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ORIGINAL ARTICLE

Lipocalin-2 deficiency attenuates neuroinflammation and brain injury after transient middle cerebral artery occlusion in mice

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Lipocalin-2 (LCN2) is a secreted protein of the lipocalin family, but little is known about the expression or the role of LCN2 in the central nervous system. Here, we investigated the role of LCN2 in ischemic stroke using a rodent model of transient cerebral ischemia. Lipocalin-2 expression was highly induced in the ischemic brain and peaked at 24 hours after reperfusion. After transient middle cerebral artery occlusion, LCN2 was predominantly expressed in astrocytes and endothelial cells, whereas its receptor (24p3R) was mainly detected in neurons, astrocytes, and endothelial cells. Brain infarct volumes, neurologic scores, blood–brain barrier (BBB) permeabilities, glial activation, and inflammatory mediator expression were significantly lower in LCN2-deficient mice than in wild-type animals. Lipocalin-2 deficiency also attenuated glial neurotoxicity in astrocyte/neuron cocultures after oxygen-glucose deprivation. Our results indicate LCN2 has a critical role in brain injury after ischemia/reperfusion, and that LCN2 may contribute to neuronal cell death in the ischemic brain by promoting neurotoxic glial activation, neuroinflammation, and BBB disruption.

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INTRODUCTION

Lipocalin-2 (LCN2) is a secreted protein of the lipocalin family and is involved in the transport of small hydrophobic molecules.[1,2] Lipocalin-2 regulates cell death/survival,[3] cell migration/invasion,[4] cell differentiation,[5] and iron delivery.[6,7] Furthermore, LCN2 has been implicated in chronic kidney disease,[8] cardiovascular disease,[9] cancer[10] and acute endotoxemia.[11] However, the precise role of LCN2 in the central nervous system (CNS) has not yet been defined. We previously found LCN2 is secreted by glial cells in the CNS, and that it regulates glial cell death/survival, motility, and morphologic changes.[12,13] Lipocalin-2 has been found to act as a chemokine inducer in the CNS,[14] and we and others have reported that LCN2 contributes to neuronal cell death.[15,16] Lipocalin-2 binds to its receptor (24p3R),[7] which is expressed at high levels in the brain.[17] Recently, LCN2 was found to be associated with reactive astrocytosis[12,13] and various CNS injury conditions, such as, lipopolysaccharide-induced neuroinflammation,[17,18] experimental autoimmune encephalomyelitis,[19] intracerebral hemorrhage,[20] and spinal cord injury.[21] Furthermore, modulation of LCN2 expression has been observed in a mouse model of cerebral ischemia[18,22] and in stroke patients.[23,24] Moreover, increasing evidence indicates inflammatory response is closely involved in the pathogenesis and progression of ischemic stroke.[25–27]

These previous findings led us to investigate the role of LCN2 in stroke using a rodent ischemic stroke model, that is, a transient middle cerebral artery occlusion (tMCAO) model. In addition, mechanistic studies were conducted using glia/neurons cocultured under oxygen-glucose deprivation and reoxygenation (OGD + R).

MATERIALS AND METHODS

Animals

Lipocalin-2-deficient ($LCN2^{-/-}$) mice were obtained from Dr Kiyoshi Mori (Kyoto University, Japan) and Dr Shizuo Akira (Osaka University, Japan). The genetic background of the $LCN2^{-/-}$ mice was C57BL/6 as described previously.[6] Lipocalin-2 wild-type ($LCN2^{+/+}$) and $LCN2^{-/-}$ mice were back-crossed for eight to ten generations onto the C57BL/6 background to generate homozygous and heterozygous animals free of background effects on phenotypes. The absence of LCN2 was confirmed by polymerase chain reaction (PCR) analysis of genomic DNA.[28] All animal procedures were approved by the Institutional Animal Care Committee of Kyungpook National University and performed in accordance with the animal care guidelines of the National Institute of Health. All efforts were made to minimize the number of animals used and animal suffering. Mice were housed under specific pathogen-free conditions within the Kyungpook National University School of Medicine. Lipocalin-2 wild-type ($n = 85$) and $LCN2^{-/-}$ ($n = 78$) mice were randomly allocated to two groups: experimental group ($LCN2^{+/+}$ ($n = 60$), $LCN2^{-/-}$ ($n = 54$)), sham-operated controls ($n = 20$ per group). The mice killed during surgery were excluded ($LCN2^{+/+}$ ($n = 5$), $LCN2^{-/-}$ ($n = 4$)). Masking was done in all experiments, except the neuron-astrocyte coculture. In the neuron-astrocyte coculture experiments, all different treatment groups were labeled and included in a single plate to reduce the variation. The experiments were performed in accordance with the ARRIVE guidelines.

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Transient Middle Cerebral Artery Occlusion

Male LCN2$^{-/-}$ and LCN2$^{+/+}$ control littermates (~30 g; 12–14-week-old) were anesthetized with 2% isoflurane (induction) and maintained by the intranasal inhalation of 1% isoflurane in 2/3 N_2O and 1/3 O_2 using a vaporizer. Mice were placed on a homeothermic heat blanket (Harvard Apparatus, South Natick, MA, USA) at 37 °C to maintain body temperature during the procedures. The ischemic stroke model involved middle cerebral artery occlusion, as described previously.[29] Briefly, a midline neck incision was made and the left common carotid artery (LCCA) was carefully separated without harming the vagus nerve. A silicon-coated 7–0 monofilament suture (the diameter of silicone-coated tip: 0.23 ± 0.01 mm; Doccol, Albuquerque, NM, USA) was then inserted from the LCCA to the internal carotid artery and advanced to the Circle of Willis to occlude the left middle cerebral artery at its origin. After 60 minutes of ischemia, the suture was gently withdrawn to permit reperfusion, the LCCA was ligated, and the wound closed. For the sham operation, the LCCA was dissected in the same manner, but no suture was inserted. Mice were sacrificed under deep anesthesia achieved with an overdose of ether by inhalation.

Hypoxic-Ischemic Brain Injury

The hypoxic-ischemia was induced by the Rice–Vannucci method[30] with some modifications. Left common carotid artery of 9-day-old mice was permanently occluded with a coagulator. Afterwards, mice were returned to their dam and allowed to recover for 60 minutes and then placed in a hypoxic chamber circulated with a humidified gas mixture (8 ± 0.1% oxygen in nitrogen) at 37 °C for 60 minutes. After hypoxia, the pups were returned to their dam.

Measurement of Physiologic Parameter

All physiologic parameters were measured in LCN2$^{+/+}$ and LCN2$^{-/-}$ mice before and 24 hours after tMCAO. Body temperature was measured by a rectal thermocouple probe (Harvard Apparatus). A polyethylene catheter was placed into the LCCA, to draw 200 μL blood samples, which were then analyzed for pH, oxygen saturation, pCO_2 by using the Critical Care Xpress (Nova biomedical, Waltham, MA, USA). Blood pressure in the conscious animal was measured by using CODA2 mouse tail-cuff system (Kent Scientific, Torrington, CT, USA).

