

Increased Blood–Brain Barrier Permeability and Brain Edema After Focal Cerebral Ischemia Induced by Hyperlipidemia

Role of Lipid Peroxidation and Calpain-1/2, Matrix Metalloproteinase-2/9, and RhoA Overactivation

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Background and Purpose—Hyperlipidemia is a highly prevalent risk factor for ischemic stroke. Its impact on brain injury and blood–brain barrier permeability, so far, has not been assessed in animal models of ischemic stroke.

Methods—Wild-type and apolipoprotein E^{-/-} mice, fed with normal or cholesterol-rich high-fat food, were subjected to 30 minutes of middle cerebral artery occlusion. Ischemic injury, brain edema, IgG extravasation, lipid peroxidation, calpain-1/2, matrix metalloproteinase-2/9, and RhoA activation, and occludin expression were evaluated 24 hours after reperfusion.

Results—Cholesterol-rich food, but not apolipoprotein E deficiency, increased IgG extravasation and brain edema without influencing infarct area and the density of DNA fragmented cells. Increased lipid peroxidation and low-density lipoprotein oxidation were noticed in the brain of hyperlipidemic mice and were associated with increased activation of calpain-1/2 and matrix metalloproteinase-2/9, overactivation of RhoA and its guanine exchange factor leukemia-associated guanine exchange factor, and downregulation of the tight junction protein occludin in cerebral microvessels.

Conclusions—That postischemic blood–brain barrier permeability and brain edema are increased during hyperlipidemia points toward the importance of the recognition and adequate treatment of this highly prevalent condition. Translational studies should more adequately mimic risk factors prevalent in human stroke. (*Stroke*. 2011;42:3238–3244.)

Key Words: apolipoprotein E ■ hyperlipidemia ■ lipid peroxidation ■ middle cerebral artery occlusion ■ Rho-GTPase

Stroke therapies have made little progress in recent years. Several pharmacological compounds stabilizing blood–brain barrier (BBB) integrity or promoting neuronal survival that had been successful in animal studies were tested in human patients with ischemic stroke.^{1,2} Apart from thrombolytics, none of these compounds were effective under clinical conditions.^{1,2} A major limitation of animal stroke studies is that mostly young animals without risk factors are evaluated.² Such studies barely mimic the pathophysiology of ischemic stroke in humans, which often affects elderly subjects with unfavorable life habits or cerebrovascular risk factors.²

Hyperlipidemia induced by excess or a high-cholesterol diet is a highly prevalent condition in ischemic stroke patients. In large clinical trials or patient registries, ≈45% to 60% of patients exhibit elevated serum cholesterol levels.^{3,4} Cholesterol levels >7.0 mmol/L are associated with an elevated stroke risk,⁵ which in clinics may successfully be

reduced by cholesterol-lowering drugs.⁶ In view of the huge importance of hyperlipidemia for stroke pathology, it is surprising that not much is known from experimental studies about how hyperlipidemia affects brain injury once a stroke has occurred.

In apolipoprotein E (ApoE)^{-/-} mice that exhibit enhanced atherosclerosis, increased BBB permeability has previously been reported on cortex trauma and aging when animals were placed on a cholesterol-rich Western diet.^{7,8} After cortex trauma, the authors did not observe increased BBB permeability in hyperlipidemic wild-type mice,⁷ suggesting that excessive cholesterol levels or preexisting atherosclerosis are needed for vascular permeability changes to be induced. Plasma cholesterol levels are ≈5- to 8-times higher in ApoE^{-/-} than in wild-type mice.^{9–11} Whether hyperlipidemia influences BBB integrity after ischemic stroke was unknown.

To elucidate effects of hyperlipidemia on ischemic injury and BBB integrity, we submitted wild-type and ApoE^{-/-}

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mice that were kept on a regular or Western diet to focal cerebral ischemia. After showing that Western diet, but not ApoE^{-/-}, increased BBB permeability, thus resulting in enhanced brain swelling, we performed a more detailed analysis of mechanisms contributing to BBB dysfunction in which we investigated lipid deposition and oxidation, the activation of calpain-1/2, matrix metalloproteinase (MMP)-2/9, and Rho GTPase RhoA, and the expression of the tight junction protein occludin.

Materials and Methods

Animal Groups

Experiments were performed with government approval (Bezirksregierung Düsseldorf) according to the National Institutes of Health guidelines for the care and use of laboratory animals. Male C57BL6/j or ApoE^{-/-} mice that were fed with regular or cholesterol-rich high-fat chow (ie, Western diet; TD88137; Harlan Teklad) for 6 weeks starting at the age of 6 weeks were submitted to 30 minutes of middle cerebral artery (MCA) occlusion followed by 24 hours reperfusion. One set of animals (n=6/group) was used for histological analysis and molecular biological studies using brain capillary extracts. Another set of animals (n=4/group) was used for lipid peroxidation measurements using whole brain homogenates and for total cholesterol level measurements in blood.

