SUPPLEMENTAL MATERIAL

Lipoprotein receptor-related protein-6 protects the brain from ischemic injury

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Material and Methods.

Middle cerebral artery occlusion

Procedures for transient MCA occlusion were identical to those previously described and are only summarized.[1-3] Mice were anesthetized with isoflurane (1.5% to 2%). Fiber optic probes were glued to the parietal bone and connected to a laser Doppler flowmeter (Periflux System 5010) for cerebral blood flow (CBF) monitoring. One probe was placed in the center of the ischemic territory (ischemic core; 2 mm posterior and 5 mm lateral to bregma) and another probe at the periphery (2 mm posterior and 2 mm lateral to bregma), in a region recruited into infarction in LRP6+/- mice. A 6.0 monofilament surgical suture was inserted into the external carotid artery, advanced into the internal carotid artery, and wedged into the circle of Willis to obstruct the origin of the MCA. The filament was left in place for 40 minutes and then withdrawn. Only animals that exhibited a 85% reduction in CBF during MCA occlusion and in which CBF recovered by 80% after 10 min of reperfusion were included in the study.[2-5] In all mice, rectal temperature was kept at 37.0 ± 0.5°C during surgery and in the recovery period until animals regained consciousness.

Assessment of Motor Function and Measurement of Infarct Volume

Motor function and infarct volume were assessed on day three after reperfusion. Motor function was assessed by the hanging wire test, as described in detail elsewhere.[2] For assessment of infarct volume, brains were removed, frozen, and sectioned (thickness, 30 μm) in a cryostat.[2-5] Brain sections were collected at 600 μm intervals and stained with cresyl violet. Infarct volume was determined using an image analyzer (MCID; Imaging Research Inc). To minimize the contribution of postischemic edema to the volume of injury, infarct volumes were corrected for swelling as previously described.[2-5]

Cerebrovascular regulation

Procedures for testing cerebrovascular regulation in mice have been described previously.[6] Briefly, under isoflurane anesthesia, the femoral artery was cannulated for recording of arterial pressure and collection of blood samples. Mice were then intubated and artificially ventilated with an oxygen–nitrogen mixture adjusted to provide an arterial pO_2 of 120 –140 mm Hg. Rectal temperature was maintained at 37°C using a thermostatically controlled rectal probe connected to a heating device. After surgery, isoflurane was gradually discontinued, and anesthesia was maintained with urethane (750 mg/kg, i.p.; Sigma-Aldrich) and chloralose (50 mg/kg, i.p.; Sigma-Aldrich). The stability of the level of anesthesia was checked by testing corneal reflexes and motor responses to tail pinch. The somatosensory cortex was exposed through a small craniotomy (2 x 2 mm). The dura was removed, and the site was superfused with a modified Ringer's solution (37°C; pH 7.3–7.4). CBF was continuously monitored at the site of superfusion with a laser-Doppler probe (Vasamedic, St. Paul, MN) positioned stereotaxically on the somatosensory cortex, a region supplied by the MCA. CBF changes were expressed as percentage increase relative to the resting level. To study the increase in CBF produced by neural activity (functional hyperemia), the somatosensory cortex was activated by gently stroking the contralateral whiskers with a cotton-tipped applicator for 60 s. Endothelium-dependent vasodilation was tested by topical superfusion of acetylcholine (10 μM; Sigma) or bradykinin (50 μM; Sigma) for 3–5 min, and the CBF increase was recorded. The CBF response to adenosine (400 μM; Sigma), an agent that produces vasodilation by acting directly on vascular smooth muscles, was also tested.

