Hyperlipidemia Attenuates Vascular Endothelial Growth Factor–Induced Angiogenesis, Impairs Cerebral Blood Flow, and Disturbs Stroke Recovery via Decreased Pericyte Coverage of Brain Endothelial Cells

Anil Zechariah, Ayman ElAli, Nina Hagemann, Fengyan Jin, Thorsten Roland Doeppner, Iris Helfrich, Günter Mies, Dirk Matthias Hermann

Objective—Therapeutic angiogenesis aims at the promotion of vascular growth, usually under conditions of atherosclerosis. It was unknown how hyperlipidemia, a risk factor that is closely associated with atherosclerosis of brain vessels in humans, influences vascular endothelial growth factor–induced angiogenesis and stroke recovery.

Approach and Results—Wild-type and apolipoprotein-E (ApoE)−/− mice were kept on regular or cholesterol-rich diet for mimicking different severities of hyperlipidemia. Mice were treated intracerebroventricularly with recombinant human vascular endothelial growth factor for 21 days (0.02 µg/d) and subsequently subjected to 90-minute middle cerebral artery occlusion followed by 1 or 24 hours of reperfusion. Histochemical, autoradiographic, and regional bioluminescence techniques were used to evaluate effects of blood lipids on postischemic angiogenesis, histopathologic brain injury, cerebral blood flow, protein synthesis and energy state, and pericyte coverage of brain endothelial cells. Hyperlipidemia dose-dependently attenuated vascular endothelial growth factor–induced capillary formation and pericyte coverage of brain endothelial cells, abolishing the improvement of cerebral blood flow during subsequent stroke, resulting in the loss of the metabolic penumbra and increased brain infarction. The enhanced angiogenesis after vascular endothelial growth factor treatment was accompanied by increased expression of the adhesion protein N-cadherin, which mediates endothelial-pericytic interactions, in ischemic brain microvessels of wild-type mice on regular diet that was blunted in wild-type mice on Western diet and ApoE−/− mice on either diet.

Conclusions—The compromised vessel formation and hemodynamics question the concept of therapeutic angiogenesis in ischemic stroke where hyperlipidemia is highly prevalent. (Arterioscler Thromb Vasc Biol. 2013;33:1561-1567.)

Key Words: ATP bioluminescence imaging ■ cerebral blood flow autoradiography ■ hypercholesterolemia ■ metabolic penumbra

Major efforts have been made in human patients with atherosclerosis to stimulate angiogenesis by delivery of vascular endothelial growth factor (VEGF) or of viral vectors or plasmids containing the VEGF transgene.1–4 Functionally relevant improvements were noticed only in some,5–10 but not other,11–13 clinical studies, in which VEGF was delivered for vascular endothelial growth factor 21 days (0.02 µg/d) and subsequently subjected to 90-minute middle cerebral artery occlusion followed by 1 or 24 hours of reperfusion. Histochemical, autoradiographic, and regional bioluminescence techniques were used to evaluate effects of blood lipids on postischemic angiogenesis, histopathologic brain injury, cerebral blood flow, protein synthesis and energy state, and pericyte coverage of brain endothelial cells. Hyperlipidemia dose-dependently attenuated vascular endothelial growth factor–induced capillary formation and pericyte coverage of brain endothelial cells, abolishing the improvement of cerebral blood flow during subsequent stroke, resulting in the loss of the metabolic penumbra and increased brain infarction. The enhanced angiogenesis after vascular endothelial growth factor treatment was accompanied by increased expression of the adhesion protein N-cadherin, which mediates endothelial-pericytic interactions, in ischemic brain microvessels of wild-type mice on regular diet that was blunted in wild-type mice on Western diet and ApoE−/− mice on either diet.

Conclusions—The compromised vessel formation and hemodynamics question the concept of therapeutic angiogenesis in ischemic stroke where hyperlipidemia is highly prevalent. (Arterioscler Thromb Vasc Biol. 2013;33:1561-1567.)