Induction of Focal Cerebral Ischemia

MCA occlusion was induced using an intraluminal filament technique, as previously described,^{12,13} whereas laser Doppler flow was monitored above the intact skull overlying the MCA territory. Twenty-four hours after stroke, animals were euthanized by transcardiac perfusion with normal saline. Brains were removed and cut on a cryostat into coronal 20- μ m sections that were retrieved at the rostrocaudal level of the bregma, which is the level at which MCA infarcts are most reproducible. Tissue samples were collected from the ischemic and contralateral nonischemic MCA territory (striatum and overlying cortex) for lipid oxidation, Western blotting, immunoprecipitation studies, and protease activity assays.

Cholesterol Concentration Measurements

To investigate the effect of the high-fat chow on cholesterol levels in the blood, serum samples from individual animals (n=4/group) were analyzed using the cholesterol/cholesteryl ester quantitation kit (ab65359; Abcam). Serum cholesterol levels were 56.6 \pm 17.4 mg/dL for wild-type/normal diet, 220.4 \pm 58.0 mg/dL for wild-type/Western diet, 470.9 \pm 276.9 mg/dL for ApoE^{-/-}/normal diet, and 1279.1 \pm 528.2 mg/dL for ApoE^{-/-}/Western diet, similar to previous reports.^{9–11}

Analysis of Ischemic Injury, Brain Swelling, and IgG Extravasation

Representative brain sections were stained with 0.5% cresyl violet. On these sections, the lesion area and brain swelling were outlined as described.^{12,13} Briefly, stained sections were digitized and infarct borders between infarcted and noninfarcted tissue were outlined using an image analysis system (Image J; National Institutes of Health). Infarct size was measured by subtracting the area of the nonlesioned ipsilateral hemisphere from that of the contralateral hemisphere, and brain swelling size was measured by subtracting the area of ipsilateral hemisphere from that of contralateral hemisphere. Adjacent sections were processed for immunohistochemistry for extravasated IgG, which was studied as an endogenous marker of BBB permeability, using biotinylated antimouse IgG antibody (Santa Cruz Biotechnology) that was revealed with an avidin peroxidase kit (Vectastain Elite; Vector Labs) and diaminobenzidine (Sigma).¹³

Sections were digitized and evaluated for areas exhibiting IgG extravasation.

Analysis of DNA Fragmentation

Brain sections obtained from the midstriatum were fixed for 20 minutes at 4°C with 4% paraformaldehyde/0.1 mol/L phosphate-buffered saline. Terminal transferase dUTP nick end labeling was performed as described.¹⁴ Briefly, after labeling with terminal deoxynucleotidyl transferase mix, containing 12.5 mg/mL terminal deoxynucleotidyl transferase (Boehringer-Mannheim) and 25 mg/mL biotinylated dUTP (Boehringer-Mannheim), sections were stained with streptavidin-FITC, counterstained with 4',6-diamidino-2-phenylindole, and cover-slipped. DNA-fragmented cells were microscopically evaluated by counting the density of transferase dUTP nick end labeling-positive profiles in 3 regions of interest of the lateral ischemic striatum that were 250 μ m apart (each regions of interest measuring 62 500 μ m²). Mean values were calculated for all areas. The striatum was selected because 30 minutes of MCA occlusion results in striatal but not cortical injury.^{12,14}

Lipid Deposition and Oxidation Studies

For visualization of lipid deposits, brain sections of animals kept under normolipidemia and hyperlipidemia were fixed in 4% paraformaldehyde/0.1 mol/L phosphate-buffered saline, washed for 15 minutes in water, immersed in 60% isopropanol, and incubated for 15 minutes in 0.3% (wt/vol) oil red-O staining solution in 60% isopropanol. Sections were washed with 60% isopropanol, immersed in hematoxylin solution, washed with water, and cover-slipped.

To investigate the effect of hyperlipidemia on brain lipid oxidation, brain tissue samples from individual animals were homogenized and supplemented with 5% protease inhibitor cocktail (P8340; Sigma) and 1% phosphatase inhibitor cocktail-2 (P5726; Sigma). In these samples, the concentration of malondialdehyde, an end product formed during the degradation of lipids during lipid peroxidation, and of oxidized low-density lipoprotein were measured using the OxiSelect malondialdehyde Adduct (STA-332; Cell Biolabs) and oxidized low-density lipoprotein (CSB-E07933m; Cusabio Biotech) enzyme-linked immunosorbent assay kits.

Microvessel Isolation and Protein Extraction

Tissue samples from animals belonging to the same group were pooled and homogenized in ice-cold microvessel isolation buffer supplemented with 5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail-2.¹² Homogenates were centrifuged. Pellets were resuspended in 20% dextran (molecular weight, 64 000–76 000; D4751; Sigma) in microvessel isolation buffer, centrifuged, again resuspended, and filtered through 2 nylon filters using mesh sizes of 100 μ m and 30 μ m (Millipore). Isolated microvessels were homogenized,¹² supplemented with 5% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail-2, and sonicated. Protein concentrations were measured using the Bradford assay kit.

