Heart Rate Contributes to the Vascular Effects of Chronic Mental Stress

Effects on Endothelial Function and Ischemic Brain Injury in Mice

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Background and Purpose—Vascular effects of mental stress are only partially understood. Therefore, we studied effects of chronic stress and heart rate (HR) on endothelial function and cerebral ischemia.

Methods—129S6/SvEv mice were randomized to the I(f)-channel inhibitor ivabradine (10 mg/kg per day) or vehicle and underwent a chronic stress protocol for 28 days.

Results—Stress increased HR from 514±10 bpm to 570±14 bpm, this was prevented by ivabradine (485±7 bpm). Endothelium-dependent relaxation of aortic rings was impaired in mice exposed to stress. HR reduction restored endothelial function to the level of naive controls. Vascular lipid hydroperoxides were increased to 333%±24% and vascular NADPH oxidase activity was upregulated to 223±38% in stressed mice, which was prevented by ivabradine. Stress reduced aortic endothelial nitric oxide synthase mRNA expression to 84%±3% and increased AT1 receptor mRNA to 168%±18%. Both effects were attenuated by HR reduction. In brain tissue, stress resulted in an upregulation of lipid hydroperoxides to 140%±11%, which was attenuated by HR reduction. Ivabradine increased brain capillary density in naive and in stressed mice. Mice exposed to chronic stress before induction of ischemic stroke by transient middle cerebral artery occlusion exhibited increased lesion size (33.7±2.3 mm³ versus 23.9±2.4 mm³). HR reduction led to a marked reduction of the infarct volume to 12.9±3.3 mm³.

Conclusions—Chronic stress impairs endothelial function and aggravates ischemic brain injury. HR reduction protects from cerebral ischemia via improvement of endothelial function and reduction of oxidative stress. These results identify heart rate as a mediator of vascular effects induced by chronic stress. (Stroke. 2011;42:1742-1749.)

Key Words: endothelial dysfunction ■ focal brain ischemia ■ heart rate reduction ■ mental stress

Psychological distress, depression, and anxiety are independently associated with cardiovascular disease and are predictors of ischemic stroke.1-4 Clinical studies suggest that several forms of psychological stress may contribute to atherosclerotic disease via direct vascular effects.5,6 Posttraumatic stress disorders and stress provoked by standardized mental stress tests can induce endothelial dysfunction, a prerequisite and precursor of atherogenesis and the formation of atherosclerotic plaque.7,8

The underlying pathophysiological mechanisms that link stress to increased risk for stroke are poorly understood. Clinical investigations characterize an increased cardiovascular reactivity—an abrupt increase in blood pressure and heart rate in response to stress—as a risk factor for hypertension and heart disease and demonstrate an increase in resting heart rate in response to mental stress.9,10 Evidence for an association between resting heart rate, cardiovascular disease, and mortality has been documented in a number of epidemiological and clinical studies.11 Ivabradine, an inhibitor of the I(f) current in the sinoatrial node, reduces resting and exercise heart rates without affecting cardiac contractility or blood pressure. Therefore, the compound is useful as a novel tool to study the effects of heart rate on vascular biology.12,13 We recently observed that heart rate reduction with ivabradine improves endothelial function and reduces atherosclerotic plaque formation in aortic vessels of apolipoprotein E-deficient mice.14

The vascular effects of chronic mental stress are only partially understood and the role of heart rate for the pathogenesis of ischemic stroke remains elusive. We hypothesized that mental stress may negatively affect stroke out-
come. Therefore, the aim of this study was to investigate the effects of chronic stress and heart rate reduction on the histomorphological outcome after ischemic stroke in mice and to delineate the mechanisms involved.

Subjects and Methods

Animals
Animal experiments were conducted in accordance with institutional guidelines and the German animal protection law. Eight-week-old male 129S6/SvEv mice (BIR, Berlin, Germany) were used for this study. The animals were maintained in a 22°C room with a 12-hour light/dark cycle and received drinking water ad libitum. Mice were randomized to vehicle or oral ivabradine treatment via chow pellets supplemented with commercially available ivabradine (Procoralan; Servier) with a daily dose of 10 mg/kg body weight for 6 weeks. After 2 weeks of drug treatment, 2 groups of mice (n=10) were subjected to a 28-day stress paradigm.

