to IL-6R, which is composed of 2 major subunits, a ligand binding α subunit (IL-6Ra) and a β subunit (gp130), which is a transmembrane glycoprotein with signal transduction capability. The binding of IL-6 to IL-6Ra induces homodimerization of gp130 and consequently recruits the Janus kinase (JAK) to the cytosolic part of gp130. Recruited JAK becomes phosphorylated and phosphorylates multiple tyrosine residues on the cytoplasmic part of gp130, thereby creating docking sites for signal transducer and activator of transcription 3 (STAT3). The recruited STAT3 is then phosphorylated at Y705 residues and phosphorylated STAT3 (p-STAT3; Y705) dimerizes and translocates to the nucleus, where it binds to the promoter of target genes to regulate transcription.

STAT3 is involved in neuroprotection against various brain injuries, including cerebral ischemia. After brain ischemia, superoxide anions and reactive oxygen species (ROS) are produced in mitochondria. These ROS induce the mitochondrial-dependent apoptotic pathways. These ROS, however, are removed by manganese-containing superoxide dismutase (Mn-SOD or SOD2), a primary cellular defense antioxidant enzyme specific to superoxide. STAT3 regulates transcription of the Mn-SOD gene in the mouse cerebral cortex and cortical neurons. In this study, we elucidated the molecular mechanism of neuroprotection by IL-6 in relation to IL-6R-mediated STAT3/ROS regulation in mouse ischemic brains.

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Materials and Methods

Animals

All procedures in experiments with animals were performed in accordance with National Institutes of Health guidelines and were approved by Stanford University’s Administrative Panel on Laboratory Animal Care. Male CD1 mice (30–35 g, 2 months old) were purchased from Charles River Laboratories (Wilmington, MA). Heterozygous SOD1 knockout mutant mice (CD1/SV129 back-crossed with CD1 for 10 generations) and their WT littermates with an identical genetic background were used. Heterozygous copper/zinc-superoxide dismutase transgenic (SOD1 TG) mice (SOD1 TGHS/SF-218-3 strain with a CD1 background) and their WT littermates with an identical genetic background were also used.

Focal Cerebral Ischemia

Forty-five minutes of transient focal cerebral ischemia (tFCI) were induced by middle cerebral artery occlusion. CD1 mice were anesthetized with 1.5% isoflurane in 70% nitrous oxide and 30% oxygen using a face mask and maintained with 1.5% isoflurane during surgery. Rectal temperature was kept at 37°C with a homeothermic blanket. An 11.0-mm 6-0 surgical monofilament nylon suture, blunted at the tip, was introduced into the left internal carotid artery through the external carotid artery stump. After 45 minutes of middle cerebral artery occlusion, the nylon suture was withdrawn, blunted at the tip, was introduced into the left internal carotid artery and maintained for 4 hours in the ischemic brain and cortical neurons. Injections of IL-6 (50 ng in 2 μL of phosphate-buffered saline) were administered 30 minutes before and 15 minutes after middle cerebral artery occlusion in mice. We then examined the change in p-STAT3 and Mn-SOD expression caused by IL-6 in ischemic cerebral tissue. Injections of IL-6 significantly recovered p-STAT3 and Mn-SOD expression, which was reduced by 3 hours of reperfusion in the ischemic cerebral cortex (Figure 1A). The Mn-SOD mRNA level also recovered after these injections (data not shown). In addition, recovery of p-STAT3 and Mn-SOD expression (Figure 1B) and the Mn-SOD mRNA level (data not shown) were administered 30 minutes before and 15 minutes after middle cerebral artery occlusion in mice.

Statistical Analysis

All results were obtained from 3 to 5 independent experiments and are presented as mean±SEM. Data are expressed using Student t test. Differences were considered statistically significant at a probability value <0.05.

Detailed descriptions of our methods appear in the Supplemental Material (http://stroke.ahajournals.org). These methods include, primary cortical neuron culture, oxygen–glucose deprivation and reoxygenation, IL-6 treatments, coimmunoprecipitation assay, immunofluorescence staining and confocal microscopy, detection of protein oxidation, small interfering RNA (siRNA) transfection, Western blot, chromatin immunoprecipitation assay, luciferase activity assay, determination of infarction, and cell death assay.