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Major efforts have been made in human patients with atherosclerosis to stimulate angiogenesis by delivery of vascular endothelial growth factor (VEGF) or of viral vectors or plasmids containing the VEGF transgene.1–4 Functionally relevant improvements were noticed only in some,5–10 but not other,11–13 clinical studies, in which VEGF was delivered for the prevention of coronary heart or peripheral occlusive artery disease. Thus, the translation of therapeutic angiogenesis from bench to bedside still hampers.

Reflecting the deposition of lipids into vessel walls either because of dietary habits or genetic predisposition, atherosclerosis is frequently accompanied by lipid abnormalities. In ischemic stroke, about half of the patients exhibit hyperlipidemia,11,14 and subjects with hyperlipidemia in turn have an elevated stroke mortality.10,15 Chronically increased cholesterol levels trigger a number of vascular events, such as oxidative stress, endothelial dysfunction, blood–brain barrier disturbances, and vascular inflammation.16–19

Although the consequences of elevated cholesterol levels for vascular integrity are well established and spontaneous angiogenesis is compromised,16,20,21 it is still unknown how hyperlipidemia influences angiogenesis in response to VEGF treatment. Because of severe hemodynamic abnormalities, patients with intracranial atherosclerosis, which is particularly strongly associated with dyslipidemia,22,23 might specifically be eligible for angiogenic therapies. Thus, the question how hyperlipidemic blood vessels respond to VEGF possesses high-clinical relevancy.

To elucidate effects of hyperlipidemia on VEGF-induced angiogenesis, we herein treated normolipidemic

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From the Departments of Neurology (A.Z., F.J., N.H., T.R.D., A.E., D.M.H.), and Dermatology (I.H.), University Hospital Essen, Germany; and Max-Planck-Institute for Neurological Research, Cologne, Germany (G.M.).

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Correspondence to Dirk M. Hermann, MD, Department of Neurology, University Hospital Essen, Hufelandstrasse 55, D-45122 Essen, Germany. E-mail dirk.hermann@uk-essen.de

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or hyperlipidemic C57BL6 wild-type and apolipoprotein-E (ApoE)−/− mice by intracerebroventricular infusion with normal saline or VEGF for 21 days and exposed these animals to focal cerebral ischemia. We subsequently analyzed changes in brain capillary density, regional cerebral blood flow (CBF), cerebral protein synthesis and energy state, and histological brain injury.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

VEGF-Induced Capillary Formation Is Blunted by Hyperlipidemia

To examine how the delivery of VEGF influences microvascular networks in wild-type and ApoE−/− mice, brain capillary density was analyzed by CD31 immunohistochemistry. VEGF delivered at a dose of 0.02 µg/day24 increased capillary density in wild-type mice on regular, that is, normal diet and cholesterol-rich, that is, Western diet and ApoE−/− mice on normal diet but not in ApoE−/− mice on Western diet (Figure 1A and 1B), which exhibit highest plasma cholesterol levels.17 Three-way ANOVA showed a significant interaction effect for VEGF×ApoExdietary status on capillary density (F1,39=4.444; P<0.05), demonstrating that angiogenesis was prevented by hyperlipidemia, most strongly in ApoE−/− mice on Western diet.

Hyperlipidemia Abolishes VEGF-Induced Neuroprotection and Blood-Brain Barrier Integrity

To evaluate the effects of hyperlipidemia on histopathologic brain injury and blood–brain barrier integrity, mice treated with vehicle or VEGF were exposed to 90-minute middle cerebral artery occlusion followed by 24 hours reperfusion. Laser Doppler flow recordings taken during and after middle cerebral artery occlusion revealed reproducible ischemia that did not differ between vehicle- and VEGF-treated mice (Figure 2A and 2B). Postischemic Laser Doppler flow values were lower in animals on Western diet than in animals on normal diet (Figure 2A and 2B).