Chronic Stress Procedure
The chronic stress procedure was performed as described by Strekalova et al15 with minor modifications. The procedure consists of exposure to rat, restraint stress, and tail suspension, which were applied in the following order: days 1 to 7, exposure to a rat; days 8 to 10, restraint stress; days 11 to 14, tail suspension; days 15 to 21, exposure to rat; days 22 to 25, restraint stress; and days 26 to 28, tail suspension. Heart rate and blood pressure were measured by a computerized tail-cuff system after termination of the stress protocol as described previously (BP-2000; Visitech Systems).

Heart Rate and Blood Pressure Measurements by Radiotelemetry
Additional C57BL/6N mice (Charles River Laboratories, Sulzfeld, Germany) were studied using a radiotelemetry system (PA-C10; Data Science International). Briefly, a pressure-sensing catheter was implanted into the left carotid artery. The transducer unit was inserted into a subcutaneous pouch along the right flank. After a recovery period of 10 days, heart rate and blood pressure recordings were collected, stored, and analyzed with the Dataquest ART software 3.0. Data for blood pressure and heart rate were collected continuously in 5-second intervals; values were averaged over intervals of 1 minute (tail suspension, day 11 after implantation, n=5 mice), 5 minutes (restraint, day 12, n=5), and 30 minutes (exposure to a rat, day 13, n=3). Starting from day 18, mice were treated with ivabradine (10 mg/kg per day). The stress protocol was repeated on days 20 to 22.

Aortic Ring Preparations and Tension Recording
The detailed preparation and the protocol of the tension recording of aortic rings are described in the online Data Supplement. The preparation was performed as described. Measurement of Lipid Peroxidation
Aortic and brain tissue (block of brain from bregma −2.12 to bregma −4.6) were homogenized in phosphate-buffered saline (pH 7.4) containing butylated hydroxytoluene (4 mmol/L). Lipid hydroperoxides were determined with the Lipid Peroxidation Assay Kit II (Calbiochem) as described.

Measurement of NADPH Oxidase Activity
NADPH oxidase activity was measured in aortic and brain tissue (block of brain from bregma −2.12 to bregma −4.6) by a lucigenin-enhanced chemiluminescence assay as previously described.16

Cultured Brain Endothelial Cells
Brain endothelial cells (bEnd.3, ATCC, CRL-2299) were cultured and grown to confluence using DMEM (ATCC-30-2002). Cellular mRNA expression of endothelial nitric oxide synthase (eNOS) was quantified with the TaqMan polymerase chain reaction system (Abi Science International). Briefly, a pressure-sensing catheter was implanted into the left carotid artery. The transducer unit was inserted into a subcutaneous pouch along the right flank. After a recovery period of 10 days, heart rate and blood pressure recordings were collected, stored, and analyzed with the Dataquest ART software 3.0. Data for blood pressure and heart rate were measured by a computerized tail-cuff system after termination of the stress protocol as described previously (BP-2000; Visitech Systems). Please refer to the online Data Supplement for the detailed method.

Vascular and Brain Gene Expression
The primer sequences and polymerase chain reaction protocols are provided in the online Data Supplement. The detailed method is provided in the online Data Supplement.

Immunofluorescence Analysis
Immunofluorescence studies were performed on paraffin-embedded 5-μm brain sections (block of brain from bregma 0 to −2.12) applying a monoclonal antibody against CD31 (PECAM-1, CD31; Santa Cruz) as described previously. The detailed method is provided in the online Data Supplement.

Cerebral Ischemia and Measurement of Physiological Parameters
After 6 weeks of treatment including 4 weeks of stress procedures, mice were subjected to left middle cerebral artery occlusion for 30 minutes, followed by reperfusion (72 hours) as described previously. The full method is provided in the online Data Supplement.