Western Blot Analysis

Mice were deeply anesthetized with an ether overdose and perfused with sterile saline through the aorta to remove blood. Brains were then rapidly dissected out and divided into ipsilateral and contralateral hemispheres. Excised tissues were homogenized in ice-cold lysis buffer containing 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 1% triton X-100, 2 mmol/L EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40, 100 mmol/L Na_3VO_4, 100 mmol/L NaF, and protease inhibitor. Homogenates were centrifuged at 12,000 r.p.m. for 30 minutes at 4 °C, and supernatants (the protein fractions) were subjected to the Bradford protein assay (Bio-Rad, Hercules, CA, USA) to determine protein concentrations. Equal amounts of protein (20 or 50 μg per sample) were separated by SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), and transferred to nitrocellulose filter membranes (Millipore, Billerica, MA, USA), which were then blocked with 5% skimmed milk in Tris-buffered saline for 60 minutes and incubated with primary antibodies diluted in the Tris-buffered saline containing 0.05% Tween 20 and 5% skimmed milk overnight at 4 °C. Blots were detected by enhanced chemiluminescence using goat anti-LCN2 (1:500; R&D Systems, Minneapolis, MN, USA), mouse anti-claudin-5 (1:1,000; Invitrogen, Carlsbad, CA, USA), mouse anti-β-catenin (1:500; Invitrogen), and mouse anti-α-tubulin (1:1,000; Sigma-Aldrich, St Louis, MO, USA) antibodies, and horseradish peroxidase-conjugated anti-goat (Santa Cruz, Dallas, TX, USA) and -mouse IgG secondary antibodies (Thermo Scientific, Rockford, IL, USA).

Reverse Transcription-Polymerase Chain Reaction

Mice were deeply anesthetized with an ether overdose and perfused with diethylpyrocarbonate-treated phosphate-buffered saline (PBS) through the aorta to remove blood. Brains were rapidly excised and divided into ipsilateral and contralateral hemispheres, which were immediately homogenized in Trizol reagent (Invitrogen). Total RNA (2 μg) from each sample was reverse-transcribed into cDNA using a First Strand cDNA Synthesis kit (MBI Fermentas, Hanover, Germany). Real-time reverse transcription–PCR (RT–PCR) was performed using the one-step SYBR PrimeScript RT–PCR kit (Perfect Real Time; Takara Bio, Tokyo, Japan) and the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. The $2^{-\Delta\Delta CT}$ method was used to calculate relative changes in gene expressions determined by Real-time RT–PCR. β-actin was used as an internal control.[31] Traditional PCR amplification using specific primer sets was carried out at an annealing temperature of 55–60 °C for 25–32 cycles. PCR was performed using a C1000 Touch Thermal Cycler (Bio-Rad). For the analysis of PCR products, 10 μL of the reaction was separated in agarose gel and detected under ultraviolet light after ethidium bromide staining. The nucleotide sequences of the primers used were based on published cDNA sequences (Supplementary Table I).

Immunofluorescence Staining

Mice were killed by ether inhalation, and subjected to intracardiac perfusion-fixation using 0.9% NaCl and 4% paraformaldehyde dissolved in 100 mmol/L PBS (pH 7.4). Isolated brains were immersion-fixed in 4% paraformaldehyde for 72 hours and cryoprotected by incubation in 30% sucrose diluted in 0.1 M PBS for 72 hours. They were then embedded in OCT compound (Tissue-Tek, Torrance, CA, USA) for frozen section and cut into 20 μm-thick coronal sections, which were permeabilized in 0.1% Triton X-100 and blocked with 1% bovine serum albumin and 5% normal donkey serum for 60 minutes at room temperature. Brain sections were then incubated with primary antibodies (goat anti-LCN2 (1:500), rabbit anti-24p3R (1:200; Sigma-Aldrich), mouse anti-NeuN (1:200; Millipore), rabbit anti-GFAP (1:500; DakoCytomation, Glostrup, Denmark), mouse anti-GFAP (1:200; BD, Franklin Lakes, NJ, USA), rat anti-CD31 (1:200; BD), rabbit anti-Iba-1 (1:500; Wako, Tokyo, Japan), and rat anti-Ly6G (1:200; eBioscience, San Diego, CA, USA)) overnight at 4 °C, washed in PBS containing 0.1% Tween 20, and then incubated with fluorescein isothiocyanate- or Cy3-conjugated secondary antibodies (1:200; Jackson ImmunoResearch, West Grove, PA, USA). Sections were then mounted and counterstained using gelatin containing DAPI (4′,6-diamidino-2-phenylindole). Tiled images of each section were captured with a CCD color video camera (Olympus D70) through an objective lens attached to a fluorescence microscope (Olympus BX51, Hicksville, NY, USA). Three squares (500 μm × 500 μm) were placed in the same location of each coronal brain section ($n = 6$). Cells in these three squares were counted and statistically analyzed as previously described using the NIH image J program.[32]

Determination of Cerebral Vascular Structures

The cerebral vascular structures of the brains of LCN2$^{+/+}$ and LCN2$^{-/-}$ mice were compared by 4% Trypan blue staining. Trypan blue dye (Sigma-Aldrich) was injected into a femoral vein (5 mL/kg of body weight). At 60 minutes after reperfusion, brains were carefully isolated, and large blood vessels around the Circle of Willis were examined.

Measurement of Infarct Volume

At 24 hours after reperfusion, mice were perfused with ice-cold saline solution through the ascending aorta. Brains were removed and sectioned coronally into 2-mm-thick slices, and the slices were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37 °C for 10 minutes and then left in 4% paraformaldehyde overnight. Infarct volume was measured using the Image J program and the following formula: ((volume of the contralateral hemisphere − volume of the non-ischemic ipsilateral hemisphere)/volume of the whole ipsilateral hemisphere) × 100. Unstained areas (pale colored) were defined as ischemic lesions.

Assessment of Neurologic Deficits

After recovery from anesthesia, neurologic functioning was assessed by a masked investigator. Neurologic deficits were graded on a scale of 0–5, as previously described,[33] using the following criteria: grade 0 = no observable neurologic deficit; grade 1 = failed to extend right forepaw; grade 2 = circled to the right; grade 3 = fell to the right; grade 4 = could not walk spontaneously; grade 5 = dead.