Results

IL-6 Increases p-STAT3 and Mn-SOD Expression in the Ischemic Brain and Cortical Neurons

To investigate the neuroprotective effect of IL-6, 2 intracerebroventricular injections of IL-6 (50 ng in 2 μL of phosphate-buffered saline) were administered 30 minutes before and 15 minutes after middle cerebral artery occlusion in mice. We then examined the change in p-STAT3 and Mn-SOD expression caused by IL-6 in ischemic cerebral tissue. Injections of IL-6 significantly recovered p-STAT3 and Mn-SOD expression, which was reduced by 3 hours of reperfusion in the ischemic cerebral cortex (Figure 1A). The Mn-SOD mRNA level also recovered after these injections (data not shown). In addition, recovery of p-STAT3 and Mn-SOD expression (Figure 1B) and the Mn-SOD mRNA level (data not shown) were detected after treatment with IL-6 (50 ng/mL) in primary cortical neurons subjected to 2.5 hours of oxygen–glucose deprivation/4 hours of reoxygenation.

Disruption of IL-6R by Ischemic Reperfusion in Mouse Cerebral Cortex and Cortical Neurons

To elucidate the neuroprotective role of IL-6R by IL-6 in cerebral ischemic injury, we first examined the conformational status of IL-6R using a coimmunoprecipitation assay of ischemic mouse brain tissue. We found that there was an interaction between IL-6Rα and gp130 in IL-6R with sham.
surgery under normal physiological conditions in mouse brain cortices (Figure 1C). The specificity of each antibody used in this assay was confirmed using normal rabbit IgG as a negative control (Supplemental Figure IA–B). Interestingly, this interaction dissociated within 3 hours of reperfusion after tFCI (Figure 1C). The association between IL-6Rα and gp130 subsequently causes phosphorylation of JAK, and phosphorylated JAK phosphorylates multiple tyrosine residues on the cytoplasmic part of gp130.8 However, the dissociation between IL-6Rα and gp130 after reperfusion in cerebral ischemic injury caused suppression of JAK2 phosphorylation (Y1007/1008) and then finally blocked phosphorylation of STAT3 (Y705; Supplemental Figure IC). This sequential disruption blocked recruitment of STAT3 to the cytosolic domain of gp130 (Figure 1D) and then finally blocked phosphorylation of STAT3 (Y705; Supplemental Figure IC). However, IL-6 injections restored the association between IL-6Rα and gp130 and reinstated recruitment and phosphorylation of STAT3 (Figure 1C–D). The dissociation of IL-6R after reperfusion after tFCI in vivo and the recovery of the IL-6R association by IL-6 were also confirmed using a confocal microscope (Supplemental Figure II). Moreover, the same disruption of IL-6R was revealed in cerebral cortical neurons within 4 hours of reoxygenation after 2.5 hours of oxygen–glucose deprivation (Supplemental Figure IIIA–B). This disruption of IL-6R suppressed recruitment of STAT3 to the cytosolic domain of gp130 (Supplemental Figure IIIC), and treatment with IL-6 restored the assembly in the IL-6R component (Supplemental Figure III). We found a continuous association between IL-6Rα and gp130 in the mouse cerebral cortex and cortical neurons under normal conditions (Figure 1C; Supplemental Figures II and III). This continuous association led to the recruitment of STAT3 to the cytosolic domain of gp130 and phosphorylation of STAT3 (Y705), even under normal conditions (Figure 1D; Supplemental Figures IIIC and IIID). These results explain why STAT3 had high activity, even in normal brain tissue and cortical neurons, in our previous report.11

Oxidation of Each Component of IL-6R Caused by Reperfusion in the Mouse Cerebral Ischemic Cortex and Cortical Neurons