![Graph A](image1.png)

**Figure 1.** Hyperlipidemia prevents vascular endothelial growth factor (VEGF)-induced brain capillary formation. A and B, CD31 immunohistochemistry showing an increased capillary density in VEGF-treated wild-type mice kept on normal and Western diet and ApoE−/− mice on normal diet. Capillary formation is blunted in ApoE−/− mice on Western diet that exhibit the highest plasma cholesterol levels between all groups. Representative microphotographs from wild-type mice kept on normal diet and ApoE−/− mice on Western diet are also shown. Data are means±SD (n=6–7 animals/group). *P<0.05/**P<0.01 compared with corresponding vehicle. Bar, 100 µm.

![Graph B](image2.png)

**Figure 2.** Laser Doppler flow during and after 90-minute middle cerebral artery occlusion. A and B, Laser Doppler flow (LDF) recordings confirming the reproducibility of middle cerebral artery occlusions. Note that LDF values after reperfusion are lower in animals kept on Western diet than in animals kept on normal diet. This effect was significant in ApoE−/− mice (B). Data are means±SD (n=6–7 animals/group). *P<0.05 for vascular endothelial growth factor (VEGF)-treated normal diet compared with vehicle-treated normal diet; †P<0.05 for vehicle-treated Western diet compared with vehicle-treated normal diet.
Infarct measurements on cresyl violet stainings at 24 hours after reperfusion revealed that VEGF reduced infarct volume in the wild-type mice kept on normal diet (Figure 3A), which is in line with earlier results of our group, and in wild-type mice kept on Western diet (Figure 3A). Conversely, VEGF did not influence infarct size in ApoE−/− mice on normal diet or ApoE−/− mice on Western diet (Figure 3B). The attenuation of brain injury was associated with reduced immunoglobulin G extravasation in wild-type mice on normal diet, but not in any of the other groups (Figure 3C and 3D).

**Hyperlipidemia Prevents VEGF-Induced Extracellular Matrix Preservation**

To assess how hyperlipidemia influences extracellular matrix integrity after VEGF treatment, matrix metalloproteinase-9 activity was analyzed by gelatin zymography. In line with a

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Hyperlipidemia abolishes vascular endothelial growth factor (VEGF)-induced neuroprotection and blood-brain barrier preservation. **A** and **B**, Infarct volume of mice submitted to 90-minute middle cerebral artery occlusion followed by 24 hours reperfusion showing VEGF-induced reduction of ischemic injury in wild-type mice on normal diet and wild-type mice on Western diet, but not in ApoE−/− mice on normal diet or ApoE−/− mice on Western diet that exhibit more pronounced hyperlipidemia. **C** and **D**, Immunohistochemistry of the same animals demonstrating decreased serum immunoglobulin G (IgG) extravasation by VEGF in wild-type mice on normal diet, but not in wild-type mice on Western diet and ApoE−/− mice on normal or Western diet. **E** and **F**, Gelatin zymography of the same animals exhibiting deactivation of matrix metalloproteinase (MMP)-9 by VEGF in wild-type mice on normal diet that is attenuated in wild-type mice on Western diet and ApoE−/− mice on normal or Western diet. In **C**–**F**, tissue samples derived from the ischemic middle cerebral artery territory (striatum and overlying cortex) were analyzed. Representative images from wild-type mice kept on normal diet and ApoE−/− mice on Western diet are also shown. Data are mean±SD (n=9–11 animals/group for analysis of ischemic injury, and n=6–7 animals/group for all other readouts). *P<0.05/**P<0.01 compared with corresponding vehicle. ††P<0.01 compared with corresponding normal diet.
Hyperlipidemia abolishes vascular endothelial growth factor (VEGF)-induced improvement of regional cerebral blood flow, promoting secondary breakdown of brain energy state. A, Regional cerebral blood flow (CBF) autoradiography showing increased blood flow values in ischemic brain tissue of VEGF-treated wild-type mice on normal diet that are attenuated in ApoE<sup>−/−</sup> mice on Western diet that exhibit hyperlipidemia. B, Metabolic penumbra, defined as tissue area in which cerebral protein synthesis is suppressed, but ATP preserved, exhibiting the viability of the metabolic penumbra as a result of the enhanced blood flow in VEGF-treated wild-type mice on normal diet, but not in hyperlipidemic ApoE<sup>−/−</sup> mice on Western diet. C, Tissue area exhibiting ATP depletion, revealing reduced breakdown of the cerebral energy state in VEGF-treated normolipidemic wild-type mice, but not in hyperlipidemic ApoE<sup>−/−</sup> mice. Animals were exposed to 90-minute middle cerebral artery occlusion, followed by 60 minutes reperfusion. Tissue samples derived from the ischemic middle cerebral artery territory (striatum and overlying cortex) were evaluated. Representative autoradiographs and bioluminescence images are also shown. For wild-type/normal diet animals, see also reference 26. Data are mean±SD (n=6 animals/group). *P<0.05/**P<0.01 compared with corresponding vehicle. CPS indicates cerebral protein synthesis.