Evans Blue Extravasation

Blood–brain barrier (BBB) integrity was investigated by measuring the extravasation of Evans blue into tissues after ischemia/reperfusion (I/R). Evans blue dye (2% diluted in saline) (Sigma-Aldrich) was injected into a femoral vein (5 mL/kg of body weight) immediately after reperfusion. At

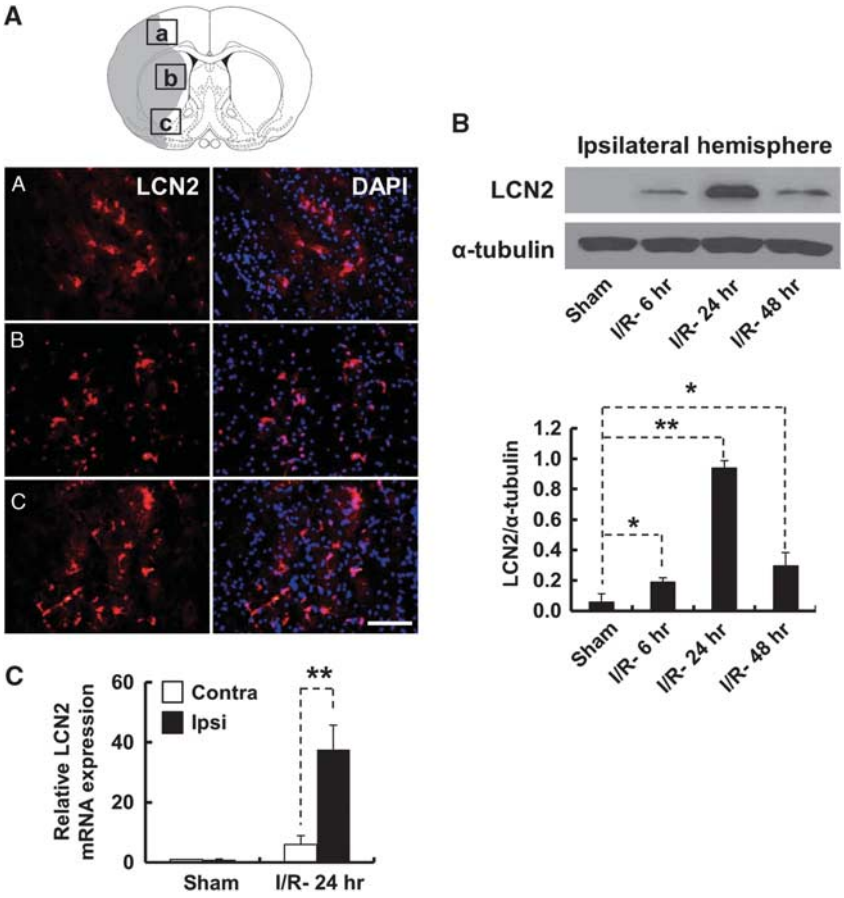

Figure 1. Induction of LCN2 protein in ipsilateral hemispheres after ischemia/reperfusion. (**A**) Immunofluorescence staining revealed that LCN2 expression (red) was induced in ipsilateral hemispheres at 24 hours after reperfusion. The shaded area indicates the infarcted region. Scale bar, 200 μm. (**B**) Western blot analyses showed the induction of LCN2 protein in ipsilateral hemispheres at 6, 24, and 48 hours after reperfusion (top). Results were normalized to α-tubulin and presented as means ± s.d. ($n = 5$) (bottom), $^*P < 0.05$, $^{**}P < 0.01$. (**C**) Lipocalin-2 mRNA was also induced in ipsilateral hemispheres at 24 hours after reperfusion. Results are means ± s.d. ($n = 5$), $^{**}P < 0.01$. Contra, contralateral hemisphere; Ipsi, ipsilateral hemisphere; LCN2, lipocalin-2.

24 hours after reperfusion, saline was perfused transcardially until colorless perfusion fluid was obtained from the right atrium under anesthesia. Excised brains were weighed, homogenized in 1.0 mL of 100 mmol/L PBS, and centrifuged at 15,000 r.p.m. for 30 minutes. An aliquot (0.5 mL) of the supernatant obtained was then added to an equal volume of trichloroacetic acid, incubated overnight at 4 °C, and centrifuged at 15,000 r.p.m. for 30 minutes. Supernatant absorbance was then measured at 615 nm using a spectrophotometer. Dye levels in brains were expressed as relative extravasation values in tissue (μg/mL) using a standard graph. Evans blue extravasation was normalized to the infarct volume, as described previously.[34]

Cell Cultures

Neonatal astrocyte cultures were prepared from mixed glial cultures, as previously described with minor modification.[13] In brief, the whole brains of 2–3-day-old mice were chopped and mechanically disrupted using a nylon mesh. The cells obtained were seeded in culture flasks and grown at 37 °C in a 5% CO_2 atmosphere in Dulbeco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 U/ml penicillin-streptomycin (Gibco-BRL, Rockville, MD). Culture media were changed initially after 5 days and then every 3 days, and cells were used after being cultured for 14–21 days. Primary astrocytes were obtained by shaking mixed glial cultures at 250 rpm overnight. Culture media were discarded, and astrocytes were dissociated using trypsin-EDTA (Gibco-BRL) and then collected by centrifuging at 1,200 rpm for 10 minutes. Primary astrocyte cultures were grown and maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin. Primary cultures of dissociated cerebral cortical neurons were prepared from embryonic day 15 (E15) mice, as described previously.[16,35] Briefly, mouse embryos were decapitated, and brains were rapidly removed and placed in a culture dish containing cold PBS. Cortices were isolated, transferred to a culture dish containing 0.25% trypsin-EDTA in PBS for 30 minutes at 37 °C, and washed twice in serum-free neurobasal media (Gibco-BRL). Cortical tissues were mechanically dissociated by gentle pipetting, and the dissociated cortical cells obtained were seeded onto plates coated with poly D-lysine (Sigma-Aldrich) in neurobasal media containing 2 mmol/L glutamine (Sigma-Aldrich), penicillin-streptomycin, nerve growth factor (Invitrogen), N2 supplement (Gibco-BRL), and B27 supplement (Gibco-BRL). Cells were used for experiments after being cultured for 7 days. The bEnd.3 mouse brain endothelial cell line was purchased from the American Type Culture Collection. Cells were cultured in DMEM containing 10% FBS at 37 °C in a humidified 5% CO_2 incubator. Cells were plated at a density of 1×10^6 cells per well in 6-well plates, and allowed to settle at 37 °C in a 5% CO_2 atmosphere. After 3 days, they were treated with recombinant mouse LCN2 protein (R&D Systems) at different concentrations (0.1 or 1.0 μg/ml) for 24 hours.

