Original Article

Upregulation of lipoprotein receptors on brain endothelial cells and neurons in the early phase of ischemic stroke in mice

Fuying Li, Satoru Ishibashi, Eri Iwasawa, Motohiro Suzuki, Keiko Ichinose and Takanori Yokota

Department of Neurology and Neurological Science, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University

Abstract

Current understanding on the mechanisms of acute stroke highlights the importance of targeting brain endothelial cells (ECs), which regulate blood-brain barrier (BBB) disruption, neuronal cell death, and immune cell infiltration. Because the transcellular pathway through ECs is activated immediately after stroke onset, understanding transporter expression should facilitate development of an efficient drug delivery system to ischemic ECs. Here, we examined BBB leakage profiles and expression of three lipoprotein receptors, low-density lipoprotein receptor (LDLR), scavenger receptor class B member 1 (SRB1), and LDLR-related protein 1 (LRP1), in a mouse model of permanent middle cerebral artery occlusion (MCAO). Evans blue staining showed a biphasic BBB disruption with one peak at 6 h and the other at 3 days after MCAO. The tight junction protein occludin in the ischemic cortex significantly decreased at 3 days, but not 6 h, after MCAO. LDLR, SRB1, and LRP1 mRNA levels were significantly up-regulated early after MCAO. LDLR and SRB1 proteins were colocalized predominantly in brain ECs, whereas LRP1 was localized in neurons in the ischemic cortex. The early overexpression of lipoprotein receptors after stroke suggests that lipoprotein-associated lipids could be suitable ligands for drug delivery into ECs and neurons in the acute ischemic brain.

Key words: Acute Stroke, Blood-Brain Barrier, Tight Junction, Low-Density Lipoprotein, High-Density Lipoprotein

Introduction

Stroke remains a major cause of mortality and long-term disability worldwide. Over the past few decades, major advances in acute ischemic stroke therapy involving the use of intravenous recombinant tissue plasminogen activator (rtPA) alone or in combination with endovascular therapy have significantly improved the chances of stroke recovery and survival; however, the use of these procedures is severely restricted owing to the extensive exclusion criteria and short therapeutic time window. Therefore, an effective stroke therapy is imperative, and research has been directed to reducing the brain ischemic lesions and neurological deficits produced by acute stroke. Since neuroprotection trials for stroke have mostly failed, the emerging concept of the “neurovascular unit (NVU)” suggests that focusing on protecting neurons alone is not sufficient.

Brain endothelial cells (ECs) form the interface between brain tissue and circulating blood, and maintain the homeostasis of the brain microenvironment by closely interrelating with the NVU, which comprises neurons and glial cells. Interrupting the blood supply to the brain after acute ischemia results in oxygen and glucose deprivation (OGD) and, consequently, reduced energy available for brain cell functions. Brain EC dysfunction leads to blood-brain barrier (BBB) disruption (i.e., increased permeability) resulting in brain edema, hemorrhage, inflammation, oxidative stress, and loss of neuronal connection, ultimately increasing brain dysfunction. The NVU performs a coordinated response after an ischemic insult to maintain and re-establish...
blood flow, thus reducing damage to tissue and neurons. Because brain ECs play a central role in maintaining NVU function, they are a key therapeutic target in the setting of BBB disruption after acute ischemic stroke.

Brain ECs form a transcellular and paracellular barrier to many blood-borne solutes via scarce endocytic vesicles and disrupted tight junctions (TJs). In ischemic stroke, the BBB primarily formed by ECs disrupts from as early as 3 h, allowing substances that do not pass during the steady state to pass from blood into the brain parenchyma. Transcellular pathways, both endocytosis and transcytosis, of brain ECs increase as early as 3–12 h post-stroke following middle cerebral artery occlusion (MCAO) in rodents. However, TJ morphology and TJ proteins remain normal during the early phase even after 24 h of MCAO, despite profuse extravasation of tracers from blood vessels in rats: disruption of TJ structure starts at 48 h post-stroke, a relatively late time point after BBB breakdown in mice. Although large molecules can be delivered via the paracellular pathway into the brain parenchyma after 48 h post-stroke, ischemic brain cells are irreversibly damaged by this time; therefore, acute stroke therapy should be better to focus on the transcellular pathway of brain ECs to improve the chances of recovery.