Hyperlipidemia Attenuates VEGF-Induced Improvement of Regional CBF
To elucidate the effect of VEGF-induced angiogenesis on brain hemodynamics during a subsequent stroke, CBF autoradiograms were analyzed at 60 minutes after middle cerebral artery occlusion. Increased regional CBF was noted in the middle cerebral artery territory of VEGF-treated wild-type mice on normal diet but not in VEGF-treated ApoE<sup>−/−</sup> mice on Western diet (Figure 4A), indicating a lack of functional hemodynamic improvements in animals on hyperlipidemia. Two-way ANOVA demonstrated a significant effect of VEGF (F<sub>1,20</sub>=8705; P<0.01), but no interaction effect with ApoE/dietary status on regional CBF.

Hyperlipidemia Abolishes VEGF-Induced Stabilization of Metabolic Penumbra
To evaluate how the improvement of regional CBF influences regional cerebral energy state, cerebral protein synthesis and ATP bioluminescence images were assessed. A stabilization of the metabolic penumbra, defined as brain tissue in which cerebral protein synthesis was suppressed but ATP preserved, was noticed in VEGF-treated wild-type mice on normal diet but not in ApoE<sup>−/−</sup> mice on Western diet (Figure 4B). Two-way ANOVA showed a significant VEGF×ApoE/dietary status interaction effect (F<sub>1,14</sub>=4.663; P<0.05), indicating that hyperlipidemia promotes secondary brain infarction. Indeed, the tissue area exhibiting ATP depletion after 60 minutes of reperfusion was considerably smaller in normolipidemic wild-type than hyperlipidemic ApoE<sup>−/−</sup> mice (Figure 4C), although this difference still failed to show significance at this time point.

Hyperlipidemia Prevents VEGF-Induced Pericyte Alignment on Brain Capillaries
To examine the functionality of cerebral blood vessels in addition to structural growth, pericyte coverage was analyzed in brain tissue samples submitted to focal cerebral ischemia. In the ischemic tissue, the percentage of cerebral microvessels surrounded by pericytes was ≈60% in 20-μm-thick cryostat sections (Figure 5A and 5B). Interestingly, VEGF treatment increased the percentage of pericyte± capillaries in wild-type mice on normal diet (relatively by ≈30%), but not in wild-type mice on Western diet or ApoE<sup>−/−</sup> mice on normal or Western diet (Figure 5A and 5B). Three-way ANOVA revealed a significant VEGF×ApoE×dietary status interaction effect (F<sub>1,41</sub>=4.487; P<0.05). To further corroborate these findings, we subsequently performed a confocal data analysis, in which pericyte volumes determined in 3D stacks were related to volumes of CD31± cerebral capillaries. This study again revealed an increased
pericyte coverage of brain endothelial cells in VEGF-treated wild-type mice on normal diet, but not in wild-type mice on Western diet or any of the ApoE−/− mice (Figure 5C and 5D). Three-way ANOVA again confirmed a VEGF×ApoE×dietary status interaction effect ($F_{1,41}=7325; P<0.05$), confirming that hyperlipidemia disturbed the alignment of pericytes with endothelial cells.