Measurement of Transendothelial Electrical Resistance using an *In Vitro* Blood–Brain Barrier Model

For astrocyte and bEnd.3 monocultures, astrocytes (4×10^4 cells) were seeded on the abluminal sides of 12-well transwell inserts (0.4 μm pore size; Corning, Corning, NY, USA) and grown for 2 days. bEnd.3 cells were then seeded on the luminal sides of the filters at a density of 1×10^5 cells per filter and grown for 3 days. After 3 days of coculture, inserts were

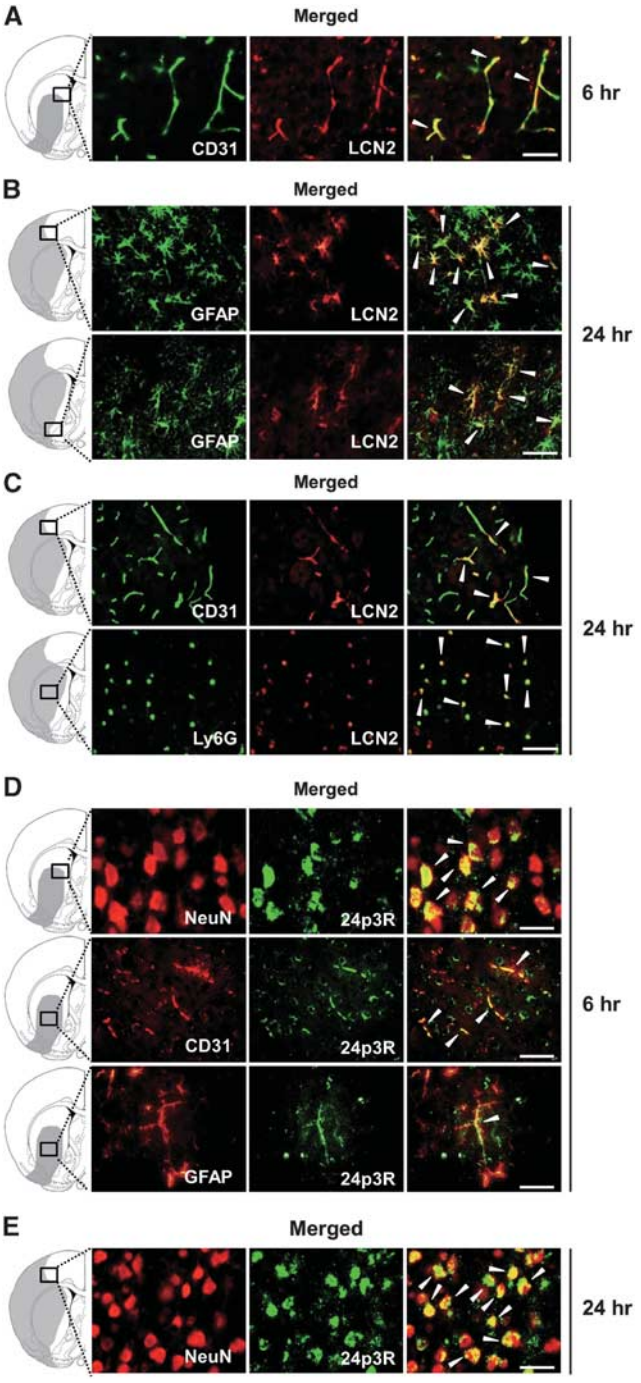

Figure 2. Immunolocalization of LCN2 and 24p3R within ipsilateral hemispheres after ischemia/reperfusion. Double-immunofluorescence staining for LCN2 or 24p3R with CD31 (endothelial cell marker), GFAP (astrocyte marker), Ly6G (neutrophil marker), or NeuN (neuron marker) revealed the localization of LCN2 in endothelial cells at 6 hours after reperfusion (**A**), or in astrocytes (**B**), endothelial cells and neutrophils (**C**) at 24 hours after reperfusion. 24p3R expression was localized in neurons, endothelial cells, and astrocytes at 6 hours after reperfusion (**D**), or neurons only at 24 hours after reperfusion (**E**). Scale bar, 100 μm. The shaded area indicates the infarcted region. Results are representative of more than three independent experiments. Colocalization is indicated by arrowheads. GFAP, glial fibrillary acidic protein; LCN2, lipocalin-2.

transferred to a cellZscope instrument (nanoAnalytics GmbH, Munster, Germany) and treated with recombinant mouse LCN2 protein (0.1 or 1.0 μg/ml) for 24 hours. Transendothelial electrical resistance and capacitance were continuously measured over 24 hours for each insert using an automated monitoring system (cellZscope, nanoAnalytics GmbH, Munster, Germany).

Oxygen-Glucose Deprivation and Reperfusion

Cells were subjected to OGD for 60 minutes by placing cultures in an anaerobic chamber (Thermo Scientific) with an O_2 tension of less than 0.5% (5% CO_2, 10% H_2, and 90% N_2) in the deoxygenated glucose-free DMEM (Sigma-Aldrich). After 60 minutes, cultures were re-oxygenated by adding normoxic glucose-containing media. Cultured cells were harvested at different times after commencing reoxygenation.

Astrocyte/Neuron Cocultures

For astrocyte/neuron cocultures, cortical neurons were plated at a density of 2×10^5 cells per well in 24-well companion plates, and allowed to settle at 37 °C in a 5% CO_2 atmosphere for 7 days. Primary astrocytes were separately plated at 2×10^5 cells per well in cell culture inserts (0.4 μm pore size; BD) and allowed to settle at 37 °C in a 5% CO_2 atmosphere overnight. For OGD coculture, cell culture inserts containing astrocytes were transferred to wells containing cortical neuron cultures in an anaerobic chamber. Then, cell cultures were subjected to OGD for 60 minutes at an O_2 tension of less than 0.5% in deoxygenated glucose-free DMEM. After 60 minutes of OGD, cultures were re-oxygenated by adding normoxic neurobasal media, incubated for 6 or 24 hours, and neuron viabilities were measured.

Assessment of Cell Viability using an MTT Assay

Cortical neurons were cocultured with astrocytes under OGD + R conditions. After the indicated times, culture inserts containing astrocytes were removed and the viabilities of neurons were measured using an MTT assay. In brief, after culture media were removed, MTT (0.5 mg/mL; Sigma-Aldrich) was added, and neurons were incubated at 37 °C in a 5% CO_2 incubator. Insoluble formazan crystals were completely dissolved in dimethyl sulfoxide, and absorbance at 570 nm was measured using a microplate reader (Anthos Labtec Instruments, Wals, Austria).

Statistical Analyses

Data are presented as the means ± s.d. of three or more independent experiments, unless otherwise stated. Two-group comparisons were done by two-tailed Student's *t*-test. Multiple comparisons were analyzed by one-way analysis of variance followed by Dunnett's test. The neurologic deficit score was analyzed using the Mann–Whitney test. The survival rate was analyzed by the log-rank test. The statistical analyses were performed using SPSS version 17.0K (SPSS; Chicago, IL, USA). Statistical significance was accepted for *P*-values < 0.05. The power calculations were performed to estimate proper sample size using G*Power 3.1.[36] The proper sample sizes were calculated for a power of 0.8 in all tests.