Brain ECs allow efficient transport of select molecules, such as glucose, amino acids, albumin, and electrolytes, and larger molecules such as low- and high-density lipoproteins (LDL and HDL), into the central nervous system (CNS) by vesicular endocytosis and transcytosis regulated by highly specialized substrate-specific transport proteins. More broadly, enhancement of endothelial endocytosis and transcytosis across the BBB after stroke has been proposed as a drug delivery tool to the ECs or brain parenchymal cells. Among these transport proteins, LDL receptor (LDLR) family members such as LDLR and LDLR-related protein 1 (LRP1) and a major HDL receptor called scavenger receptor class B member 1 (SRB1) are highly expressed within the CNS on brain ECs; these receptors are involved in receptor-mediated endocytosis and transcytosis of macromolecules including lipoproteins. Furthermore, LDLR and LRP1 are present on neurons and glial cells as well as brain ECs, and LRP1 on neurons has a role in transporting LDLs and/or HDLs under normal conditions. In this sense, brain lipoprotein receptors should be a promising target for drug delivery to NVU cells including brain ECs, neurons, and glial cells in the ischemic brain. However, in the setting of acute ischemic stroke, the role and temporal profile of these lipoprotein receptors remain largely unknown.

We hypothesized that lipoprotein receptors, such as LDLR, SRB1, and LRP1, would be up-regulated in the ischemic brain in parallel with enhanced transcellular transport in the early phase of stroke. Here, we investigated the BBB disruption and distribution of lipoprotein receptor expression from the very acute stage of ischemic stroke (3 h after onset) to 7 days after onset in a mouse stroke model.

**Materials and Methods**

**Experimental Mice**

Male C57Bl/6NcrSlc mice (Sankyo Laboratory Animal Center, Tokyo, Japan; N = 84) and male knockout (KO) mice on a C57BL/6J background (The Jackson Laboratory, Bar Harbor, ME; N = 3) aged 8 to 10 weeks were used. Of these mice, 75 were subjected to permanent MCAO, and 16 underwent a sham operation. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Permit number: A2017-223C and 2016-030C2).

**Permanent MCAO (pMCAO) Model**

An investigator blinded with respect to the treatment group performed all assessments. Mice were anesthetized, body temperature was monitored, and the left MCA was visualized and occluded as described previously, except that a microbipolar electrocoagulator (VETROSON V10, Summit Hill Laboratories, Tinton Falls, NJ, USA) was used. After replacing the temporal muscle, the skin was sutured and the mice were kept in a recovery cage until they recovered from anaesthesia. Sham surgery was performed by using the same process but without occlusion.

**Measurement of cerebral blood flow by laser Doppler flowmetry**

A laser Doppler flow meter (TBF-LN1; Unique Medical Company, Tokyo, Japan) and probe (Type-CS; Advance Co. Inc, Tokyo, Japan) were used to monitor relative changes in cerebral blood flow (CBF). CBF was recorded over the left lateral skull by placing the laser Doppler probe at a position 2.0 mm posterior and 2.5 mm lateral to bregma. Mice were anesthetized with 2% isoflurane and CBF was measured immediately before and after the occlusion, and at 3 days after operation. CBF values are expressed as percentages of the pre-operation value.
Evaluation of BBB permeability by using Evans blue

Evans blue (EB, 2% in saline, 4 mL/kg), which binds to albumin, was intravenously injected via the tail vein 3 h before the mice were euthanized. BBB permeability was evaluated by measuring the area of extravasated EB as described previously20. Sham-operated and pMCAO mice (n = 4 for each group) were transcardially perfused with 4% paraformaldehyde and brains were quickly removed and cut in 1-mm-thick coronal sections by using a mouse brain slice matrix (Harvard Apparatus, Holliston, MA, USA) apart from 2.2 mm anterior to 2.2 mm posterior to bregma. The photographs of four serial sections were taken through a stereoscopic microscope (Olympus SZX7, Olympus, Tokyo, Japan). The area of EB extravasation in the ipsilateral hemisphere and the area of contralateral hemisphere were measured with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The total area of EB extravasation in the ipsilateral hemisphere was calculated as a percentage of the relative area of the contralateral hemisphere.