Calpain-1/2 and MMP-2/9 Activity Assays

To investigate calpain-1/2 and MMP-2/9 activities in brain microvessels, we established caseinase and gelatinase microplate assays, which evaluate caseinase and gelatinase activities in the presence of their endogenous inhibitors (Supplemental Methods, <http://stroke.ahajournals.org>). In addition, MMP-2/9 activity was evaluated by gelatin zymography,¹³ which differs from microplate assays in which the endogenous inhibitors of MMP-2/9 are absent during the digestion process. Each assay was performed 4 times. Activity was evaluated by measuring absorbance after incubation with G-250 dye with the iMark microplate reader (caseinase and gelatinase assays) or by densitometry after staining with Coomassie brilliant blue R-250 (Bio-Rad).

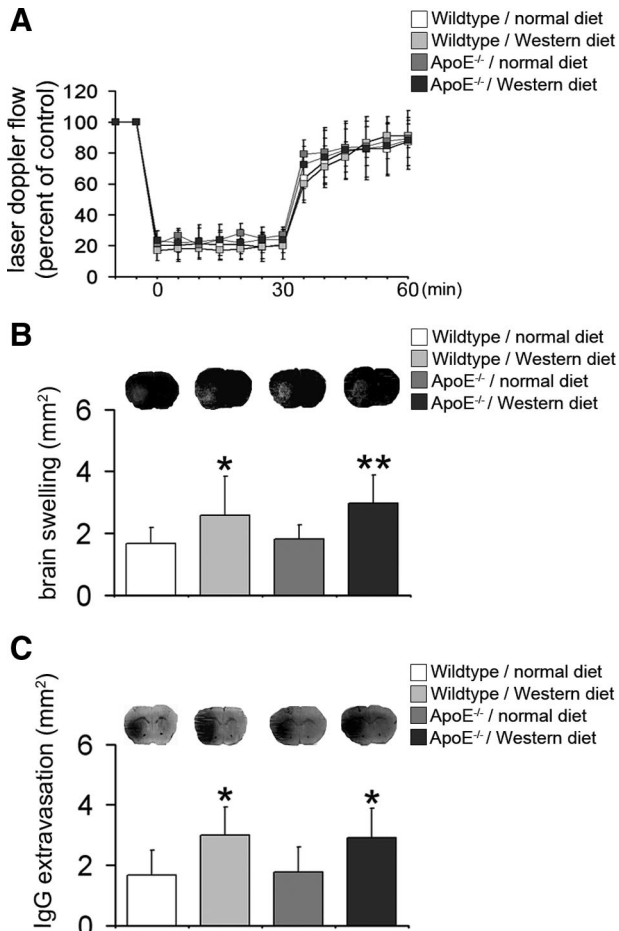


Figure 1. Blood-brain barrier (BBB) permeability is increased in hyperlipidemic mice after focal cerebral ischemia, accompanied by lipid accumulation and oxidation. Laser Doppler flow (LDF) above the core of the middle cerebral artery (MCA) territory (A), brain swelling (B), and serum IgG extravasation (C) measurement in brain tissue 24 hours after reperfusion in wild-type and apolipoprotein E (ApoE)^{-/-} mice receiving normal diet or cholesterol-rich (Western) diet. Note that LDF did not differ between groups (A) and that ApoE^{-/-} does not influence brain swelling (B) and serum IgG extravasation (C). Data are means±standard deviation (SD) (n=6 animals/group). **P*<0.05/***P*<0.01 compared with normal diet (1-way analysis of variance [ANOVA] followed by least significant difference [LSD] tests).

Western Blotting, Pull-Down Assays, and Coprecipitation Studies

For protein expression studies, samples were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis followed by Western blot analysis using primary antibodies diluted 1:1000 in 5% skim milk (Sigma) and 0.1 mol/L Tris-buffered saline-Triton X-100.¹² RhoA activity was evaluated by Rho GTPase affinity binding (pull-down) assay.¹⁵ RhoA binding to the guanine exchange factor leukemia-associated guanine exchange factor was assessed by coprecipitation experiments.¹⁵ All used antibodies and histochemicals are described in the Supplemental Methods section.

Statistics

Data were analyzed by 1-way analysis of variance followed by least significant differences tests. Two-way analysis of variance was also computed and results were reported in the text. Data are presented as means±standard deviation *P*<0.05 were considered significant.