Hyperlipidemia Attenuates N-Cadherin Expression in Cerebral Microvessels

The physical interaction of pericytes with endothelial cells is mediated by the junctional protein N-cadherin. To evaluate whether N-cadherin expression was altered by blood lipids, Western blots were prepared with capillary extracts obtained from normolipidemic and hyperlipidemic wild-type mice (Figure 5E and 5F). The expression of N-cadherin was increased in VEGF-treated wild-type mice on normal diet, but not in hyperlipidemic wild-type mice on Western diet or ApoE−/− mice. Animals were submitted to 90-minute middle cerebral artery occlusion followed by 24 hours reperfusion. Tissue samples derived from the ischemic middle cerebral artery territory (striatum and overlying cortex) were evaluated. Representative microphotographs or blots are also shown (A and B; CD31 in green/desmin in red). Data are means±SD (n=6–7 animals/group [in A–E]/n=4 independently processed blots [in E and F]).

*P<0.05**P<0.01 compared with vehicle. Bar (A and B), 100 µm.
and ApoE−/− mice. This study revealed that VEGF potently increased N-cadherin levels in ischemic microvessels obtained from wild-type mice on normal diet, but not in any of the hyperlipidemic groups (Figure 5E and 5F). The lack of N-cadherin expression provides a mechanism for the disturbed pericyte coverage of endothelial cells in hyperlipidemia.

Discussion

Using a multiparametric approach combining histochemical, autoradiographic, bioluminescence, and molecular biological studies in vivo, we herein show that VEGF-induced angiogenesis is compromised by hyperlipidemia, translating into the loss of hemodynamic improvements after subsequent stroke injury, the breakdown of the metabolic penumbra, and brain infarction. N-cadherin expression was reduced on cerebral microvascular cells exposed to hyperlipidemia that was associated with decreased coverage of endothelial cells with pericytes, demonstrating that blood vessels were dysfunctional. In this study, wild-type and ApoE−/− mice fed with regular or cholesterol-rich chow were used to induce different degrees of hyperlipidemia. Our data might provide an explanation for the poor effects of angiogenic therapies in human patients with symptomatic atherosclerosis.2–4 These patients frequently suffer from hyperlipidemia and particularly brain vessels are affected by atherosclerotic plaques.22,23

That VEGF induces brain angiogenesis is well established, whereas evidence for the hemodynamic relevance of these effects is less clear.2 In an MRI study, acute VEGF infusion was previously shown to induce transient CBF increases in ischemic brain tissue lasting up to 3 hours,25 which however was a consequence of VEGF-induced vasorelaxation rather than of induced vascular growth. In mice expressing human VEGF chronically in the whole brain under a neuron-specific enolase promoter, regional CBF was observed to be reduced in ischemic brain areas as a consequence of a hemodynamic steal flow.25 The exogenous delivery of VEGF differs from transgenic VEGF expression, because it increases regional CBF in ischemic brain tissue.26 Importantly, both the VEGF-induced angiogenesis and the enhancement of blood flow were blunted in animals exhibiting hyperlipidemia.

Impaired spontaneous angiogenesis has previously been noted under conditions of hyperlipidemia.16,20,21 Thus, reduced vessel densities have been reported in hyperlipidemic rats exposed to hindlimb ischemia, which was attributed to reduced NO activity in hyperlipidemic ischemic tissue.16 Consequences of hyperlipidemia on brain angiogenesis and on the responses of blood vessels to VEGF were so far unknown. In vitro studies reported that endothelial proliferation and migration, 2 fundamental processes required for angiogenesis were blunted in animals exhibiting hyperlipidemia.

Of blood-brain barrier properties and controlling vascular reactivity.27 Indeed, the preservation of blood-brain barrier integrity of the VEGF was abolished under conditions of hyperlipidemia, which was reflected by the enhanced access of serum IgG to the brain parenchyma, while matrix metalloproteinase-9 activity at the same time was increased. In line with the disturbed pericyte coverage, the junctional protein N-cadherin, which mediates physical interactions between endothelial cells and pericytes and which was upregulated by VEGF in normolipidemic microvessels, did not respond to VEGF in hyperlipidemic mice. That hyperlipidemia prevents the pericyte alignment with endothelial cells is noteworthy. It provides a new, hitherto unknown mechanism for disturbances of vascular reactivity in hyperlipidemia.