Cell culture and OGD procedures

Mouse brain ECs (bEnd.3 cell line; American Type Culture Collection, Manassas, VA, USA) were maintained at 37 °C in a 5 % CO2 /95% air atmosphere in Dulbecco’s modified Eagle’s medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing glucose (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Gibco Laboratories, Gaithersburg, MD, USA) and 1% antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin); the medium was changed every 3 to 4 days. To mimic the ischemic conditions in vitro, bEnd.3 cells were subjected to OGD as described previously21. Briefly, bEnd.3 cells were cultured in 6-well culture plates at a density of 3 × 10^4 cells/cm^2 and incubated with glucose-free DMEM in a closed chamber (Billups-Rothenberg Inc., San Diego, CA) filled with OGD gas mixture (5% CO2, 95% N2) for 2, 4, or 6 h at 37 °C before collection for a quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay. To simulate pMCAO rather than transient MCAO, we did not provide re-oxygenation.

qRT-PCR assay

Total RNA was extracted from mouse brains and bEnd.3 cells by using Isogen (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer’s protocol. For brains, 27 mice subjected to pMCAO (n = 3 for each group) and three mice subjected to sham operations were deeply anesthetized and perfused with cold phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO) through the left cardiac ventricle. The ipsilateral cerebral ischemic cortex and contralateral non-ischemic cortex were separately dissected, immediately frozen in liquid nitrogen, homogenized in isotonic buffer, and subjected to RNA extraction. For cells, Isogen buffer was added directly into the culture plates after OGD, and cells were lysed mechanically, and subjected to RNA extraction. Reverse transcription of 100 ng RNA to cDNA was performed with Transcriptor Universal cDNA Master (Roche Diagnostics, Mannheim, Germany) by using the following conditions: 25 °C for 5 min, 55 °C for 10 min, and 85 °C for 5 min. Quantitative PCR of the resultant DNA was performed by using the TaqMan probe master and a Light Cycler 480 Real Time PCR Instrument (Roche Diagnostics) with the primers for mouse LDLR (Mm00440169_m1), SRB1 (Mm00450234_m1), LRP1 (Mm00464608_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 4352932E) supplied in the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). Relative LDLR, SRB1, and LRP1 mRNA levels were calculated in comparison to GAPDH mRNA levels as an internal control.

Preparation of the microvascular fraction of the brain

The microvascular fraction (i.e., EC fraction) of the brain was prepared as described previously22. Briefly, after either the sham operation or pMCAO, ipsilateral brain were separately homogenized in PBS and centrifuged for 5 min at 800 × g at 4 °C. The pellet was suspended in a 15% dextran solution and centrifuged for 10 min at 4500 × g at 4 °C. The pellet was solubilized and homogenized in Radioimmunoprecipitation (RIPA) buffer (50 mmol/L Tris HCl (pH 8.0), 150 mmol/L sodium chloride, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), and 1.0% (w/v) NP-40 substitute), with 1× Complete protease inhibitor cocktail (Roche Diagnostics) for western blot analysis.

Western blot analysis

The ipsilateral brain after either the sham operation or pMCAO were dissected and homogenized in RIPA buffer with 1× Complete protease inhibitor cocktail (Roche Diagnostics). Homogenates were centrifuged at 16,000 × g at 4 °C for 20 min. Protein concentrations of the supernatants were determined with a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA) using bovine serum albumin as the standard.
Samples (40 μL) were mixed with 10 μL of 5× Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA), and then denatured at 95 °C for 30 min. Proteins were separated by electrophoresis in a 5%–20% SDS-polyacrylamide gel (ATTO Corporation, Tokyo, Japan), and transferred to a polyvinylidene difluoride (PVDF) membrane. Blots were probed with a mouse monoclonal antibody against occludin (Thermo Fisher Scientific) at a dilution of 1:1000, a rabbit polyclonal antibody against LDLR (Abcam, Cambridge, UK) at a dilution of 1:5000, a rabbit monoclonal antibody against SRB1 (Abcam) at a dilution of 1:5000, a Jackson ImmunoResearch, Jennersville, PA, USA labelled with horseradish peroxidase, or probed with anti-β-actin antibody (1:2000, Wako Pure Chemical Industries, Osaka, Japan) labelled with horseradish peroxidase. Blots were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and by using the ChemiDoc Touch Imaging System (Bio-Rad Laboratories). Band intensities were quantified by using Image Lab software.