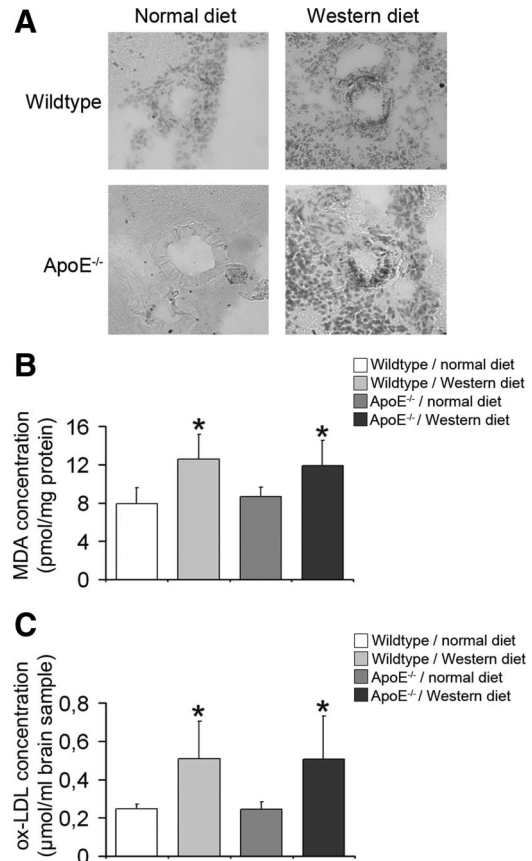


Figure 2. Lipids are accumulated in brain arterioles of hyperlipidemic mice, accompanied by increased lipid oxidation in the ischemic brain. Lipid accumulation in brain arterioles (A) as well as lipid oxidation evaluated by malondialdehyde (MDA) (B) and oxidized low-density lipoprotein (ox-LDL) (C) measurement in brain tissue 24 hours after reperfusion in wild-type and apolipoprotein E (ApoE)^{-/-} mice receiving normal diet or Western diet. Note that ApoE^{-/-} does not influence lipid accumulation in brain arterioles (A), lipid oxidation (B), and ox-LDL (C). Data are means±standard deviation (SD) (n=6 animals/group [A]/n=4 animals/group [C]). **P*<0.05 compared with normal diet (1-way analysis of variance [ANOVA] followed by least significant difference [LSD] tests).

Results

Increased Brain Swelling and IgG Extravasation in Ischemic Animals Receiving Cholesterol-Rich Diet

To evaluate the reproducibility of ischemia, laser Doppler flow measurements were analyzed. In all groups, laser Doppler flow decreased to ≈20% of baseline on MCA occlusion (Figure 1A). During reperfusion, blood flow rapidly resumed to baseline values. Laser Doppler flow recordings did not exhibit any differences between animals receiving regular (ie, normal) and cholesterol-rich (ie, Western) diet (Figure 1A).

At 24 hours after reperfusion, focal ischemic injury was observed mainly in the striatum. As such, mild brain swelling (Figure 1B) associated with modest IgG extravasation (Figure 1C) was noticed in animals on normal diet. Western diet markedly increased brain swelling and IgG extravasation (Figure 1B, C).