Statistical Analysis
Results are presented as mean±SEM. ANOVA and 2-way ANOVA were used for multiple comparisons. Post hoc comparisons were performed with Newman-Keuls and Tukey tests. P<0.05 was considered statistically significant.

Results
Heart Rate and Blood Pressure
Twelve-week-old male 129S6/SvEv mice were randomized to oral ivabradine (10 mg/kg body weight per day) or vehicle treatment for 6 weeks. After 2 weeks of treatment, 2 groups of mice (n=10 per group) were subjected to a 28-day stress paradigm. The Table shows mean heart rates and systolic and diastolic blood pressures after 4 weeks of stress. Chronic mental stress increased heart rate from 514±10 bpm to...
Treatment with ivabradine decreased heart rate by 14.9% in the stressed mice (485 ± 7 bpm; \( P<0.001 \)). To characterize acute effects of the 3 stress protocols on heart rate in vehicle-treated and ivabradine-treated mice, telemetric measurements were conducted in 5 mice. Figure 1 depicts that all 3 forms of stress provoked a marked and abrupt increase in heart rate immediately after initiation of stress. Tail suspension increased heart rate by ≈150 bpm. Restraint stress and exposure to rat induced an increase of ≈250 and ≈200 bpm, respectively. After tail suspension, mice recovered to the baseline heart rate within minutes, whereas restraint stress and exposure to rat induced an accelerated heart rate persisting considerably longer than the duration of stress application. Ivabradine treatment reduced baseline heart rate by ≈100 bpm and notably attenuated the basal heart rate during all 3 stress protocols. Telemetric measurement of mean arterial blood pressure was not different between the groups.

**Vascular Function**

Endothelium-dependent relaxation of aortic rings by carbachol was markedly impaired in mice exposed to the stress protocol (n=10 per group; \( *P<0.05 \) versus naive vehicle). Heart rate reduction by ivabradine improved endothelial function in stressed mice almost to the level of naive controls (\( P<0.05 \) versus stress; Figure 2A). Endothelium-independent vasorelaxation induced by glyceroltrinitrate and vasoconstriction induced by phenylephrine and KCl were similar in all groups (Figure 2B).

**Vascular Oxidative Stress**

Reactive oxygen species impair vascular function. As a global parameter of oxidative stress, lipid peroxidation of the aortic wall was quantified. Stressed animals displayed a 3-fold upregulation of vascular lipid hydroperoxides to 333% ± 24% compared with nonstressed littermates (\( P<0.05 \)). Heart rate reduction with ivabradine reduced lipid peroxidation in stressed mice to 187% ± 58% (\( P<0.05 \); Figure 3A). NADPH oxidase is a major source of superoxide radicals in the vascular wall.23 Figure 3B shows that NADPH oxidase activity in the aorta was upregulated to 222% ± 38% in the stressed mice (\( P<0.05 \)) and significantly reduced by ivabradine treatment (169% ± 43%; \( P<0.05 \)).

**Vascular Gene Expression**

The hallmark of endothelial dysfunction and atherogenesis is impaired nitric oxide-dependent endothelial vasodilation.24 To further address the effects of chronic stress on the vascular
phenotype, mRNA expression of vascular eNOS was quantified in aortic homogenates. In our model, chronic stress was associated with a downregulation of eNOS mRNA expression in the aorta to 84% compared to naive controls (P\textless 0.05). Concomitant ivabradine treatment of stressed mice prevented eNOS downregulation (Figure 3C). The local vascular renin angiotensin system participates in all stages of vascular disease. Upregulation of the vascular angiotensin II type 1 receptor expression is linked to the onset and progression of endothelial dysfunction and atherosclerosis. Repetitive stress led to a marked upregulation of angiotensin II type 1 receptor mRNA to 168% compared to naive controls (P\textless 0.05). Heart rate reduction with ivabradine attenuated stress-induced up-regulation of the angiotensin II type 1 receptor (112%\textless P\textless 0.05 versus stress; Figure 3D). The expression of vascular adhesion molecules (intercellular adhesion molecule-1, vascular cell adhesion molecule-1) and monocyte chemotactic protein-1 are regulated by oxidative stress and endothelial nitric oxide. Ivabradine treatment reduced mRNA expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 to 70%\textpm 11% and 58%\textpm 7% and reduced monocyte chemotactic protein-1 to 55%\textpm 12% in the aorta of stressed compared to vehicle-treated stressed mice (P\textless 0.05 for all comparisons).