Translational stroke studies should be aware that hyperlipidemia alters responses of cerebral blood vessels to angiogenic growth factors. The lack of vascular growth responses after VEGF treatment questions the concept of therapeutic angiogenesis in ischemic stroke under conditions of hyperlipidemia.

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Disclosures

None.

References

Hyperlipidemia Attenuates VEGF-Induced Angiogenesis


**Significance**

By using wild-type and apolipoprotein-E (ApoE)−/− mice that were kept on regular or cholesterol-rich diet, we show that vascular endothelial growth factor–induced angiogenesis is attenuated in the brains of hyperlipidemic mice, whereas pericyte coverage of brain endothelial cells is impaired, resulting in the lack of cerebral blood flow improvement during subsequent middle cerebral artery occlusion, followed by the loss of the metabolic penumbra, which is defined as tissue at risk that is still amenable to therapeutic interventions, and secondary brain infarction. The compromised new vessel formation and disturbed cerebral hemodynamics question the concept of therapeutic angiogenesis in ischemic stroke, where hyperlipidemia is highly prevalent.
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Anil Zechariah, Ayman ElAli, Nina Hagemann, Fengyan Jin, Thorsten Roland Doeppner, Iris Helfrich, Günter Mies, Dirk Matthias Hermann

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Materials and methods

Experimental groups
All experiments were performed with government approval. Male C57BL6/j and ApoE\(^{-/-}\) mice were used that were fed with either normal (i.e., regular) or cholesterol-rich (Western) diet for six weeks starting at the age of 3 weeks. We have previously analysed plasma cholesterol levels following this diet exposure, showing that plasma cholesterol levels were 56.6±17.4 mg/dl in wildtype animals kept on regular diet, 220.4±58.0 mg/dl in wildtype animals on Western diet, 470.9±276.9 mg/dl in ApoE\(^{-/-}\) animals on normal diet and 1279.1±528.2 mg/dl in ApoE\(^{-/-}\) animals on Western diet in the end of the 6-week period.\(^1\) Hyperlipidemic animals exhibited lipid deposits in cerebral blood vessels in this study.\(^1\)

The animals were randomly assigned into different groups to exclude data bias, investigators being blinded to experimental conditions in all phases of data collection and analysis. In a first set of studies, male wildtype mice and ApoE\(^{-/-}\) mice that were fed with normal or Western diet were assigned to eight groups receiving miniosmotic pump implantation into the left lateral ventricle at the age of six weeks (i.e., three weeks after the initiation of the dietary regimen), via which vehicle (0.9% NaCl) or recombinant human VEGF\(_{165}\) (0.02 µg/day) were administered over 21 days. At the age of nine weeks, when animals had a body weight of 20-25 g, animals were exposed to 90 minutes middle cerebral artery (MCA) occlusion followed by 24 hours reperfusion. These animals were used for histochemical analysis of angiogenesis, pericyte coverage, molecular biological studies and ischemic injury (n= 9-11 animals/ group evaluated for infarct volumetry, at least 6 animals/ group used for all other histochemical and molecular biological studies).

A second set of C57BL6/j and ApoE\(^{-/-}\) mice kept on normal or Western diet were assigned to four groups treated with vehicle or VEGF\(_{165}\) over 21 days in an identical way. At the age of nine weeks, animals were submitted to 90 minutes MCA occlusion followed by 60 minutes of reperfusion. These animals were used for CBF and cerebral protein synthesis (CPS) double autoradiography and for regional ATP bioluminescence imaging (6 animals/ group).