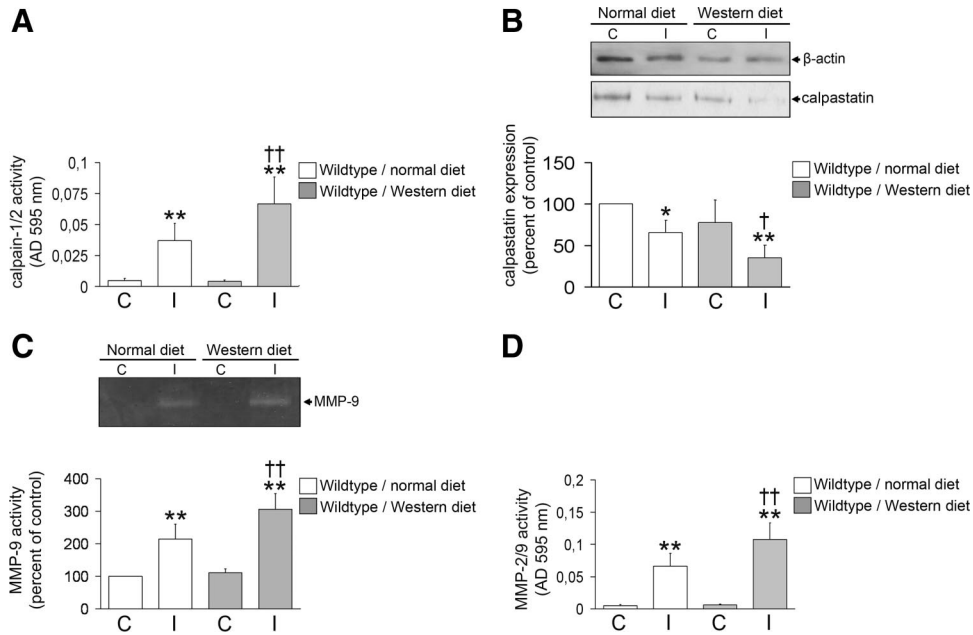


Figure 3. The proteases calpain-1/2 and matrix metalloproteinase (MMP)-2/9 are overactivated in ischemic cerebral microvessels of hyperlipidemic mice. Caseinase microplate assay for calpain-1/2 (A), Western blot analysis for the endogenous inhibitor of calpain 1/2, calpastatin (B), gelatinase zymography for MMP-9 (C), and gelatinase microplate assay for MMP-2/9 (D) using cerebral microvessel extracts of normolipidemic and hyperlipidemic mice 24 hours after middle cerebral artery (MCA) occlusion. Note that in parallel with the activation of calpain-1/2 (A), the endogenous inhibitor of calpain 1/2, calpastatin (B), is downregulated under conditions of hyperlipidemia. Data are means±standard deviation (SD) (n=4 assays, zymographies, or Western blots/ group). C, contralateral nonischemic; I, ischemic. **P*<0.05/***P*<0.01 compared with corresponding nonischemic/†*P*<0.05/††*P*<0.01 compared with ischemic normal diet (1-way analysis of variance [ANOVA] followed by least significant difference tests [LSD]).

It is noteworthy that ApoE deficiency did not have any effect on brain swelling and IgG extravasation (Figure 1B, C). The 2-way analysis of variance revealed an effect of Western diet, but not of ApoE^{-/-}, on brain swelling (*F*_{1,20}=9.784; *P*<0.01) and IgG extravasation (*F*_{1,20}=10.888; *P*<0.01).

Infarct area did not differ between groups (wild-type/normal diet, 5.0±1.5; wild-type/Western diet, 5.3±1.3; ApoE^{-/-}/normal diet, 5.1±1.6; ApoE^{-/-}/Western diet, 5.8±1.8 mm²). Similarly, DNA fragmentation analysis did not reveal any relevant changes (wild-type/ normal diet: 17.5±8.1; wild-type/ Western diet: 23.8±7.8; ApoE^{-/-}/ normal diet: 17.7±5.1; ApoE^{-/-}/ Western diet: 22.5±7.6 profiles/regions of interest).

Hyperlipidemia Induces Lipid Deposits in Brain Arterioles, Promoting Lipid Peroxidation After Focal Cerebral Ischemia

To characterize histopathologic changes induced by hyperlipidemia, oil red stainings were evaluated. Lipid deposits were noticed in brain arterioles of hyperlipidemic, but not in normolipidemic mice that were similar in wild-type and ApoE^{-/-} animals (Figure 2A). No lipid deposits were detected in brain capillaries.

In peripheral arteries, hyperlipidemia induces low-density lipoprotein oxidation, thus inducing endothelial dysfunction.¹⁶ To evaluate the contribution of lipid peroxidation to the development of BBB leakage, malondialdehyde and oxidized low-density lipoprotein were examined in brain tissue. Increased lipid peroxidation was noticed in ischemic tissue samples of hyperlipidemic compared with normolipi-

dem mice that did not differ between wild-type and ApoE^{-/-} animals (Figure 2B, C). Our results showed that although ApoE^{-/-} increases vascular dysfunction in peripheral arteries, thus promoting atherogenesis,^{17,18} its effect on lipid peroxidation in the ischemic brain is negligible.

Overactivation of Calpain-1/2 and MMP-2/9 in Ischemic Microvessels of Hyperlipidemic Mice

In the ischemic brain, calpain-1/2 and MMP-2/9 are known to contribute to the development of BBB breakdown.^{19,20} To evaluate how hyperlipidemia influences the activation of these proteases, calpain-1/2 and MMP-2/9 activities were studied by microplate assay and gelatin zymography in mice receiving normal and Western diets. Calpain-1/2 and MMP-2/9 activities did not differ in nonischemic microvessel extracts of normolipidemic and hyperlipidemic mice (Figure 3A, C, D).

On ischemia, calpain-1/2 activity increased in cerebral microvessels of normolipidemic mice (Figure 3A). Hyperlipidemia further elevated the calpain-1/2 activity (Figure 3A). Because the microplate assay evaluates protease activity in the presence of endogenous inhibitors, we checked the expression of the endogenous inhibitor of calpain-1/2, calpastatin, by Western blots. Calpastatin was downregulated by ischemia, more strongly in animals fed the cholesterol-rich than those fed normal diet (Figure 3B).

Similar to calpain-1/2, MMP-2/9 activity was increased in ischemic microvessels, as shown in gel zymography (Figure 3C) and microplate assay (Figure 3D). This activation was stronger in hyperlipidemic than in normolipidemic animals.