### Brain Oxidative Stress

Chronic stress may induce alternations in the cerebral expression profile of genes associated with oxidative stress. Therefore, lipid peroxidation and NADPH oxidase activity were quantified in brain homogenates. Stressed mice displayed upregulation of brain lipid hydroperoxides to 140% compared to naive controls (P\textless 0.05). Heart rate reduction with ivabradine prevented the stress induced increase (94%\textpm 12%; P\textless 0.05; Figure 4A). Brain NADPH oxidase activity remained unchanged (Figure 4B).

### Brain Gene Expression

To determine cerebral effects of chronic stress, eNOS and angiotensin II type 1 receptor mRNA expressions were quantified in the brain. Mice subjected to stress exhibited downregulation of eNOS mRNA to 70% (Figure 4C). Angiotensin II type 1 receptor mRNA expression was decreased to 64%\textpm 9% compared to naive controls (P\textlt 0.05 for each comparison). Concomitant ivabradine treatment did not significantly prevent these effects. Moreover, ivabradine would not significantly prevent these effects. Moreover, ivabradine...
treatment was associated with a nonsignificant trend to reduce the mRNA expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 to 85% and 86% and monocyte chemotactic protein-1 to 85% in the brains of stressed mice.

**Brain Capillary Density**

An increase in brain capillary density is associated with improved stroke outcome in mice.28 Chronic stress did not affect density of CD31-positive cells in the brain. Heart rate reduction with ivabradine increased the number of CD31-expressing cells in naive mice from $4503 \pm 501$ to $6794 \pm 485$ (P<0.05). In stressed mice, ivabradine increased CD31-expressing cells from $4417 \pm 280$ to $5345 \pm 698$ (P<0.05; Figure 4 D). The ratio of CD31-positive cells per nuclei increased from 0.27±0.03 in naive to 0.39±0.01 in ivabradine-treated naive mice and from 0.23±0.02 in stressed to 0.36±0.04 in ivabradine-treated stressed mice (P<0.05 for each comparison; Figure 4E).

**Potential Direct Effects of Ivabradine on Brain Endothelial Cells**

To investigate potential direct effects of ivabradine on gene expression in brain endothelial cells, cultured bEnd.3 cells were treated with ivabradine (0.2–200 μM for 16 hours), and eNOS mRNA expression was examined by polymerase chain reaction analysis. As depicted in Figure 4F, eNOS mRNA expression was not altered by increasing doses of ivabradine.

**Effect of Chronic Stress and Heart Rate Reduction on Cerebral Lesion Size After Middle Cerebral Artery Occlusion**

Findings from epidemiological studies suggest that psychological distress is a predictor of ischemic stroke.1–3 To determine whether chronic stress affects lesion size after stroke male 129S6/SvEv mice were subjected to middle cerebral artery occlusion for 30 minutes followed by 72 hours of reperfusion, a model of mild cerebral ischemia. Lesion volumes were quantified by computer-assisted volumetry on 20-μm hematoxylin-stained cryostat sections. In mice exposed to chronic stress, cerebral lesion sizes after 4 weeks were increased in contrast to nonstressed controls (33.7±2.3 versus 23.9±2.4 mm$^3$; P<0.05). Chronic heart rate reduction with ivabradine potently reduced ischemic lesion size in stressed mice (33.7±2.3 versus 12.9±3.3 mm$^3$; P<0.05; Figure 5A, B).

**Discussion**

The data identify heart rate as an important mediator of vascular effects induced by chronic stress. Chronic mental stress impairs endothelial function, augments vascular and brain oxidative stress, and increases cerebral lesion size in mice. Heart rate
reduction by the I(f) current inhibitor ivabradine restores endothelial function, reduces oxidative stress, and protects from focal brain ischemia by a marked reduction of cerebral lesion size.