Delivery of recombinant human VEGF\(_{165}\)
Cannulae linked to miniosmotic pumps (Alzet 2004; Palo Alto, CA, U.S.A.) were implanted under 1% isoflurane anaesthesia (30% O\(_2\), remainder N\(_2\)O) into the left lateral ventricle (1 mm lateral to bregma/ 2.5 mm below brain surface) at the age of six weeks (i.e., three weeks after the initiation of the dietary regimen) for administration of normal saline or rhVEGF\(_{165}\) (Peprotech, Hamburg, Germany; 0.02 µg/day).\(^2\) This dose has previously been shown to induce angiogenesis following intracerebroventricular administration.\(^2\) After implantation, wounds were carefully sutured, anaesthesia discontinued and animals returned to their cages.

Induction of focal cerebral ischemia
Focal cerebral ischemia was induced during 1% (30% O\(_2\), remainder N\(_2\)O) isoflurane anaesthesia by intraluminal MCA occlusion using a silicon-coated microfilament as previously described.\(^3,4\) Briefly, a midline neck incision was made and the left common and external carotid arteries were isolated and ligated. A microvascular clip (Aesculap, Tuttlingen, Germany) was temporarily placed on the internal carotid artery and a silicon-coated monofilament (Doccol, Sharon, MA, U.S.A.) was directed
through the internal carotid artery until the origin of MCA. The monofilament was left in place for 90 minutes and then withdrawn to facilitate reperfusion. Rectal temperature was maintained between 36°C and 37°C using a feedback-controlled heating system throughout the experiments. Laser Doppler flow (LDF) was measured during the experiments up to 30 minutes after reperfusion using a flexible fiberoptic probe attached to the skull overlying the core of the MCA territory. In the first set of studies, anesthesia was discontinued after this procedure. Wounds were sutured and animals returned to their cages. Animals were sacrificed 24 hours later by transcardiac perfusion with normal saline. In the second set of studies, animals remained under anesthesia for delivery of radioactive tracers.

**Evaluation of brain capillary density**

20 µm cryostat sections were prepared from brains of animals sacrificed 24 hours after reperfusion. In sections obtained from the rostrocaudal level of the mid-striatum, i.e., the site of the maximum extension of the MCA territory, new vessel formation was evaluated by fluorescence immunohistochemistry using a rat anti-CD31 antibody (BD Biosciences, San Diego, CA, U.S.A.). Stainings were evaluated by counting the number of CD31 positive vessel profiles intersecting the horizontal and vertical lines of a 500 µm x 500 µm grid. A total of four regions of interest (ROI) in the lateral striatum and three ROI in the overlying parietal cortex (all inside the MCA territory) were evaluated. Means were calculated for all ROI that were used for further analysis.

**Analysis of infarct volume**

Cryostat sections 1 mm apart were stained with cresyl violet. The border between infarcted and healthy tissue was outlined using image analysis software (Image J; National Institutes of Health) and the area of infarction was quantified by subtracting the area of nonlesioned ipsilateral hemisphere from that of the contralateral side. Infarct areas from various rostrocaudal brain levels were integrated for infarct volume analysis.

**Serum IgG extravasation studies**

Brain sections obtained from the mid-striatum were processed for serum IgG immunohistochemistry. Stained sections were scanned and converted into gray values. Sections were densitometrically analyzed by evaluating a sample of 1 x 1 mm in the core of the MCA territory (striatum and overlying cortex), from which background staining in the contralateral MCA territory was subtracted. A total of four sections were evaluated for each animal, for which mean values were calculated.

**Zymography for matrix metalloproteinase (MMP)-9**

Brain samples obtained from the ischemic and contralateral non-ischemic MCA territory were homogenized, lysated, supplemented with 5% protease inhibitor cocktail, and sonicated. Protein concentrations were determined using the Bradford assay. MMP-9 (gelatinase-B) activity was assessed by zymography as described. Protein extracts of all animals belonging to the same group were pooled. A total of four independent gelatin gels were prepared. These gels were scanned, converted into gray values and densitometrically analyzed.