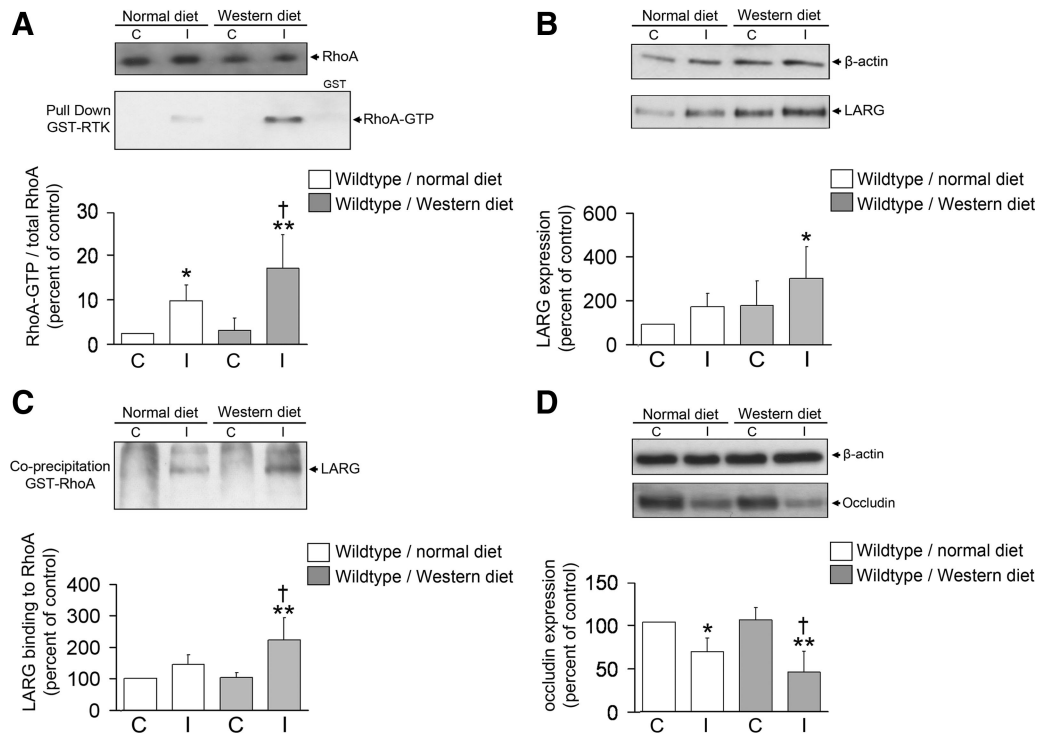


Figure 4. The RhoGTPase RhoA is overactivated and the tight junction protein occludin is downregulated in ischemic microvessels of hyperlipidemic mice. Western blot analysis and pull-down assay for RhoA (A), Western blot analysis for the guanine nucleotide exchange factor (GEF) of RhoA, leukemia-associated guanine exchange factor (LARG) (B), coprecipitation study for RhoA and LARG (C), and Western blot analysis for occludin (D) using cerebral microvessel extracts of normolipidemic and hyperlipidemic mice 24 hours after middle cerebral artery (MCA) occlusion. Note that LARG is overexpressed in ischemic microvessels of hyperlipidemic mice (B) and directly interacts with RhoA (C), which explains the overactivation of RhoA that is known to destabilize tight junctions. Data are means \pm standard deviation (SD) (n=4 Western blots, pull-down assays, or coprecipitation studies/group). C, contralateral nonischemic; I, ischemic. * $P < 0.05$ ** $P < 0.01$ compared with corresponding nonischemic/† $P < 0.05$ compared with ischemic normal diet (1-way analysis of variance [ANOVA] followed by least significant difference [LSD] tests).

Our data suggest that both MMP-2/9 and calpain-1/2 contribute to the elevated BBB permeability induced by hyperlipidemia.

Cholesterol-Rich Diet Enhances the Activation of RhoA and Its Guanine Nucleotide Exchange Factor Leukemia-Associated Guanine Exchange Factor in Ischemic Brain Microvessels

RhoA is a member of the Rho GTPases proteins family that previously has been shown to destabilize tight junctions, thus promoting BBB breakdown.²¹ To elucidate whether RhoA and its guanine nucleotide exchange factor, leukemia-associated guanine exchange factor, was responsible for the induction of BBB permeability by hyperlipidemia, Western blots, pull-down assay, and coprecipitation studies were used. Western blots and pull-down assays showed that whereas RhoA expression did not differ between groups, RhoA activity increased on ischemia in brain microvessels of normolipidemic mice (Figure 4A). Hyperlipidemia further elevated RhoA activity (Figure 4A).

Using Western blots and coprecipitation studies, we observed that leukemia-associated guanine exchange factor was overexpressed in ischemic brain microvessels of hyperlipidemic mice (Figure 4B) and that on ischemia leukemia-associated guanine exchange factor directly interacted with RhoA (Figure 4C). Our data indicated that leukemia-

associated guanine exchange factor catalyzes the RhoA activation induced by hyperlipidemia.