RESULTS

Lipocalin-2 Expression in Ipsilateral Hemispheres after Ischemia/Reperfusion Injury

Lipocalin-2 expression was not detected in the ipsilateral or contralateral hemispheres of sham-operated control mice (data not shown). A significant LCN2 expression was not detected in the contralateral hemispheres at 24 hours after I/R (Supplementary Figure I). However, LCN2 expression was strongly induced in the penumbra of ipsilateral hemispheres 24 hours after I/R (Figure 1A; Supplementary Figure I). Western blot analysis revealed that the induction of LCN2 protein in ipsilateral hemispheres began as early as 6 hours, peaked at 24 hours, and subsided at 48 hours after reperfusion (Figure 1B). Strong induction of LCN2 mRNA expression in ipsilateral hemispheres was confirmed by Real-time RT–PCR (Figure 1C). The penumbra refers to the area of the brain tissue that is damaged but not yet dead.[37] The penumbra has been considered as a therapeutic target in stroke patients. In our results, LCN2 expression was detected in the penumbra of ipsilateral hemispheres. These results suggest that LCN2 might participate in brain I/R injury.

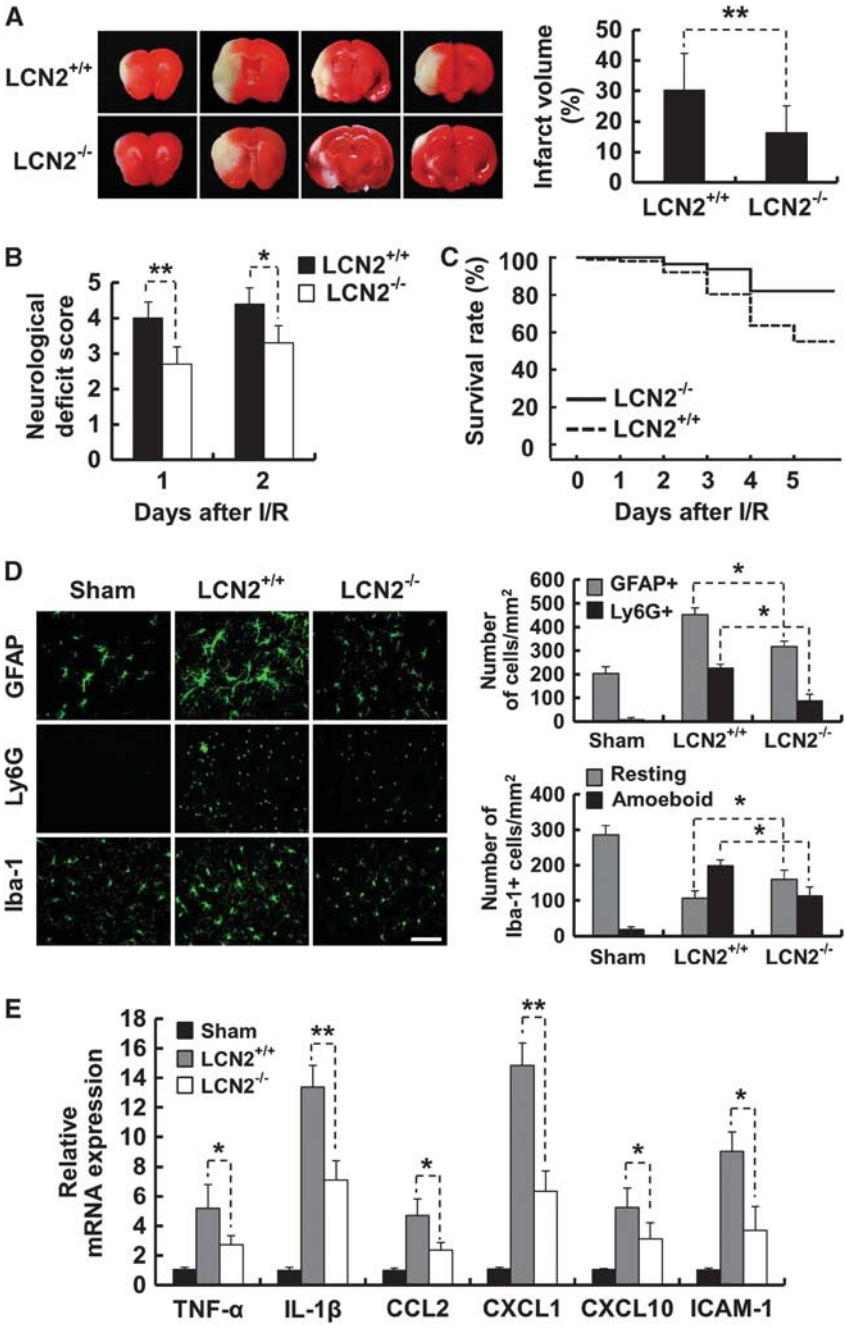

Figure 3. Lipocalin-2 deficiency attenuated infarct volume, neurologic deficit, and inflammatory response after ischemia/reperfusion (I/R). (**A**) At 24 hours after reperfusion, infarct volumes were compared in $LCN2^{+/+}$ and $LCN2^{-/-}$ mice by TTC staining coronal sections ($n = 10$ per group). (**B**) The neurologic deficit scores of $LCN2^{+/+}$ and $LCN2^{-/-}$ mice were compared on 1 and 2 days after reperfusion ($n = 10$ per group). (**C**) Kaplan–Meier curve of survival rate in $LCN2^{+/+}$ ($n = 60$) and $LCN2^{-/-}$ ($n = 54$) mice ($P < 0.05$). (**D**) Immunofluorescence staining for GFAP, Ly6G, and Iba-1 (a microglia/macrophage marker) was performed to determine the number of cells expressing GFAP, Ly6G, and Iba-1 in penumbras and infarct cores ($n = 5$ per group). Scale bar, 200 μm. (**E**) Relative mRNA expression of proinflammatory cytokines (TNF-α and IL-1β), chemokines (CCL2, CXCL1, and CXCL10), and adhesion molecules (ICAM-1) in ipsilateral hemispheres at 24 hours after reperfusion was determined by Real-time RT–PCR ($n = 5$ per group). Results are means ± s.d., $^{*}P < 0.05$, $^{**}P < 0.01$. GFAP, glial fibrillary acidic protein; IL, interleukin; LCN2, lipocalin-2; RT–PCR, polymerase chain reaction with reverse transcription; TNF, tumor necrosis factor; TTC, 2,3,5-triphenyltetrazolium chloride.