Real-time PCR

The mRNA for proinflammatory genes was examined after ischemia using real-time PCR as previously described.[3, 7] A group of genes that have been previously shown to be regulated after MCAo and have been implicated in neuronal cell death and post-ischemic inflammation were included in the mRNA expression screen. The following genes were studied: Cyclooxygenase-2 (COX-2, *Ptgs2*), prostaglandin E synthase 1 (PTGES, *Ptges*), interleukin-1β (IL-1β, *IL1b*), tumor necrosis factor (TNF, *Tnf*), monocyte chemotactic protein-1 (MCP-1, *Ccl2*), 10 kDa interferon gamma-induced protein (IP-10, *Cxcl10*), RAS-related C3 botulinum substrate 2 (Rac2, *Rac2*), NADPH oxidase 2 (NOX2, *Cybb*), endothelial-leukocyte adhesion molecule-1 (ELAM1, *Sele*), intercellular adhesion molecule-1 (ICAM1, *Icam1*), and LRP6. Mice were sacrificied 0, 2, 6, 24, and 72 hr after ischemia, and their brains were removed. A 2-mm-thick coronal brain slice was cut at the level of the bregma, and the infarcted (right) cortex was collected and frozen in liquid nitrogen. Total RNA was isolated using Trizol reagent (Invitrogen) and oligo-dT-primed cDNA was generated using Superscript II reverse transcriptase according to manufacturers suggestions (Invitrogen). Quantitative determination of mRNA levels was performed using Chromo 4 thermocycler (MJ Research, Watertown, MA). Two µl of diluted cDNA (1:10) were amplified by Platinum SYBR green qPCR supermix-UDG (Invitrogen). The reactions were incubated at 50°C for 2 min and then at 95°C for 10 min. A PCR cycling protocol consisting of 15 s at 95°C and 1 min at 60°C for 45 cycles was required for quantification.[3] Data were first normalized to the mouse HPRT housekeeping gene, and then expressed relative to the respective control condition.[3] Primer details are presented in Supplemental Table 1.

Double-label immunohistochemistry

For identification of the cell types expressing LRP6, mice were anesthetized with sodium pentobarbital (120 mg/kg) and perfused transcardially with 4% paraformaldehyde. Brains were removed, frozen, and sectioned through the parietal cortex. Brain sections were first processed for LRP6 immunocytochemistry, followed by incubation with antibodies against the neuronal marker NeuN (1: 100; Millipore), the endothelial cell marker CD31 (1:100; BD Biosciences, San Diego, CA), the astrocytic marker glial fibrillary acidic protein (GFAP) (1:1000; Sigma-Aldrich) or the microglial marker ionized calcium-binding adaptor molecule 1(Iba1)(1:500; WaKo). Sections were then incubated with cyanine dye (Cy5)-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The specificity of the immunolabel was assessed by omitting the primary antibodies or by preadsorption with the antigen.[8] Images of double-labeled neocortex were sequentially acquired using a Leica (Mannheim, Germany) TCS SP5 confocal laserscanning microscope. LRP6 signals were pseudocolored green, whereas Cy5 signals were pseudocolored red.

Western blot analysis

In GSK-3β phosphorylation studies, mice were sacrificed 0, 1, 3, 6 h after ischemia-reperfusion, and their brains were removed. The cortex of the ischemic hemisphere was sampled and homogenized in lysis buffer containing protease and phosphotase inhibitors. After protein concentration determination with DC reagents (BioRad, Hercules, CA), equal amount of proteins were gel-separated and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with specific antibodies in appropriate dilutions overnight after blocking with 5% dry milk in PBS for 1 hour at room temperature. The blots were probed with phospho-GSK-3β-Ser9 or phospho-GSK-3β-Y216 specific antibodies (Cell Signaling, Danvers, MA) and re-probed with pan-GSK-3β antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or β -actin-specific antibody (Sigma, St. Louis, MO) to verify equal loading. The membranes were washed 3 times with PBST (PBS + 0.1% Tween-20) and incubated with appropriate secondary antibodies for one hour, washed 3 times with PBST, and developed by the enhanced chemiluminescence system (ECL, Amersham, Arlington Heights, IL). Protein band images were captured on digital

files by using a Kodak Digital Imaging Station. The protein band intensity of each lane was obtained using Kodak 1D imaging software following instructions. For β-catenin detection, protein lysates were made from the cortex of right hemisphere at each time point in isotonic lysis buffer containing protease and phosphatase inhibitors. Briefly, tissue was homogenized in a Dounce glass homogenizer on ice by applying 20 strokes with the tight fitting pestle. After the lysates were cleared by centrifugation at 1500rpm in a tabletop centrifuge, the supernatants were further centrifuged for 30 min at 100,000 x *g* at 4°C. The supernatants were collected as cytosolic extracts for Western blotting analysis with anti-β-catenin antibody (Santa Cruz Biotechnology).