Endothelial dysfunction precedes vascular diseases in different vascular beds. Clinical and experimental investigations identify an association of psychological disorders and mental stress with impaired endothelial function. Animal models offer the opportunity to establish the causal sequence of events because unknown inherited or acquired factors that may be responsible for this association in humans are excluded. The data here demonstrate that exposure to recurrent mental stress impairs endothelial function in healthy wild-type mice. Monitoring during acute stress showed a pronounced stress-dependent increase in heart rate. Recordings subsequent to the 28-day period of recurrent stress demonstrated a persisting effect on heart rate in comparison to the nonstressed controls. To test the importance of this increase of heart rate for the vascular effects of chronic stress, the I(f) current inhibitor ivabradine was used. Heart rate reduction by ivabradine significantly improved endothelial function in stressed mice almost to the level of wild-type controls in absence of blood pressure-lowering. These data are in agreement with the concept of an increased resting heart rate as an independent cardiovascular risk factor and an accelerator of atherogenesis. Heart rate reduction with ivabradine improves endothelial dependent vasodilation in mouse models of lipid-induced endothelial dysfunction. Similarly, erectile dysfunction, which is caused by endothelial dysfunction, is restored by heart rate reduction. Those results extend earlier findings in cynomolgus monkeys demonstrating that endothelial injury induced by psychosocial stress was mediated via β1-adrenoceptor activation and was prevented by β-adrenergic blockade. Because ivabradine exerted no hemodynamic effects apart from heart rate reduction, and because no direct effects on isolated aortic ring preparations and vascular cells were observed, we suggest that the primary mechanism by which ivabradine exerts these vasculoprotective effects in stressed mice is the reduction of heart rate. However, the data do not exclude the possibility of pleiotropic effects of ivabradine in other circumstances.

An increased production and release of reactive oxygen species is one of the key events in the pathogenesis of endothelial dysfunction and atherosclerosis. Recent clinical evidence has shown that anxiety may increase reactive oxygen species formation independent of blood pressure in hypertensive patients. Moreover, in mice, the expression levels of the antioxidant enzymes glyoxalase-1 and glutathione reductase are correlated with anxiety-related behavior. Therefore, we hypothesized that mental stress would aggravate vascular oxidative stress and investigated lipid peroxidation, a global marker of oxidative stress, as well as the activity of the NADPH oxidase, an important source of...
vascular superoxide formation. The chronic stress paradigm led to increased vascular oxidative stress as displayed by a marked increase in lipid peroxidation and activity of NADPH oxidase. Heart rate reduction by ivabradine potently decreased NADPH oxidase activity in the aorta as well as aortic lipid peroxidation. Thus, the results of these assays show a robust antioxidative effect of ivabradine on the vasculature of stressed mice, providing an explanation for the observed effects on endothelial function. In addition, the data show that stress reduces the expression of nitric oxide synthase (eNOS), which is a major regulator of vascular function. Stress-induced downregulation was potently prevented by heart rate reduction induced by ivabradine. Consistent with these findings, ivabradine treatment of stressed mice resulted in a downregulation of the vascular adhesion molecules intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 that are nitric oxide-regulated and monocyte chemotactic protein-1 expression. Another important downstream effect of endothelial nitric oxide is angiogenesis, and brain capillary density determines stroke outcome in mice. Here, ivabradine significantly increased capillary density both in naive and in stressed mice, consistent with the importance of vascular nitric oxide for the ivabradine effects. In summary, these findings support the concept of heart rate reduction as an intervention to conserve endothelial integrity.

Several investigations have established a relevant relationship between stress and the renin-angiotensin-aldosterone system. Whereas increases in renin-angiotensin-aldosterone system activity are likely to play a role in cardiovascular adaptation during acute stress, sustained alterations of the system may contribute to the pathogenesis of cardiovascular disease during chronic stress. Angiotensin II type 1 receptor activation is involved in the multifactorial pathogenesis of endothelial dysfunction. However, to date, possible effects of stress on the vascular renin-angiotensin-aldosterone system have not been investigated. Here, the data show a marked increase in AT1 receptor expression induced by chronic stress as an indicator of local vascular renin-angiotensin-aldosterone system activation and endothelial activation. This effect was attenuated by heart rate reduction with ivabradine.