Localization of LCN2 and 24p3R in Brain after Ischemia/Reperfusion

To determine the cellular distributions of LCN2 and 24p3R in the ischemic brain, we performed double-immunostaining for LCN2 or 24p3R and for different cell markers at 6 and 24 hours after I/R. CD31, glial fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule (Iba-1), Ly6G, and neuronal-specific nuclear protein (NeuN) were used as cell type-specific markers for endothelial cells, astrocytes, microglia, neutrophils, and neurons, respectively. Lipocalin-2 expression only colocalized with endothelial cells within the ischemic penumbra at 6 hours after reperfusion (Figure 2A). In particular, LCN2 expression was not colocalized

Table 1. Physiologic parameters before and after middle cerebral artery occlusion (MCAO) in wild-type ($LCN2^{+/+}$) and LCN2-deficient ($LCN2^{-/-}$) mice

	Before MCAO		*24 h after MCAO*	
	$LCN2^{+/+}$	$LCN2^{-/-}$	$LCN2^{+/+}$	$LCN2^{-/-}$
Body temperature (°C)	37.0 ± 0.1	37.0 ± 0.1	36.9 ± 0.1	36.9 ± 0.2
Arterial blood pH	7.29 ± 0.01	7.27 ± 0.02	7.32 ± 0.03	7.30 ± 0.03
Arterial blood sO_2 (%)	99.0 ± 0.1	99.2 ± 0.1	99.4 ± 0.4	99.1 ± 0.3
Arterial blood pCO_2 (mm Hg)	45.4 ± 4.9	43.9 ± 6.5	48.4 ± 8.38	46.9 ± 0.8
Blood pressure (mm Hg)	102.7 ± 1.5	101.7 ± 2.5	103.0 ± 3.6	102.0 ± 1.7

LCN2, lipocalin-2; sO_2, oxygen saturation. Results are means ± s.d. ($n = 3$ per group). There were no significant differences between the groups in any of the parameters before and after MCAO.

with astrocytes, microglia, or neurons at 6 hours after reperfusion (Supplementary Figure IIA). Conversely, at 24 hours after reperfusion, LCN2 expression was mainly colocalized with astrocytes in the penumbra of ipsilateral hemispheres (Figure 2B). Lipocalin-2 expression was also colocalized with endothelial cells and neutrophils in the ischemic penumbra or the infarct core (Figure 2C), but not with microglia or neurons (Supplementary Figure IIB). The endothelial LCN2 was also demonstrated using bEnd.3 endothelial cells cultured under OGD conditions (Supplementary Figure IIIA). 24p3R expression was detected in both the penumbra and infarct core at 6 hours after reperfusion, and was colocalized with neurons in the penumbra, but with endothelial cells or astrocytes in the infarct core (Figure 2D). At 24 hours after reperfusion, 24p3R expression was detected in the penumbra, and found to be colocalized with neurons (Figure 2E). Furthermore, 24p3R expression was also localized to neurons in non-infarcted areas (data not shown). 24p3R expression was not detected in the microglia 6 hours after reperfusion (Supplementary Figure IIC) nor in the microglia, astrocytes, or endothelial cells 24 hours after reperfusion (Supplementary Figure IID). We also evaluated mRNA levels of LCN2 and 24p3R in different CNS cell types after OGD + R. The mRNA levels of LCN2 strongly increased in astrocytes 24 hours after OGD + R (Supplementary Figure IVA; left), and in bEnd.3 endothelial cells 6 and 24 hours after OGD + R (Supplementary Figure IVA; right). 24p3R mRNA expression was detected in the neurons, astrocytes and bEnd.3 endothelial cells at different time points after OGD + R (Supplementary Figure IVB).

Role of LCN2 in Ischemic Brain Damage and Neurologic Deficit

To determine the contribution made by LCN2 to ischemic brain damage and subsequent neurologic deficit, $LCN2^{-/-}$ mice and their littermate $LCN2^{+/+}$ mice were subjected to tMCAO, and infarct volumes were compared by TTC staining. Lipocalin-2 deficiency was found to reduce infarct volumes 24 hours after reperfusion (Figure 3A). Furthermore, an evaluation of neurologic scores indicated that I/R-induced neurologic deficit was also attenuated in $LCN2^{-/-}$ mice 1 and 2 days after reperfusion (Figure 3B). The survival rate was higher in $LCN2^{-/-}$ mice than in $LCN2^{+/+}$ mice after I/R ($P < 0.05$) (Figure 3C). An additional transient cerebral ischemia model was employed to further investigate the role of LCN2 in I/R injury of brain. Lipocalin-2 deficiency also reduced brain infarct volumes after hypoxic-ischemic injury in neonatal mice (Supplementary Figure V). These observations indicate that LCN2 contributes to brain damage and neurologic deficit after I/R. Because, anomalies of the cerebral vasculature in $LCN2^{-/-}$ mice could have affected infarct volume after tMCAO, we examined cerebral vasculatures after a Trypan blue injection. However, no significant difference was observed between $LCN2^{+/+}$ and $LCN2^{-/-}$ brains (data not shown). Also, there were no significant differences in body temperature, arterial blood pH, oxygen saturation, pCO_2, and blood pressure between $LCN2^{+/+}$ and $LCN2^{-/-}$ mice before and after tMCAO (Table 1).

Role of LCN2 in Neuroinflammation and Blood–Brain Barrier Breakdown after Ischemia/Reperfusion Injury

Glial activation and proliferation and neutrophil infiltration are the main events of neuroinflammation. To determine the role of LCN2 during inflammatory response in the ischemic brain, we examined glial activation, proliferation, and neutrophil infiltration in ischemic hemispheres by immunofluorescence staining. I/R-induced glial activation and neutrophil infiltration were found to be significantly lower in $LCN2^{-/-}$ mice than in $LCN2^{+/+}$ animals (Figure 3D). Numbers of ameboid microglia (representing an activated status) were also lower in $LCN2^{-/-}$ brains than in $LCN2^{+/+}$ brains after I/R. Real-time RT–PCR analysis revealed that LCN2 deficiency decreased the I/R-induced mRNA expression of proinflammatory cytokines (TNF-α and IL-1β), chemokines (CCL2, CXCL1, and CXCL10), and adhesion molecules (ICAM-1) (Figure 3E). Inflammatory responses in brain are often accompanied by the infiltration of inflammatory cells through the BBB, and BBB permeability is increased during ischemic stroke.[38] Furthermore, reactive gliosis and neuroinflammation are associated with BBB leakage.[39] Thus, we sought to determine whether LCN2 affects BBB breakdown after I/R injury. Evans blue extravasation assays indicated that LCN2 deficiency attenuated I/R-induced BBB leakage at 24 hours after reperfusion (Figure 4A). Evans blue extravasation normalized to the infarct size was also reduced by LCN2 deficiency. In addition, I/R-induced reductions in tight and adherens junction proteins (claudin-5 and β-catenin) were also prevented in $LCN2^{-/-}$ mice at 24 hours after reperfusion (Figure 4B). Transendothelial electrical resistance measurements using an *in vitro* BBB model indicated that LCN2 (1.0 g/mL) significantly decreased transendothelial electrical resistance and increased capacitance as compared with untreated control cells (Figure 4C). Moreover, treatment of bEnd.3 endothelial cells with LCN2 protein augmented the expression of proinflammatory mediators at 6 and 24 hours (Supplementary Figure IIIB). These results suggest that I/R-induced LCN2 acts on both glia and endothelial cells to influence neuroinflammation and BBB.