Downregulation of Tight Junction Protein Occludin by Hyperlipidemia

BBB integrity depends on the functionality of tight junctions. To evaluate whether and how the Western diet influenced tight junction proteins, Western blots were evaluated. The expression of occludin decreased on ischemia in cerebral microvessels of normolipidemic mice (Figure 4D). Hyperlipidemia further reduced occludin expression (Figure 4D).

Discussion

Using wild-type and ApoE^{-/-} mice that were kept on a cholesterol-rich diet, we showed that hyperlipidemia promotes BBB permeability after focal cerebral ischemia, thus exacerbating brain edema. Because hyperlipidemia is a highly prevalent condition that affects $\approx 50\%$ of stroke patients,^{3,4} it was surprising to us that the effects of cholesterol-rich diets on brain injury and BBB integrity never had been assessed in animal models of ischemic stroke. Studies in models of cortex trauma and aging revealed an increased BBB permeability in hyperlipidemic ApoE^{-/-}, but not wild-type mice,^{7,8} indicating that excessively high cholesterol levels or preexisting atherosclerosis are required so that BBB permeability is induced.

It was unexpected to us that ApoE^{-/-} did not influence BBB permeability after focal cerebral ischemia. In a recent study, we observed that ApoE regulates ATP-binding cassette transporters on ischemic brain capillaries via its receptor ApoE receptor 2, thus controlling the access of pharmacological compounds to the brain tissue.¹² Expecting that the regulation of ATP-binding cassette transporters and tight junctions are linked, we hypothesized that ApoE should also control paracellular BBB tightness. Contrary to our assumption, neither IgG extravasation, nor lipid accumulation in brain arterioles and lipid peroxidation differed between wild-type and ApoE^{-/-} mice. Our data suggest that consequences of hyperlipidemia for the brain differ from that of peripheral arteries, where endothelial dysfunction induced by cholesterol-rich food is markedly exacerbated by ApoE^{-/-}.^{17,18} Our interpretation is supported by recent observations of Jansen et al,²² who reported that cholesterol levels in brain tissue of hyperlipidemic wild-type and ApoE^{-/-} mice did not differ from each other, despite dramatic differences in blood cholesterol levels.

We examined a model of mild ischemia to obtain well-preserved vessels that could be used for molecular biological analysis. In screening experiments using a prolonged ischemia, ie, 90 minutes of MCA occlusion, we similarly in the present study did not find an effect of ApoE^{-/-}, but did find an effect of Western diet on brain swelling (wild-type/normal diet, 4.7±1.7 mm²; wild-type/Western diet, 6.4±1.4 mm²; ApoE^{-/-}/normal diet, 5.0±2.1 mm²; ApoE^{-/-}/Western diet, 7.5±1.5 mm² (n=6 animals/ group; F_{1,20}=8.986; P<0.01 for Western diet in 2-way analysis of variance). In that study, histological brain injury did not differ between groups. We thus conclude that our findings are also relevant for more severe ischemic stroke.

It is well-established that calpain-1/2 and MMP-2/9 are activated in response to brain ischemia,^{19,20} contributing to the development of endothelial dysfunction.^{23,24} The small GTPase RhoA is activated on stroke in the cytosol,¹⁵ inducing endothelial permeability by destabilizing cytoskeleton and tight junction proteins, such as occludin.^{21,24–26} It has already been shown in human aortic endothelial cells that lipid peroxidation activates MMP-2/9, which in turn induces RhoA activation.²⁴ By demonstrating that lipid peroxidation goes along with MMP-2/9 and RhoA activation under conditions of hyperlipidemia, we provided evidence that this pathway is also relevant in vivo. Not only RhoA, but also the guanine exchange factor of RhoA, leukemia-associated guanine exchange factor, was overactivated in ischemic microvessels of hyperlipidemic mice, suggesting that leukemia-associated guanine exchange factor is involved in the responses of brain capillaries to high cholesterol levels.

Our observation that BBB permeability and brain edema are increased in the ischemic brain by hyperlipidemia points toward the importance of the recognition and adequate treatment of this factor in human stroke patients. That BBB permeability differs between normolipidemia and hyperlipidemia raises concerns about current strategies in translational stroke research, in which mostly young animals receiving a cholesterol-restricted diet are used for the evaluation of new therapies. Because half of stroke patients have hyperlipid-

emia,^{3,4} such animals poorly reflect the pathophysiological conditions in humans. Animal studies should more closely mimic vascular risk factors in the future.

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Disclosures

None.

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Increased Blood–Brain Barrier Permeability and Brain Edema After Focal Cerebral Ischemia Induced by Hyperlipidemia: Role of Lipid Peroxidation and Calpain-1/2, Matrix Metalloproteinase-2/9, and RhoA Overactivation

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SUPPLEMENTAL MATERIALS

Caseinase microplate assay for MMP-2/9

To investigate the activation of calpain-1/2 in brain microvessels, we developed a caseinase microplate assay, which evaluates caseinase activity in the presence of calpain-1/2's endogenous inhibitors. Twenty μg of protein extracts from each sample, obtained using NP-40 lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl and 1% NP-40 (pH 7.4), were added into 96 well microplate and complemented with calibrating buffer containing 20 mM Tris-HCl, 1 mM EDTA, 100 mM KCl and 0.1% 2-mercaptoethanol (pH 7.4), resulting in volumes of 60 μl in each well.