To clarify why the association between IL-6Rα and gp130 is blocked after reperfusion after tFCI, we examined oxidative injury caused directly by reperfusion in each component of IL-6R. Under exposure to ROS, proteins are processed to oxidative modification, which can lead to conformational changes in the proteins.15 We measured the quantification of proteins modified by oxygen free radicals using 2,4-dinitrophenyl, which specifically reacted with the oxidized carbonyl residue on the protein samples.16 To detect oxidation of IL-6Rα or gp130 after reperfusion after tFCI, the oxidized proteins were immunoprecipitated by an anti-2,4-dinitrophenyl antibody and then immunoblotted using IL-6Rα or gp130 antibodies. As shown in Figure 2, the oxidation level of IL-6Rα or gp130 was significantly increased after 3 hours of reperfusion in the cerebral ischemic cortex (Figure 2A–B). However, oxidation of STAT3 was not detected (Figure 2C). Interestingly, injections of IL-6 blocked oxidation of IL-6Rα and gp130 in the cerebral ischemic cortex. Also, the oxidation level of IL-6Rα or gp130 was significantly increased after 4 hours of reoxygenation in cortical neurons subjected to 2.5 hours of oxygen–glucose deprivation, and this oxidation was blocked by IL-6 treatments (Supplemental Figure IVA–B).

To confirm whether protein oxidation in each IL-6R component causes a disassociation of IL-6R, we compared the level of disassociation of IL-6R caused by ischemic reperfusion between the SOD1 TG mice and the WT mice. The level of the disassociation between IL-6Rα and gp130 in the SOD1 TG mice was lower than in the WT mice (Supplemental Figure IVD). These results indicate that the acute influx of oxygen during reperfusion after tFCI induces the oxidation of IL-6Rα and gp130 and that these consequently cause a disassociation of each component of IL-6R.
Gene Silencing of IL-6R Causes a Decrease in Mn-SOD Expression Through STAT3 Inactivation in Cerebral Cortical Neurons

IL-6 injections restored the IL-6R association, consequently leading to the recovery of p-STAT3 (Y705) and Mn-SOD levels after reperfusion in the cerebral ischemic cortex (Figure 1). These results indicate that IL-6R is an upstream regulator in STAT3-mediated Mn-SOD expression in the mouse cerebral cortex. To clarify this, we applied the siRNA transfection system for knockdown of each component of IL-6R in mouse cerebral cortical neurons and then examined STAT3 activity and the Mn-SOD level. As shown in Figure 3, the transfection efficiency of each specific siRNA was confirmed by quantification of the total protein level for IL-6R or gp130. Inhibition of IL-6R or gp130 by transfection with each specific siRNA in cerebral cortical neurons significantly reduced STAT3 phosphorylation and Mn-SOD expression. Reduction in Mn-SOD by STAT3 inhibition was also confirmed using the STAT3 inhibitor, AG490. Moreover, the level of 3-nitrotyrosine protein nitrosylation was increased in cortical neurons transfected with IL-6R- or gp130-specific siRNA. This result indicates that IL-6R is involved in the ROS regulation system through upregulation of STAT3 and Mn-SOD.

IL-6 Recovers STAT3 Recruitment to the Mn-SOD Promoter in Ischemic Reperfusion

To confirm whether IL-6R regulates STAT3-induced transcriptional activity of the Mn-SOD promoter, we first examined recruitment of STAT3 by IL-6 to the mouse Mn-SOD promoter using a chromatin immunoprecipitation assay. As shown in Figure 4A, STAT3 was strongly recruited to the Mn-SOD promoter in chromatin from sham-operated mouse brain cortices, and this recruitment was completely blocked after reperfusion after tFCI. However, IL-6 injections reinstated the recruitment of STAT3 into the Mn-SOD promoter. Moreover, IL-6Rα or gp130 inhibition by transfection with each specific siRNA in cerebral cortical neurons completely

Figure 3. Reduction of p-STAT3 and Mn-SOD by IL-6R knockdown in mouse cerebral cortical neurons. A, Western blot analysis of IL-6Rα, gp130, p-STAT3 (Y705), Mn-SOD, 3NT, and β-tubulin in cerebral cortical neurons transfected with IL-6Rα- or gp130-specific siRNA or treated with AG490 for 24 hours. B, Summary graphs depicting the band intensity of each Western blot. *P<0.05 (n=4 per group). 3NT indicates 3-nitrotyrosine; OD, optical density; p-STAT3, phosphorylated signal transducer and activator of transcription 3; Mn-SOD, manganese–superoxide dismutase; IL-6R, interleukin-6 receptor; siRNA, small interfering RNA.