**CBF and CPS double autoradiography**
Fifteen minutes after reperfusion, 150 µCi L-[4, 5-3H] leucine (specific activity 151 Ci/mmol; Amersham, Braunschweig, Germany) was administered intraperitoneally, followed by an intraperitoneal injection of 10 µCi 4-iodo-N-methyl-[14C] antipyrine (Amersham) 43 minutes later. After additional two minutes, animals were instantly frozen in liquid nitrogen. Blood samples were obtained from the heart, in which the activity of 4-iodo-N-methyl-[14C] antipyrine was measured. Brains were removed and cut into 20 µm thick sections that were mounted on poly-l-lysine coated slides. These were exposed for 14 days, together with 14C and 3H standards on 14C-Hyperfilm (Amersham) for CBF autoradiography. Brain slices were then incubated for 24 h in 10% trichloroacetic acid (TCA) to remove labeled free leucine and metabolites other than proteins, and subsequently re-exposed for the same duration to perform 3H autoradiography of 3H-labeled proteins using Hyperfilm 3H (Amersham). Regional CBF was calculated as described by calibration with the 14C- and 3H-standards, and radioactivity values measured in the blood. In the calibrated sections, regional CBF was determined in four ROI in the ischemic lateral striatum and three ROI in the overlying parietal cortex (all inside MCA territory). For each animal, mean values were formed for all ROI.

**Regional ATP bioluminescence imaging**

For ATP measurement, frozen sections were freeze-dried and coated with a layer of frozen reaction mix containing the enzymes, coenzymes and cofactors necessary for evoking ATP-specific bioluminescence. The tissue/enzyme bilayer was thawed and light emissions were recorded using a CCD camera. The CPS-deficient and ATP-depleted area was determined on the CPS autoradiograms and ATP bioluminescence images by outlining areas with preserved CPS and ATP in both hemispheres at the level of the mid-striatum. The metabolic penumbra was calculated from these results by subtracting the ATP preserved area from the CPS deficient area.

**Evaluation of pericyte coverage of brain capillaries**

Cryostat sections from the level of the mid-striatum were processed for double immunohistochemistry using rabbit anti-desmin (Abcam, Cambridge, UK) and rat anti-CD31 (BD Biosciences) antibody. To evaluate pericyte coverage of brain capillaries, the percentage of pericyte positive microvessels was counted in four ROI in the lateral striatum and three ROI in the overlying parietal cortex (all inside MCA territory, measuring 500 µm x 500 µm, as above), out of which mean values were formed. Confocal 3D stacks were obtained using laser scanning microscope (LSM 510; Carl Zeiss MicrolImaging, Jena, Germany) using 20 µm sections scanned at 2 µm intervals, which were viewed using Zeiss LSM image browser and analyzed using ImageJ. In these stacks, CD31+ capillaries and desmin+ pericytes were outlined on various images, which were integrated throughout the stack, allowing the calculation of capillary and pericyte volumes, from which volume ratios were formed. The investigator was blinded for experimental conditions at all stages of the data analysis.

**Western blots**

Protein lysates were obtained from extracted crude microvessels using tissue samples collected from the ischemic and contralateral non-ischemic MCA territory. Lysates of different animals were pooled, resolved by SDS-PAGE and transferred into polyvinylidene fluoride membranes. Membranes were immersed in blocking
solution and incubated overnight with rabbit anti-N-cadherin (4061; Cell Signaling Technology, Frankfurt, Germany). Membranes were rinsed, incubated in secondary antibody and exposed to photoluminescence solution. Protein loading was controlled using a rabbit anti-β-actin (4967; Cell Signaling Technology) antibody. Four independent blots were prepared, scanned and densitometrically analyzed. Protein levels were corrected for β-actin loading.

**Statistics**

LDF recordings were evaluated by repeated measurement analysis of variance (ANOVA) with values determined at 15-minute intervals during MCA occlusion and at 5-minute intervals after reperfusion. Since these analyses revealed significant group x time interaction effects, one-way ANOVA were computed for various time points, for which in case of significance, least significant differences (LSD) tests were calculated as post-hoc tests. All other readouts were evaluated by one-way ANOVA followed by LSD tests. In some experiments, three-way or two-way ANOVA were also computed, from which significant interaction effects are reported in the text. All data are given as means±SD. P≤0.05 was considered significant.

**References**