Mitochondrial transmembrane potential

Crude mitochondria from mouse brain were isolated as previously described.[9] Briefly, brains of LRP6+/+ and LRP6+/- mice were quickly removed. The cerebral cortex was dissected and transferred to a pre-cooled homogenizer with 10 volume of ice-cold mitochondria isolation buffer (225 mM mannitol, 75 mM sucrose, 0.1% BSA, 1 mM EGTA, 10 mM K-HEPES, pH 7.2). The tissue was homogenized by douncing. The homogenates were centrifuged at 760 x g for 10min at 4^{o}C and the supernatant was collected. The supernatant was centrifuged for 10min (10,000 x *g*, 4^{o}C). The crude mitochondria in pellet were resuspended in 1 ml of isolation buffer and used for experiments within 4 hrs. $\Delta\Psi_m$ was measured according to the method of Liu et al.[10] as previously described.[9] Mitochondria were suspended (0.1mg/ml) in a quartz cuvette in KCl buffer (125 mM KCl, 2 mM KH_2PO_4, 20 mM Hepes-KOH, pH 7.2) supplemented with 5 μM safranine O in a total volume of 1 ml. The fluorescence was measured with excitation and emission wavelengths set at 485 and 586 nm, respectively using a Hitachi F2500 fluorescence spectrophotometer. Changes in $\Delta\Psi_m$ were induced by adding 50 nmol aliquots of $CaCl_2$. In some experiments LRP6+/+ and +/- mice were treated with the GSK-3β inhibitor SB216763 (1 mg/kg; i.p.)[11] the evening before the experiment and brain mitochondria were isolated the following morning. This effectiveness and specific of the dose of SB216763 has previously been documented.[11]

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Supplemental Table

Gene	MGI Symbol	Genbank Acc.No.	Forward primer	Reverse primer
COX2	Ptgs2	NM_011198	TGGTGCCTGGTCTGATGATG	GTGGTAACCGCTCAGGTGTTG
ELAM1	Sele	NM_011345	CTCACTCCTGACATCGTCCTC	ACGTTGTAAGAAGGCACATGG
HPRT	Hprt	NM_013556	AGTGTTGGATACAGGCCAGAC	CGTGATTCAAATCCCTGAAGT
ICAM1	Icam1	NM_010493	GCCTTGGTAGAGGTGACTGAG	GACCGGAGCTGAAAAGTTGTA
IL-1β	Il1b	NM_008361	CTCTCCACCTCAATGGACAGA	TTTTGTCGTTGCTTGGTTCTC
IP-10	Cxcl10	NM_021274	ATCACTCCCCTTTACCCAGTG	GGAGGAGTAGCAGCTGATGTG
LRP6	Lrp6	NM_008514	TCCAACAGTCCTTCCACACAT	CGGCTAGGAGCATAGTCACTG
MCP-1	Ccl2	NM_011333	AGGTGTCCCAAAGAAGCTGTA	ATGTCTGGACCCATTCCTTCT
NOX2	Cybb	NM_007807	CCAACTGGGATAACGAGTTCA	GAGAGTTTCAGCCAAGGCTTC
PTGES	Ptges	NM_022415	TTTCTGCTCTGCAGCACACT	CACATCTGGGTCACTCCTGTA
Rac2	Rac2	NM_009008	GACACCATCGAGAAGCTGAAG	GTGAGTGCAGAACATTCCAAGT
TNF	Tnf	NM_013693	TTGGAGTCATTGCTCTGTGAA	GGGTCAGAGTAAAGGGGTCAG