Role of LCN2 in Glial Neurotoxicity under Oxygen Plus Glucose-Deprived Conditions

In the immunofluorescence analysis and traditional RT–PCR, astrocytes were the major cell type that expressed LCN2. Astrocytic LCN2 was confirmed using primary astrocyte cultures exposed to OGD. Lipocalin-2 protein was markedly induced in astrocytes at 24 hours after OGD + R (Figure 5A). Because LCN2 receptor 24p3R was detected in neurons by immunofluorescence analysis, we hypothesized that astrocyte-derived LCN2 might exert

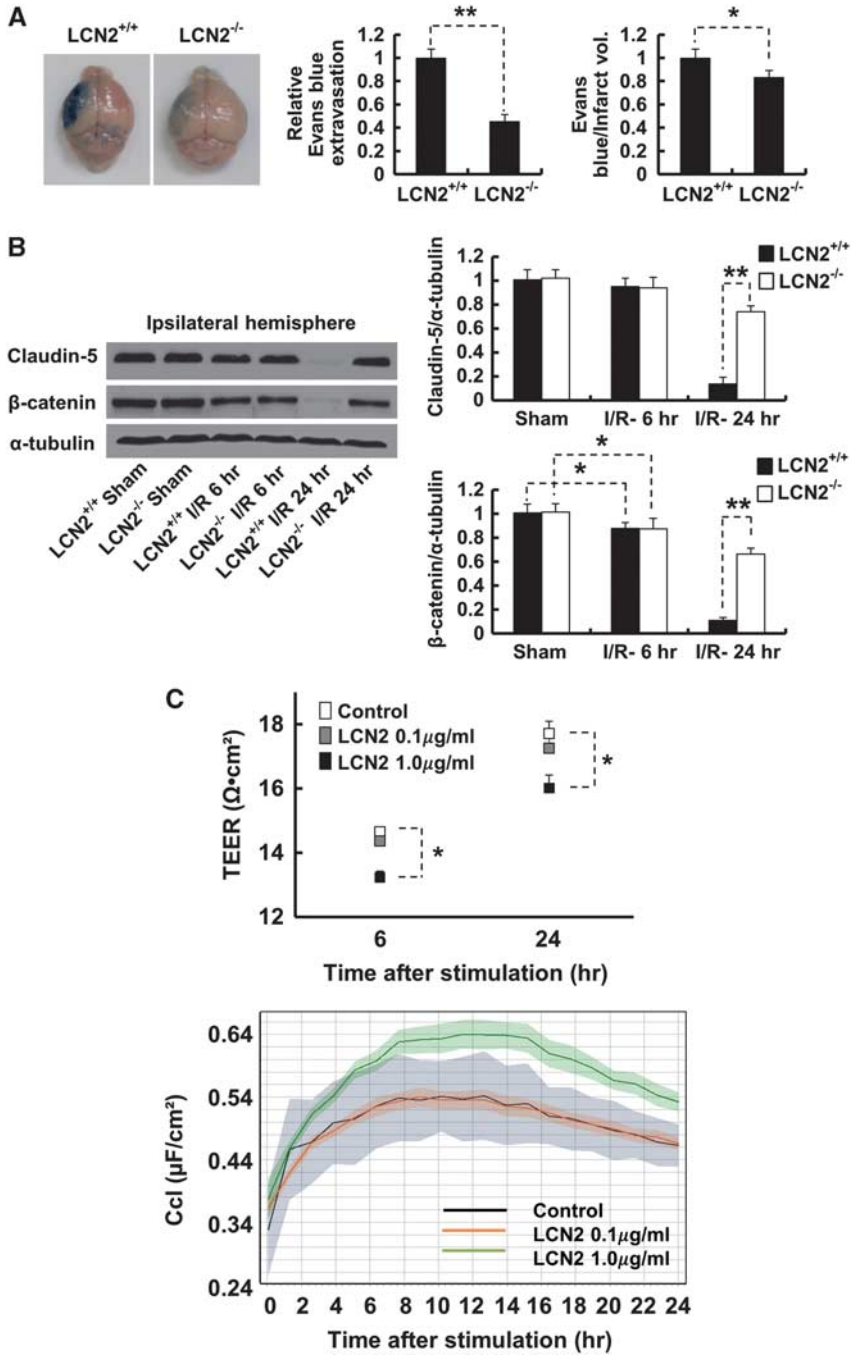

Figure 4. Effects of LCN2 on vascular permeability. (**A**) Representative images (left) and quantification of Evans blue extravasation results of $LCN2^{+/+}$ and $LCN2^{-/-}$ mice at 24 hours after I/R ($n=5$ per group) (middle). Relative Evans blue extravasation was also normalized to the infarct volume ($n=5$ per group) (right). (**B**) The levels of tight and adherens junction proteins (Claudin-5 and β-catenin) in $LCN2^{+/+}$ and $LCN2^{-/-}$ brains were compared by western blot analyses (left). Results were normalized to α-tubulin and presented as means ± s.d. ($n=5$) (right), $^{*}P<0.05$, $^{**}P<0.01$. (**C**) Using an *in vitro* BBB model, the effects of LCN2 on TEER and on corresponding Ccl values were determined ($n=3$ per group). Results are means ± s.d., $^{*}P<0.05$. The shaded areas in the graphs indicate s.d. values. Ccl, capacitance; I/R, ischemia/reperfusion; LCN2, lipocalin-2; TEER, transendothelial electrical resistance.

neurotoxic effects. This possibility was tested using astrocyte/neuron cocultures. Although coculture of $LCN2^{+/+}$ astrocytes and cortical neurons enhanced neurotoxicity 24 hours after OGD + R, coculture of $LCN2^{-/-}$ astrocytes and neurons did not (Figure 5B). These results indicate that LCN2 has a critical role in glial neurotoxicity under OGD + R.