Figure 4. IL-6R upregulates transcriptional activity of Mn-SOD through STAT3 recruitment into the Mn-SOD promoter. ChIP assay for analysis of STAT3 recruitment into the mouse Mn-SOD promoter after 3 hours of reperfusion with or without IL-6 injection in the cerebral ischemic cortex (A) or in cerebral cortical neurons transfected with IL-6Rα- or gp130-specific siRNA for 24 hours (B). C, Luciferase assay for analysis of transcriptional activity of the Mn-SOD promoter in cerebral cortical neurons transfected with pGLu-Mn-SOD and IL-6Rα- or gp130-specific siRNA or treated with AG490 or IL-6 for 24 hours (n=4 per group). Data are mean±SEM *P<0.05, **P<0.001 versus control. IR indicates ischemic reperfusion; S, sham; V, vehicle; IL-6R, interleukin-6 receptor; Mn-SOD, manganese–superoxide dismutase; STAT3, signal transducer and activator of transcription 3; ChIP, chromatin immunoprecipitation; IL-6, interleukin-6; siRNA, small interfering RNA.
blocked the recruitment of STAT3 into the Mn-SOD promoter (Figure 4B).

Decreased Transcriptional Activity of the Mn-SOD Promoter by Knockdown of the IL-6R Component in Cerebral Cortical Neurons

Using a luciferase assay, we investigated transcriptional activity of the Mn-SOD promoter in cerebral cortical neurons transfected with IL-6Rα or gp130-specific siRNA. As shown in Figure 4C, inhibition of IL-6Rα or gp130 by transfection with each specific siRNA significantly decreased luciferase activity in cortical neurons transfected with pGLu-Mn-SOD. In contrast, treatments with IL-6 significantly increased luciferase activity.

Cell Death by Knockdown of IL-6R in Cerebral Cortical Neurons

Our results indicate that IL-6R is the upstream regulator in STAT3-mediated Mn-SOD expression in cerebral cortical neurons. This may indicate that sustaining IL-6R at active status is critical for neuronal survival against oxidative stress in cerebral ischemic injury. Thus, we measured cell death by IL-6R inhibition in cerebral cortical neurons. Lactate dehydrogenase release significantly increased from cortical neurons transfected with IL-6Rα or gp130-specific siRNA for 24 hours (Figure 5A). To confirm whether this cell death was caused by reduction in Mn-SOD, we measured cell death by IL-6R inhibition in cerebral cortical neurons from SOD2−/− heterozygous knockout mice. As shown in Figure 5B, there was no significant increase in lactate dehydrogenase release in the cortical neurons transfected with IL-6Rα or gp130-specific siRNA for 24 hours in the SOD2−/− group, which expressed less Mn-SOD compared with the cortical neurons of WT mice. These results support Mn-SOD as the final target of IL-6R for neuronal survival through the ROS defense system in cerebral cortical neurons.

IL-6 Reduces Infarct Volume in the Ischemic Brain

To confirm the neuroprotective effect of IL-6 in vivo, we injected IL-6 into mouse brains before and after middle cerebral artery occlusion and measured infarction volume after 24 hours of reperfusion. The infarct volumes in the IL-6-treated mice were significantly smaller (P<0.05, n=9 per group) than in the vehicle-treated mice using 2,3,5-triphenyltetrazolium chloride staining (Figure 5C–D).

Discussion

This study has clearly elucidated the mechanism of neuroprotection by IL-6 through the IL-6R-mediated ROS defense system in mouse cerebral cortex and cortical neurons. Injections of IL-6 restored the signal transduction of STAT3-mediated Mn-SOD expression through recovery of the IL-6R association, which had been blocked by reperfusion after cerebral ischemia (Figures 1 and 4) and finally protected neuronal cells against oxidative stress (Figure 5). IL-6R was disrupted through a disassociation between IL-6Rα and gp130 caused by reperfusion after cerebral ischemia (Figure 1). This disruption was the result of the oxidation of each component of IL-6R by a direct and acute influx of oxygen during reperfusion after ischemia (Figure 2). However, in SOD1 TG mice, the disassociation between IL-6Rα and gp130 by reperfusion was attenuated compared with SOD1 WT mice (Supplemental Figure IVD). Injection of IL-6 saved each component of IL-6R from protein oxidation during reperfusion after ischemia (Figure 2). To explain why IL-6 blocks protein oxidation by reperfusion in cerebral ischemic injury, further study is needed. There are some reports that IL-6 upregulates antioxidants.17,18 Thus, IL-6 may accelerate gene expression or antioxidant activity that can block protein oxidation against oxidative stress in the ischemic brain. Also, IL-6 may play a role as an antioxidant molecule in an indirect way. The direct binding of IL-6 to IL-6Rα at the cell surface may serve as a shield from an attack of acute oxygen influx.