Histology and immunofluorescence staining
Twelve mice were subjected to pMCAO (n = 4 each for sham-operation, 6 h and 3 days after pMCAO) and four sham mice were sacrificed to assess LDLR, SRB1, and LRP1 expression after ischemic stroke. Tissues were fixed by transcardiac perfusion with 4% paraformaldehyde under deep anaesthesia. The brains were quickly removed, incubated in 4% paraformaldehyde overnight, and then incubated in 10% and 20% sucrose in PBS for over 12 h, respectively. The brains were frozen rapidly on dry ice and cut into 20-μm serial coronal sections from bregma level +1.2 mm to –2.0 mm, mounted on slides, and processed for staining. To evaluate ischemic lesions at 3 days post-ischemia, the sections were stained with cresyl violet. For double immunohistochemical staining, the sections were washed with PBS and blocked with 10% normal goat serum in PBS, and then incubated overnight at 4 °C with the two primary antibodies diluted with 10% normal goat serum. The primary antibodies used were as follows: mouse anti-occludin antibody (1:300, Thermo Fisher Scientific); rabbit anti-LDLR antibody (1:100, Abcam); rabbit anti-SRB1 antibody (1:100, Abcam); rat anti-mouse CD31 antibody (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) for detection of ECs; and mouse anti-NeuN antibody (1:100, EMD Millipore, Darmstadt, Germany) for detection of neurons. Subsequently, sections were incubated with fluorescence-labeled secondary antibodies for 1 h at room temperature. After washing in PBS, fluorescently stained sections were mounted with Vectorshield (Vector Laboratories, Burlingame, CA) and observed under a confocal laser scanning microscope (LSM510; Zeiss, Oberkochen, Germany).

Measurement of serum LDL cholesterol concentration
Blood samples were collected from mice (n = 4) prior to and 6 h after pMCAO, respectively. Serum LDL cholesterol levels were measured (SRL Inc., Tokyo, Japan).

Statistics
All values are expressed as the mean ± SEM. Statistical differences among the various groups were mainly assessed with one-way analysis of variance (ANOVA) followed by Dunnett’s one-tailed test, except for the percentage of EB extravasation area which was analyzed by Turkey-Kramer one-tailed test. The differences between two groups are compared with Student’s two-tailed t-test. The statistical software Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA) was used for statistical analyses; P values less than 0.05 were considered to be statistically significant.

Results
Dynamics of BBB disruption after pMCAO induction
Sham-operated controls did not show any detectable pathologic changes over 7 days with cresyl violet staining. In contrast, in all pMCAO mice, infarction was distributed in the ipsilateral cerebral cortex and part of the lateral striatum (Figure 1A). Immediately after pMCAO, CBF was significantly reduced to 40.5% ± 5.1% of baseline level (P < 0.001); even 3 days after the operation CBF was still significantly reduced to 59.7% ± 6.1% of baseline level (P < 0.001). (n = 6 for pMCAO group, F_{2,15} = 123.5, P < 0.0001, one-way ANOVA followed by Dunnett’s test) (Figure 1B).