Two solutions were prepared containing casein at 20 μg / ml: (i) Solution A (= activation) consisted of 20 mM Tris-HCl, 1 mM EDTA, 10 mM Ca^{2+} , 100 mM KCl and 0.1% 2-mercaptoethanol (pH 7.4), allowing to study the enzymatic activity of calpain-1/2 owing to the fact that samples contained Ca^{2+} that is required for calpain-1/2 to exert its protease activity. (ii) Solution I (= inhibition) contained 20 mM Tris-HCl, 50 mM EDTA, 100 mM KCl and 0.1% mercaptoethanol (pH 7.4). In this solution calpain-1/2 was inactive as the sample lacked Ca^{2+} ions. Experiments were conducted in triplicate for each sample, 100 μl of solution A being added to the first set of wells containing protein samples, and 100 μl of solution I being added to the next set, increasing the total volume in each well to 160 μl (60 μl + 100 μl). As such, the assay was run four times for statistical comparisons.

Each microplate was incubated for 2 hours at room temperature. Then, 120 μl of 1X G-250 dye (Quick Start Bradford Protein Assay; Biorad) were added simultaneously to each well and incubated for 5 min at room temperature, the G-250 dye forming a stable complex upon binding proteins shifting light absorbance from 470 nm to 595 nm. The dye does not bind proteolytic fragments of digested proteins like small

peptides or amino acids, assessing the enzymatic activity of calpain-1/2, the higher the activity the less absorbance values are. The absorbance was read at 595nm using the iMark microplate reader and calpain-1/2 caseinase activity was calculated for each sample by subtracting absorbances between solution I and solution A. Calpain-1/2 protease activity was expressed as absorbance difference at 595 nm units (AD_{595nm}).

Gelatine zymography

To examine the activation of MMP-2/9, we used a gelatine zymography that was previously described¹⁵ and a gelatinase microplate assay, which we established in analogy to the caseinase microplate assay. For gelatine zymography, 25 µg proteins were mixed with 5X non reducing loading buffer for 15 min at room temperature and subjected to sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using 9% acrylamide-bis gel containing 0.1% gelatine (Sigma). Gels were removed and washed, incubated for 1 hr at room temperature with slight shaking in modified enzymatic activation buffer (50 mM Tris-HCl, 6 mM CaCl₂, 1.5 µM ZnCl₂, pH 7.4) containing 2.5% Triton X-100 to remove SDS and restore gelatinase activity. Gels were incubated for 24 hr at 37°C in modified enzymatic activation buffer. Gels were stained in Coomassie brilliant blue R-250 (Bio-Rad). A total of four independent experiments were run, which were digitized and densitometrically analyzed.

Gelatinase microplate assay for MMP-2/9

To study MMP-2/9's activity in presence of their endogenous inhibitors, the caseinase microplate assay was adopted with the following changes: 5 µg of proteins were added to a 96 well microplate and complemented with a calibrating buffer containing 50 mM Tris-HCl and 6 mM CaCl₂ (pH 7.4) to 60 µl volumes. Two solutions were

prepared containing gelatine at a concentration of 20 µg/ml: (i) Solution A (= activation) (50 mM Tris-HCl, 6 mM CaCl₂, 50 mM EDTA, 1.5 µM ZnCl₂, pH 7.4) and (ii) solution I (= inhibition) (50 mM Tris-HCl, 6 mM CaCl₂, 50 mM EDTA, pH 7.4), the two samples differing in terms of the presence and absence of ZnCl₂, which is a co-factor needed for gelatinase to exert its enzymatic activity. After incubation for 2 hours at 37°C, the G-250 dye was added, the absorbance read, and MMP-2/9 gelatinase activity calculated by subtracting absorbance between solution I and solution A. MMP-2/9 protease activity was again expressed as AD_{595nm}.

Antibodies and histochemicals for Western blotting, pulldown and immunoprecipitation studies

The following antibodies and chemicals were used: anti-calpastatin (sc-20779), anti-leukemia-associated Rho guanine nucleotide exchange factor (LARG) (sc-25638), anti-RhoA (sc-418), anti-occludin (sc-27151) and protein A/G plus-agarose (sc-2003) were purchased from Santa Cruz Biotechnology. Rhotekine-RBD-GST beads (RT02) and wildtype-RhoA-GST (RHG01) were purchased from Cytoskeleton Inc. Anti-β-actin (4967) was purchased from Cell Signaling Technology. Anti-zona occludens (ZO)-1 (40-2300) was purchased from Invitrogen.