Figure 5. Knockdown of IL-6R induces cell death in cerebral cortical neurons and enhancement of IL-6R by IL-6 reduces infarct volumes in cerebral ischemic injury. A, Cell death assessed by LDH activity in a medium of cortical neurons transfected with IL-6Rα- or gp130-specific siRNA for 24 hours or treated with AG490 for 24 hours (n=4 per group). Data are mean±SEM *P<0.05, **P<0.001 versus control. B, LDH activity in a medium of cortical neurons transfected with IL-6Rα or gp130-specific siRNA for 24 hours in SOD2 WT or SOD2−/− KO mouse brains (n=4 per group). Data are mean±SEM *P<0.05 versus control. C, 2,3,5-triphenyltetrazolium chloride staining for analysis of infarct volumes after 24 hours of reperfusion after MCAO with or without IL-6 injection in male mice. D, Summary graph showing the size of infarction volumes. *P<0.05 (n=9 per group). NS indicates not significant; OD, optical density; IL-6R, interleukin-6 receptor; IL-6, interleukin-6; LDH, lactic dehydrogenase; siRNA, small interfering RNA; WT, wild-type; KO, knockout; MCAO, middle cerebral artery occlusion.
Disruption of IL-6R by transfection with IL-6α- or gp130-specific siRNA in cerebral cortical neurons significantly increased superoxide radical production through Mn-SOD reduction (Figure 3). This was caused by suppression of the transcriptional activity of the Mn-SOD promoter through blockage of STAT3 recruitment to the Mn-SOD promoter (Figure 4). Moreover, disruption of IL-6R by transfection with IL-6α- or gp130-specific siRNA induced neuronal cell death (Figure 5A). This cell death was caused by blockage of the ROS defense system. In cortical neurons from SOD2 knockout mice, cell death by transfection with IL-6α- or gp130-specific siRNA was not increased compared with a significant increase in cortical neurons from the SOD2 WT mice (Figure 5B).

STAT3 is highly phosphorylated at Y705 in the mouse brain and in cortical neurons under physiological conditions, and p-STAT3 (Y705) upregulates Mn-SOD expression.11 However, STAT3 immediately lost its activity after reperfusion in cerebral ischemic injury.11 Our present study explains why this happens. The disassembly between IL-6Rα and gp130 after ischemic reperfusion makes it impossible to recruit STAT3 into the cytosolic domain of gp130, and then STAT3 cannot be phosphorylated through sequential disruption of JAK2 phosphorylation (Y1007/1008) and phosphorylation of the cytosolic domain of gp130 (Figure 1C–D; Supplemental Figure IC). However, injections of IL-6 restored STAT3 activity through recovery of the IL-6R association against ischemic reperfusion (Figure 1). Also, enhancement of IL-6R by IL-6 recovered the pathway of STAT3-mediated Mn-SOD expression and protected neuronal cells against ischemic reperfusion (Figures 1, 4, and 5). Injection of IL-6 significantly reduced infarct volume after 24 hours of reperfusion in the ischemic brain (Figure 5C–D), although the limitations of the IL-6 effect should be considered, because the protection afforded by it may be partially lost with longer reperfusion. Thus, we suggest that IL-6 may have therapeutic potential against oxidative stress such as cerebral ischemic reperfusion and stroke.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Primary Cortical Neuron Culture

The procedure was adapted from a previously described protocol. Cerebral cortical neurons were prepared from brains of 16-day-old mouse embryos. Cerebral cortices were isolated and the meninges were carefully removed, after which the tissue was minced and treated with 0.25% trypsin in Earle’s balanced salt solution for 1 minute. After centrifugation, cortical neurons were isolated and plated on coated dishes with poly-D-lysine, and cultured in minimum essential medium (Invitrogen) containing glucose, 5% horse serum, glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 µg/ml). Two days later, the medium was changed to neurobasal medium containing B-27. Neurons were used for each experiment 7-10 days after plating.