To elucidate the time course of BBB permeability after pMCAO, extravasation of EB was evaluated at 3 h, 6 h, 1 day, 3 days, and 7 days after pMCAO. Blue-colored spots in the brain due to BBB disruption were identified in all pMCAO mice, indicating that the BBB was disrupted as early as 3 h and reached a peak at 3 days after pMCAO (Figure 1C). The area of EB extravasation in the ipsilateral hemisphere expressed as a percentage of the area of the contralateral hemisphere was significantly increased at
6 h (19.3% ± 1.6%, P = 0.001), 3 days (55.4% ± 3.0%, P < 0.001), and 7 days (25.8% ± 2.7%, P < 0.001) compared with 3 h (19.3% ± 1.6%, P = 0.001), 3 days (55.4% ± 3.0%, P < 0.001) compared with 3 h (9.9% ± 0.9%), but not at 1 day (14.3% ± 1.5%, P = 0.12) after pMCAO (n = 4 for each group. $F_{4,15} = 98.72$, $P < 0.0001$, one-way ANOVA followed by Tukey-Kramer test) (Figure 1D).

To evaluate the TJ protein at each of the biphasic peaks of BBB disruption after pMCAO, we examined the TJ protein occludin at 6 h and 3 days after pMCAO (Figure 1E-G). The occludin levels were not significantly different to the level in sham-operated control level at 6 h after pMCAO (0.78 ± 0.02 times the control level, $P = 0.11$), but were significantly decreased at 3 days after pMCAO (0.55 ± 0.03 times control level, $P = 0.002$) (n = 3 for each group. $F_{2,6} = 20.23$, $P = 0.002$, one-way ANOVA followed by Dunnett’s test; Figure 1E, 1F). By immunofluorescence staining, occludin signals were detected on CD31-positive ECs in the ischemic lesion at 6 h after pMCAO, but the signals were remarkably decreased at 3 days after the operation (Figure 1G).

**Changes in lipoprotein receptor mRNA levels in brain ECs after OGD**

To evaluate SRB1, LDLR, and LRP1 mRNA levels after permanent ischemia in vitro, mouse brain ECs (bEnd.3 cells) were subjected to OGD for 2, 4, or 6 h without reperfusion. The qRT-PCR assays showed that the LDLR mRNA level significantly increased after 2 h of OGD to reach 1.56 ± 0.03 times the non-OGD (control) level ($P < 0.001$); the level then significantly decreased by 6 h of...
OGD to become 0.59 ± 0.08 times the control level \((P = 0.004)\) (\(n = 4\) for each group, \(F_{3,12} = 46.51, P < 0.0001\), one-way ANOVA followed by Dunnett’s test; Figure 2A).

Similarly, the SRB1 mRNA level significantly increased after 2 h of OGD to reach 1.29 ± 0.03 times the control level \((P < 0.001)\), but significantly decreased after 6 h of OGD to become 0.72 ± 0.04 times the control level \((P < 0.001)\) (\(n = 4\) for each group, \(F_{3,12} = 54.10, P < 0.0001\), one-way ANOVA followed by Dunnett’s test; Figure 2B).

In contrast, LRP1 mRNA levels in mouse brain ECs did not change significantly at 2, 4, or 6 h after OGD compared with those in the controls (\(n = 4\) for each group, \(F_{3,12} = 3.38, P = 0.05\), one-way ANOVA followed by Dunnett’s test) (Figure 2C).

Lipoprotein receptor mRNA levels were upregulated in the ischemic brain in the early phase after pMCAO

We quantified mRNA levels of lipoprotein receptors, LDLR, SRB1, and LRP1, in sham-operated (control) and pMCAO groups after surgery. The qRT-PCR analyses showed that the LDLR mRNA levels increased significantly at 6 h and 9 h after pMCAO to reach 2.19 ± 0.24 and 2.98 ± 0.85 times \((P = 0.004\) and \(P < 0.001\), respectively) than the sham control level, respectively (\(n = 3\) for each group, \(F_{9,20} = 10.81, P < 0.0001\), one-way ANOVA followed by Dunnett’s test; Figure 3A). The SRB1 mRNA levels increased significantly at 6 h, 9 h, and 15 h after pMCAO to reach 2.35 ± 0.04, 2.44 ± 0.29, and 1.92 ± 0.13 times \((P < 0.001, P < 0.001\) and \(P = 0.006\), respectively) than the sham control level, respectively (\(n = 3\) for each group, \(F_{9,20} = 10.81, P < 0.0001\), one-way ANOVA followed by Dunnett’s test; Figure 3B). The LRP1 mRNA levels did not change significantly at 2, 6, or 12 h after pMCAO compared with those in the controls (\(n = 3\) for each group, \(F_{2,6} = 1.44, P = 0.27\), one-way ANOVA followed by Dunnett’s test; Figure 3C).
Figure 2. Quantification of mRNA levels of lipoprotein receptors in bEnd.3 cells subjected to oxygen and glucose deprivation (OGD). (A) Relative LDLR mRNA levels increased in bEnd.3 cells after 2 h of OGD, but decreased after 6 h of OGD compared with the control (non-OGD). (B) Relative SRB1 mRNA levels increased in bEnd.3 cells after 2 h of OGD, but decreased after 6 h of OGD compared with the control. (C) Relative LRP1 mRNA levels in bEnd.3 cells at 2, 4, and 6 h after OGD did not differ significantly from the control. **P < 0.01, ***P < 0.001 compared with the control, one-way ANOVA followed by Dunnett’s test).