DISCUSSION

Our findings indicate that LCN2 participates in brain injury after I/R, and are consistent with the pathogenic roles of LCN2 in I/R injury of other body organs, such as, kidney[40] and heart.[41] Lipocalin-2 deficiency attenuated brain damage 24–48 hours after I/R (Figure 3), and similar results were obtained 5 days after

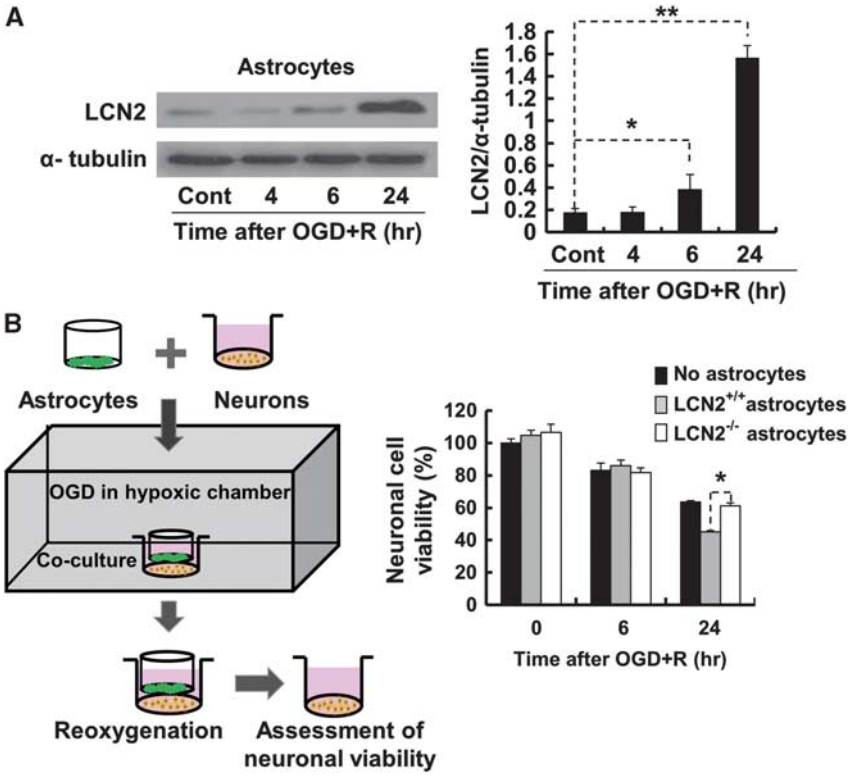

Figure 5. Neurotoxicity of glia-derived LCN2. (**A**) Western blot analyses showed the induction of LCN2 protein in primary astrocyte cultures after OGD + R (left). Control astrocytes did not undergo OGD. Results were normalized to α-tubulin and presented as means ± s.d. ($n = 3$) (right), $^*P < 0.05$, $^{**}P < 0.01$. (**B**) Astrocytes isolated from $LCN2^{+/+}$ or $LCN2^{-/-}$ mice were cocultured with cortical neurons under OGD conditions for 60 minutes, and neuronal viabilities were measured using an MTT assay at 6 and 24 hours after reoxygenation. Results are means ± s.d. ($n = 3$), $^*P < 0.05$. Coculture schemes are illustrated on the left panels. LCN2, lipocalin-2; OGD, oxygen-glucose deprivation; OGD + R, oxygen-glucose deprivation and reoxygenation.

reperfusion (unpublished data, Jin M and Suk K). On the basis of the results obtained from tMCAO and hypoxic-ischemia in $LCN2^{-/-}$ mice and from glia/neuron cocultures under OGD, we suggest LCN2 contributes to ischemic brain damage in two ways, namely, via direct neurotoxic effects and via the enhancement of neuroinflammation (Supplementary Figure VI).

Lipocalin-2 may exert direct toxic effects on neurons during I/R injury in brain. We found that LCN2 protein was increased in ipsilateral hemispheres after I/R, and that its induction began within 6 hours and peaked at 24 hours. Lipocalin-2 expression was localized in endothelial cells at 6 hours after reperfusion, whereas its expression was predominantly induced in astrocytes at 24 hours after reperfusion. The LCN2 receptor 24p3R was mainly expressed in neurons at 6 and 24 hours after reperfusion. Thus, astrocyte- or endothelial cell-derived LCN2 may act on neurons, and thus, mediate I/R damage. The neurotoxic activity of LCN2 has been previously demonstrated in cultured cortical neurons[16] and *in vivo*.[15] Furthermore, our previous results indicated that recombinant LCN2 protein is toxic to cultured cortical neurons and to several neuroblastoma cell lines.[16] Recently, Bi *et al*[15] confirmed that LCN2 produced from reactive astrocytes promotes neuronal death. Iron metabolism and BCL-interacting mediator of cell death protein had a critical role in the LCN2-induced cytotoxic sensitization toward neurons.[16] Changes in intracellular iron level were involved in the LCN2–24p3R-mediated apoptosis.[7] In the current study, coculture of astrocytes and neurons under OGD further supported the neurotoxic activity of glia-derived LCN2. In particular, astrocytes isolated from $LCN2^{-/-}$ mice were less toxic to cocultured neurons than were astrocytes from $LCN2^{+/+}$ mice after OGD + R.

Lipocalin-2 also seems to contribute to neuronal cell death via the enhancement of neuroinflammation after I/R. Despite the involvements of different mechanisms in stroke, increasing evidence indicates that inflammation has an important role in the pathogenesis of ischemic stroke.[26,27] Inflammatory response has been reported to be initiated by the accumulation of inflammatory cells and mediators in the ischemic brain.[27] After ischemia onset, inflammatory cells, such as, neutrophils,[26] microglia, and astrocytes are activated and accumulated within the ischemic region.[27] Lipocalin-2 as a key regulator of brain inflammation,[14,17] appears to have a central role in inflammatory infiltration and glial activation during I/R injury in brain, because I/R-induced glial activation, neutrophil infiltration, and proinflammatory cytokine/chemokine levels were significantly lower in the $LCN2^{-/-}$ brain. Although 24p3R was mainly expressed in neurons, it was also found to be expressed by astrocytes and endothelial cells, which suggest LCN2 might act on these cell types to promote neuroinflammation and BBB disruption in the ischemic brain. Reactive astrocytes have been reported to contribute to BBB breakdown,[39] and under ischemic stroke conditions, reduced tight junction integrity was found to result in increased BBB permeability.[38] In the current studies, I/R-induced increases in vascular permeability were attenuated by LCN2 deficiency, and I/R-induced downregulation of tight and adherens junction proteins was similarly prevented in $LCN2^{-/-}$ mice. These results strongly indicate that LCN2 is a critical mediator of neuroinflammation and BBB breakdown during I/R injury in the brain.

In summary, LCN2 appears to have a dual role during cerebral ischemia and reperfusion. That is, LCN2 may induce neurotoxicity by directly acting on neurons, or it may enhance

neuroinflammation and thus promote glial activation, BBB disruption, and inflammatory infiltration, and indirectly contribute to ischemic brain damage. These findings indicate that LCN2 should be regarded a novel therapeutic target in ischemic stroke.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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