OGD and Reoxygenation

For OGD in primary cortical neurons, the medium was replaced with buffered salt solution without glucose, and cortical neurons were placed in a gas-tight humidified anoxic chamber (PlasLabs) at 37°C for 2.5 hours, after which the cell medium was changed to a neuronal medium with glucose and reoxygenated for 4 hours.

IL-6 Treatment

IL-6 from mouse recombinant was purchased from Sigma-Aldrich. Two intracerebroventricular injections of IL-6 (50 ng in 2 µl of PBS) were administered 30 minutes before and 15 minutes after MCAO because the half-life of IL-6 in the brain is short. Two treatments with IL-6 (50 ng/ml) were also administered 30 minutes before and after OGD in primary cortical neurons.
Co-IP Assay

A Pierce CO-IP Kit (Thermo Scientific) was used for examination of the change in association between IL-6Rα and gp130 after 3 hours of reperfusion in whole cell extracts from cerebral cortices of male mice that were administered two intracerebroventricular injections of the vehicle (2 μl of PBS) or IL-6 (50 ng in 2 μl in PBS) 30 minutes before and 15 minutes after 45 minutes of MCAO. A Co-IP assay was also performed for analysis of the association between IL-6Rα and gp130 after 4 hours of reoxygenation in whole cell extracts from cerebral cortical neurons that were administered two treatments of the vehicle or IL-6 (50 ng/ml) before and after 2.5 hours of OGD. A general procedure was followed using the manufacturer's guidelines. Protein extracts were precipitated by an anti-IL-6Rα or an anti-gp130 antibody, and then immunoblotted by an anti-gp130 or an anti-IL-6Rα antibody. To be certain of the antibody specificity, protein extracts of sham samples were precipitated with anti-normal rabbit IgG (a negative control), and then immunoblotted with an anti-gp130 antibody or an anti-IL-6Rα antibody. To analyze for recruitment of STAT3 to the cytosolic domain of gp130, protein extracts were precipitated with anti-p-STAT3 (Y705) and then immunoblotted with an anti-gp130 antibody.

Immunofluorescence Staining and Confocal Microscopy

Anesthetized animals were perfused with heparinized (10 U/ml) saline and subsequently with 4% formaldehyde in PBS after 3 hours of reperfusion following tFCI. After fixation of brain tissue for 24 hours, the brains were sectioned at 50 μm using a vibratome and were washed three times with Tris-buffered saline (TBS). After using a TBS-blocking solution (1% bovine serum albumin, 0.2% skim milk, and 0.3% Triton X-100 in TBS) for 1 hour, the sections were incubated overnight in primary antibodies in TBS-blocking solution on a shaker at 4°C. The primary
antibodies we used were rabbit anti-IL-6Rα (1:100; Santa Cruz Biotechnology) and mouse anti-gp130 (1:100; R&D Systems). After washing three times, the sections were incubated in secondary antibodies conjugated with Alexa dyes (1:250; Invitrogen) for 2 hours. The sections were mounted and covered with mounting medium containing 4’,6 diamidino-2-phenylindole (Vector Laboratories) and were imaged on a confocal microscope (Zeiss LSM 510).

Detection of Protein Oxidation

An OxyBlot™ protein oxidation detection kit (Chemicon International) was used for examination of direct oxidative injury to proteins. A general procedure was followed using the manufacturer's guidelines. Briefly, 2,4-dinitrophenyl (DNP) reacted specifically with the oxidized carbonyl residue on the oxidized protein samples. The DNP-derivatized protein samples were immunoprecipitated using an anti-DNP antibody, and then immunoblotted using an anti-IL-6Rα antibody, an anti-gp130 antibody, or an anti-STAT3 antibody.

siRNA Transfection

We obtained siRNA probes targeted to mouse IL-6Rα or gp130, and non-targeting siRNA for use as a control (Qiagen). The target sequences for the mouse-specific IL-6Rα siRNA mixture were as follows: ACGAAGCGTTCACAGCTTAA (SI00193774), CAGCTTTCGATACCGACCTGTA (SI00193788), CAGTACGAAAGTTCTACAGAA (SI02690478), CCCGGCAGGAATCCTCTGGAA (SI02737336). The target sequences for the mouse-specific gp130 siRNA mixture were as follows: ACGACTTAGGACTATCTTAAA (SI00193802), CCGCTTTTCTTATAGTCACCTA (SI00193809), CTCCCTTTGCTCTAAGAGA (SI00183816), AAGAAACTGCTTTATTATGAA
(SI02712929). Non-targeting siRNA (SI03650318) was used as a control in all siRNA transfection experiments. Primary cortical neurons grown on 24-well plates (1 × 10^5 cells/well) or 6-mm dishes (1 × 10^6 cells/dish) were transfected with 10 nM siRNA per well with HiPerFect transfection reagent (Qiagen), and after 24 hours of incubation, were subsequently analyzed for various experiments.