Figure 3. Increased expression of lipoprotein receptors during the early phase after pMCAO. Measurement of LDLR (A), SRB1 (B), and LRP1 (C) mRNA levels in the ipsilateral cortex of sham-operated and pMCAO mice by using qRT-PCR. (n = 3 for sham-operated group and each pMCAO group, *P < 0.05, **P < 0.01, ***P < 0.001 compared with sham-operated controls; one-way ANOVA followed by Dunnett’s test).
respectively) than the sham control level, respectively (n = 3 for each group, F9,20 = 9.67, P < 0.0001, one-way ANOVA followed by Dunnett’s test; Figure 3B). The LRP1 mRNA levels increased significantly in the early phase at 6 h, 9 h, and 15 h after pMCAO to reach 2.14 ± 0.20, 1.87 ± 0.12, and 1.63 ± 0.10 times (P < 0.001, P < 0.001 and P = 0.004, respectively) than the sham control level, respectively, but also remained significantly higher than in the control in the late phase at 7 days after pMCAO (1.64 ± 0.15 times the control level, P = 0.02) (n = 3 for each group, F9,20 = 6.28, P = 0.0003, one-way ANOVA followed by Dunnett’s test; Figure 3C).

Figure 4. Increase in expression of LDLR and SRB1 in brain ECs in the ipsilateral cortex after pMCAO. Confocal immunofluorescence double-labeling images with antibodies against LDLR (red, A), SRB1 (red, B), and the endothelial-specific marker CD31 (green) in sham-operated and the ipsilateral ischemic cortex at 6 h after induction of pMCAO (scale bars = 50 μm). Strong LDLR (A) and SRB1 (B) signals were detected in microvascular cells in the ischemic cortex, but not in the cortex of the sham-operated control mice. LDLR signals were also detected on neuron-like cells (blue arrowheads). (C) Representative Western blot analysis of LDLR and SRB1 levels in the purified EC fraction of brains in sham-operated and ipsilateral ischemic cortex at 6 h and 3 days after pMCAO. Reblotting with anti-β-actin was performed as a loading control. Comparison of quantitative densitometric analyses of LDLR (D) and SRB1 (E) protein levels in the brains. (n = 3 for each group. *P < 0.05, **P < 0.01, ***P < 0.001 compared with sham-operated group; One-way ANOVA followed by Dunnett’s test).