**Western Blot Analysis**

Whole cell extracts were obtained from the cerebral cortex and caudate putamen (except the hippocampus) or were obtained from primary cortical neurons. Briefly, whole cell protein extracts were run on a sodium dodecyl sulfate gel, subsequently transferred to a polyvinylidene difluoride membrane, and incubated with primary antibodies for 24 hours at 4°C, and then with secondary antibodies for 1 hour at room temperature. The primary antibodies used were monoclonal or polyclonal antibodies against p-STAT3 (Y705), phospho-JAK2 (Y1007/1008) (1:1000; Santa Cruz Biotechnology), 3-nitrotyrosine (1:1000; Exalpha Biologicals), β-tubulin (1:5000; Sigma-Aldrich), IL-6Rα (1:5000; Santa Cruz Biotechnology), gp130 (1:5000; Santa Cruz Biotechnology), phospho-tyrosine (1:1000), and Mn-SOD (1:5000; Stressgen). The signal was then detected with horseradish peroxidase-conjugated IgG with the use of a chemiluminescent kit (Amersham Biosciences).

**ChIP Assay**

Chromatin isolation from mouse brain tissue or mouse primary cortical neurons and a chromatin immunoprecipitation assay were performed according to the manufacturer's protocol using a commercially available kit (EZ-Zyme™ Chromatin prep kit, EZ-ChIP™; Millipore). After
fixation of brain tissue with 1% formaldehyde, each soluble chromatin was digested using EZ-Zyme enzymatic cocktail and isolated. The diluted chromatin solution was precleared with protein G agarose/salmon sperm DNA/preimmune serum. The precleared chromatin solution was used for immunoprecipitation assays with a p-STAT3 antibody. After several washes, the antibody-protein-DNA complex was eluted from beads. After reversal cross-link incubation, protein and RNA were removed by proteinase K and RNase A. Purified DNA was subjected to polymerase chain reaction with primer-specific putative STAT3-binding sites up-stream of the transcriptional start site. The sequences of the polymerase chain reaction primers used were:

- forward, 5’-ACTCAGCCACTGCTTCTGC-3’, reverse, 5’-TGAGACTGGGGTTGCGGACT-3’,
- or forward, 5’-AGTCCGCAACCCCAGTCTCA-3’, reverse, 5’-AAATTGGTAGAGGCGCGTGT-3’.

**Transient Transfection and Luciferase Assay**

Primary cortical neurons were cultured on 24-well plates at a density of 1 × 10^5/well and were transfected with 250 ng of pGLu-Mn-SOD promoter reporter DNA per well using Lipofectamine LTX (Invitrogen). After 24 hours of transfection, the cells were treated with 50 μM of AG490 or 10 nM of siRNA for IL-6Rα or 10 nM of siRNA for gp130 or non-targeting siRNA per well. Cells were incubated for 2 more days and subsequently analyzed for luciferase activity. A Gaussia Luciferase Assay kit (New England BioLabs) was used to detect Gaussia luciferase activity from cell culture supernatants, according to the protocol provided by the manufacturer. All experiments were performed in quadruplicate and were repeated at least five times.
Cell Death Assay

Cell viability of primary cortical neurons was quantified by a standard measurement of LDH release using an LDH assay kit (BioVision). The amount of extracellular LDH was measured in an aliquot of the medium overlying the cells following the manufacturer's guidelines.

Determination of Infarction

After 24 hours of reperfusion following tFCI, the mice were killed and the brains were isolated and chilled in ice-cold PBS. They were then sliced coronally at 1-mm intervals. Individual slices were then incubated in 2% 2,3,5-triphenyltetrazolium chloride in 0.1 mol/L PBS (pH adjusted to 7.4), followed by 3.7% formalin. The infarct area was quantified by Adobe Photoshop (Adobe Systems).
References


**Supplemental Figure 1.** A, To verify the antibody specificity, protein extracts from the cerebral cortex of sham samples were precipitated by anti-normal rabbit IgG (for negative control) or an anti-IL-6Rα antibody, and then immunoblotted by an anti-gp130 antibody (left panels). To further ascertain the antibody specificity, protein extracts were also precipitated by anti-normal rabbit IgG (for negative control) or an anti-gp130 antibody, and were then immunoblotted by an anti-IL-6Rα antibody (right panels). B, Cell protein extracts of cerebral cortical neurons were precipitated by anti-normal rabbit IgG (for negative control) or an anti-IL-6Rα antibody, and then immunoblotted by an anti-gp130 antibody (left panels). To further ascertain the antibody specificity, the protein extracts were also precipitated by anti-normal rabbit IgG (for negative control) or an anti-gp130 antibody, and were then immunoblotted by an anti-IL-6Rα antibody (right panels). C, Western blot analysis of p-JAK2 (Y1007/1008), p-STAT3 (Y705), and β-tubulin or Co-IP assay for phosphorylation of gp130 at tyrosine residues after 3 hours of reperfusion following tFCl with or without IL-6 injection. S indicates sham; C, control; IP, immunoprecipitation; WB, Western blot; V, vehicle.
Supplemental Figure 2. Immunofluorescence staining for analysis of association between IL-6Rα and gp130 after 3 hours of reperfusion in tFCI with or without IL-6 injection in mouse cerebral ischemic cortex. A through D, Sections were stained using primary rabbit anti-IL-6Rα and mouse anti-gp130 and secondary antibodies conjugated with Alexa dyes. The sections were imaged on a confocal microscope (Zeiss LSM 510). Scale bars = 20 μm in A; 5 μm in B (sham), C (IR), and D (IL-6 + IR). DAPI indicates 4’,6-diamidino-2-phenylindole; IR, ischemia reperfusion.
**Supplemental Figure 3.** Co-IP assay for analysis of association between IL-6Rα and gp130 after 4 hours of reoxygenation following 2.5 hours of OGD in cerebral cortical neurons with or without IL-6 injection. A and B, The protein extracts were precipitated by an anti-IL-6Rα antibody or anti-gp130 antibody and were then immunoblotted by an anti-gp130 antibody or an anti-IL-6Rα antibody. C, To analyze for recruitment of STAT3 to the cytosolic domain of gp130 after 4 hours of reoxygenation, protein extracts were precipitated by an anti-gp130 antibody and then immunoblotted by anti-p-STAT3 (Y705). Summary graphs depicting the band intensity of all Co-IP data. *P<0.05 vs. control, #P<0.05 vs. vehicle (n=4 per group). C indicates control; V, vehicle; OR, oxygen-glucose deprivation/reoxygenation; IP, immunoprecipitation; WB, Western blot; O.D., optical density.
Supplemental Figure 4. Co-IP assay for analysis of protein oxidation of IL-6Rα and gp130 after 4 hours of reoxygenation in whole cell extracts from cerebral cortical neurons that were administered two treatments of the vehicle or IL-6 (50 ng/ml) before and after 2.5 hours of OGD. Whole cell protein extracts were precipitated by an anti-DNP antibody and then immunoblotted by an anti-IL-6Rα antibody (A), an anti-gp130 antibody (B), or an anti-STAT3 antibody (C). Summary graphs depicting the band intensity of all Co-IP data. *P<0.05 vs. control, **P<0.05 vs. vehicle (n=4 per group). D, Co-IP assay for analysis of association between IL-6Rα and gp130 after 3 hours of reperfusion in whole cell extracts from cerebral cortices of SOD1 WT or SOD1 TG male mice that were subjected to MCAO for 45 minutes. Whole cell protein extracts were precipitated by an anti-IL-6Rα antibody and then immunoblotted by an anti-gp130 antibody. Summary graphs depicting the band intensity of Co-IP data. *P<0.05 vs. sham (n=4 per group). C indicates control; V, vehicle; OR, oxygen-glucose deprivation/reoxygenation; IP, immunoprecipitation; WB, Western blot; NS, not significant; O.D., optical density.