**Upregulation of LDLR and SRB1 in brain ECs in ischemic cortex after pMCAO**

We immunohistochemically examined the expression patterns of LDLR and SRB1 in brains of sham-operated mice and pMCAO mice at 6 h after surgery, which is the time point of upregulation of the corresponding mRNAs. LDLR signals were not detected in the cortex of sham-operated mice (Figure 4A) or in the contralateral cortex of pMCAO mice, but were strongly detected on both the CD31-positive microvessels and/or neuron-like cells (blue arrowheads) in the ipsilateral ischemic cortex (Figure 4A). The SRB1 signals primarily colocalized with CD31-positive microvessels in the ipsilateral ischemic cortex, but were not apparent in the cortex of sham-operated mice (Figure 4B) or in the contralateral cortex of pMCAO mice.
To confirm the increased LDLR and SRB1 expression in brain ECs, we purified the EC fraction of the brains of sham-operated mice and of the ipsilateral ischemic cortex at 6 h and 3 days after pMCAO, and then measured LDLR and SRB1 protein levels by Western blots analysis. The LDLR protein level in purified brain ECs significantly increased by 6 h after pMCAO to reach 1.23 ± 0.04 times the control level (P = 0.02), but was significantly decreased by 3 days after pMCAO to become 0.25 ± 0.07 times the control level (P < 0.001). (n = 3 for each group, F2, 6 = 94.48, P < 0.0001, one-way ANOVA followed by Dunnett’s test; Figure 4C, 4D). The SRB1 protein level was significantly increased at 6 h after pMCAO (2.10 ± 0.18 times the control level, P=0.002), but was similar to the control level at 3 days after pMCAO (0.78 ± 0.10 times control level, P = 0.36) (n = 3 for each group, F2, 6 = 19.28, P = 0.0024, one-way ANOVA followed by Dunnett’s test; Figure 4C, 4E).

These results indicate that brain ischemia enhanced LDLR and SRB1 expression primarily in brain ECs immediately after the onset of ischemia.

Upregulation of LDLR and SRB1 mRNA in neurons in ischemic cortex after pMCAO

We could not detect increased LDLR mRNA expression in mouse ECs after OGD in vitro, although the LDLR mRNA levels were rapidly increased in the ischemic brain after pMCAO in vivo. Therefore, we examined what cell type, other than ECs, expresses LDLR in the ischemic brain at 6 h after pMCAO. Immunohistochemical LDLR signals were detected both in the cortex of sham-operated mice (Figure 5A) and pMCAO mice (Figure 5B), and colocalized with neuronal cell marker NeuN. The LDLR signals on neurons were markedly increased in the ischemic cortex compared with the non-ischemic cortex of sham controls (Figure 5A, B). These results indicate that brain ischemia enhanced LDLR expression primarily in neurons, but not ECs, soon after the onset of ischemia.

Decreased EB extravasation in LDLR knockout mice after pMCAO

To elucidate the different BBB permeability between C57BL/6 wild type (WT) and LDLR knockout (KO) mice in early phase of ischemic stroke, extravasation of EB was evaluated 6 h after pMCAO, respectively (Figure 6A). The area of EB extravasation in the ipsilateral hemisphere expressed as a percentage of the area of the contralateral hemisphere was obviously decreased in LDLR KO mice (11.6% ± 1.1%) compared with WT mice.
(19.3% ± 1.6%) with statistically significant at 6 h after pMCAO operation. (n = 3 for KO and n = 4 for WT groups. P = 0.009, Student’s two tailed t-test) (Figure 6B), which indicates that LDLR may play a causative role as a transporter in early phase of ischemic stroke.

**Evaluation of serum LDL cholesterol before and after pMCAO**

Because a previous study reported that LDLR expression is sensitive to serum cholesterol concentration, we measured the serum levels of LDL cholesterol before and 6 h after pMCAO. The serum LDL cholesterol levels did not differ between groups (11.0 ± 0.9, mg/dL in before pMCAO vs. 10.0 ± 0.4, mg/dL in 6 h after pMCAO, n = 4, P = 0.36, Student’s two tailed t-test).

**Discussion**

In the current study, our EB extravasation results showed a biphasic pattern of BBB disruption in the pMCAO mouse model. Extravasated EB, an indicator of transported albumin, was detected as early as 3 h after pMCAO, and the EB area was extended in ischemic cortex at 6 h, and reduced at 1 day, and subsequently extended again at 3 days. The protein level of TJ protein occludin, which limits paracellular diffusion, was preserved at 6 h but significantly decreased at 3 days after pMCAO, indicating that transcellular diffusion was the major route for albumin transport within 6 h of ischemic stroke onset. In this initial phase of ischemic stroke, we found that lipoprotein receptors of LDLR and SRB1 were significantly upregulated in ECs and LRP1 was significantly upregulated in neurons in the ipsilateral ischemic cortex, whereas expression of lipoprotein receptors decreased from 24 h after pMCAO.

**TJ disruption and paracellular diffusion after ischemic stroke**

Consistent with previous reports in a transient ischemia/reperfusion MCAO rodent model, we found a biphasic EB extravasation pattern in the pMCAO mouse model. The EB assay is a commonly used technique for detecting BBB disruption, based on the ability of EB dye to bind to serum albumin immediately following its intravenous injection. Since serum albumin does not cross the BBB under normal conditions, the blue region of EB extravasation indicates the location of vascular leakage after stroke. Since albumin crosses the BBB by both the receptor-mediated transcellular pathway and the paracellular pathway through the TJ under ischemic
Upregulation of lipoprotein receptors after acute stroke

The members of the LDL receptor family, LDLR and LRP1, and the HDL receptor SRB1 are expressed in numerous cell types and play an important role in cholesterol metabolism in the peripheral circulation. These receptors are also highly expressed within the CNS. They reside on ECs, neurons, and/or glial cells and are involved in receptor-mediated endocytosis or transcytosis of lipoprotein particles such as LDL or HDL under normal conditions. According to previous reports, LDLR was one of the cell surface lipoprotein receptors that undergo endocytosis and thus carry a cell surface-bound particle, such as the internalized LDL cholesterol, into endosome of the hepatocyte cell where it is directly fused to the lysosome for proteolytic degradation. Then, the LDLR is recycled back to the hepatocyte cell surface. There are also some evidences that LDLR is expressed at the brain ECs involved in a receptor-mediated transcytosis process from blood lumen to brain parenchyma (BBB). Furthermore, transcellular barrier impairment occurs during the first 24 h after onset, allowing albumins and other possibly selected molecules to enter the brain in a transporter-dependent manner after ischemic stroke. However, it is not still elucidated that lipoprotein receptors such as LDLR were involved in this transcellular barrier impairment at the early phase of brain ischemia. In our study, EB extravasation reduction in LDLR KO mice compared with WT mice at 6 h after pMCAO indicates that LDLR contribute to, if possibility, transcellular pathway after ischemia. These results speculate that upregulated LDLR may partially play a causative role as a transporter via LDLR-mediated transcellular pathway in early phase of ischemic stroke.

Although the temporal profile of LDLR and SRB1 expression in the acute phase of stroke has not been examined until now, LRP1 has been reported to be upregulated in both brain ECs and neurons in the ischemic cortex within 6 h of transient ischemia/reperfusion MCAO in mice. Here, by using the pMCAO model in which the MCA is occluded permanently, we showed that LRP1 mRNA levels also increased at 6-15 h after surgery, but were not upregulated in ECs after OGD in vitro. LRP1 immunohistochemical signals were primarily colocalized with the neuronal marker, not the EC marker, in the ischemic lesions. These results collectively suggest that the presence of reperfusion after ischemia may be required to upregulate the LRP1 expression on brain ECs.

A previous report showed that LDLR in the cytoplasm of neurons was upregulated in ischemic lesions at 7 and 21 days after transient-MCAO in rats, and that upregulation of ApoE signals in neurons correlated with enhanced LDLR expression. These results indicate that enhanced LDLR on neurons, but not brain ECs, in the late stage of stroke may play an important role in lipid transport. Interestingly, both LDLR mRNA and protein levels in purified brain ECs fraction decreased at 3 days after pMCAO in our study. According to previous reports, the cell membranes of ECs and neurons are disintegrated and the lipid products such as cholesterol are generated after brain ischemia. Therefore, we speculated that cell cholesterol increased and generated by the disintegrated cells membranes and then inhibit LDLR expression at 3 days and later after brain ischemia. Here, we found that the major three lipoprotein receptors of the brain, which are involved in LDL and HDL endocytosis and transcytosis across the BBB, LDLR and SRB1 were colocalized predominantly in brain ECs, whereas LRP1 was localized in neurons in the ischemic cortex at this early phase of ischemic stroke. Utilization of lipoprotein receptor-mediated endocytosis...
and transcytosis, which are significantly driven by ischemic stress, may enable early effective intervention in the early phase of ischemic stroke.


References

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