Examination of brain homogenates revealed downregulation of eNOS expression and increased markers of oxygen free radicals induced by the stress protocol. These data are in agreement with studies in rodents showing that chronic stress induces brain oxidative stress, which may predispose to increased damage after ischemia. Ivabradine showed no effect on NADPH oxidase, AT1 receptor, and eNOS in the brain homogenates, suggesting that the systemic vascular regulation of these parameters is even more relevant. Importantly, ivabradine prevented upregulation of reactive oxygen species induced by stress in the brain tissue, which was associated with reduced stroke susceptibility.

Psychological distress has been investigated as a potential risk factor for cardiovascular and cerebrovascular disease in community cohorts and as a risk factor and predictor for cardiac events, stroke, and mortality in patients with established coronary or cerebrovascular disease. Several mechanisms have been proposed to explain the increased risk of cardiovascular disease in mental distress. However, to date, a definite association between stress and stroke could not be substantiated. A relevant and novel finding of our study is the evidence of a causal relation between a predefined multimodal stress exposure and the morphological outcome after ischemic stroke in healthy wild-type mice. Repetitive stress increased stroke volume after middle cerebral artery occlusion followed by reperfusion. These data identify chronic mental stress as a cause of impaired outcome in ischemic stroke.

Measures that improve endothelial-dependent vasorelaxation such as statin treatment or physical activity protect from ischemic stroke. Therefore, we hypothesized that selective heart rate reduction by ivabradine might affect stroke outcome and randomized stressed mice to ivabradine treatment. Heart rate reduction potently protected mice subjected to stress from cerebral ischemia. Because the physiological parameters such as blood pressure were not affected by ivabradine, the decrease in infarct volume is most likely mediated by heart rate reduction. Conservation of endothelial homeostasis represents a prerequisite and a basic mechanism of stroke protection in mice, as shown by a multitude of experimental data. Our data are supported by recent evidence showing that deterioration of the endothelial dilator function of cerebral arteries associated with dyslipidemia in mice is prevented by heart rate reduction with ivabradine.

Conclusions

In conclusion, our experiments show that heart rate is an important mediator of the stress-induced vascular damage. Heart rate reduction restores endothelial function, reduces oxidative stress, and protects from ischemic brain injury. These data suggest heart rate as a potential novel target for the improvement of cerebrovascular function that may be worth prospective testing in clinical studies.

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Disclosures

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Chronic Stress Procedure
The chronic stress procedure was carried out as described by Strekalova et al. with minor modifications. The procedure consists of exposure to rat, restraint stress, and tail suspension which were applied in the following order: days 1-7: exposure to a rat; days 8-10: restraint stress; days 11-14: tail suspension; days 15-21: exposure to rat; days 22-25: restraint stress; and days 26-28: tail suspension.

Exposure to rat: At the beginning of the dark phase of the light cycle two mice were placed inside a cage with diameters of 16x14x22cm which was then placed inside a rat cage with diameters of 33x19x55cm. Subsequently a male wistar rat (Charles River) was introduced into the rat cage and remained there for 15 hours (19:30 – 10:30). To improve olfactory contact between the animals, mice cages contained holes (diameter 0.7 cm) in the side wall. After the termination of the procedure animals were housed in their home cages for the rest of the day.

Restraint stress: Plastic restrainers were constructed by making air holes on 50 ml syringes. Animals were placed inside the restraining syringe (internal diameter 30 mm) for 2.5 h during the dark phase of the light cycle.

Tail suspension stress: Approximately 1 cm from the end, each mouse's tail was taped (3M Durapore tape) to a piece of metal tubing fixed to a wall. Mice were suspended by the tail approximately 80 cm above the floor for 6 min/day during the dark phase of the light cycle.

Heart rate and blood pressure were measured by a computerized tail-cuff system after termination of the stress protocol as described previously (BP-2000, visitech Systems, Apex, NC) in conscious animals.

Aortic Ring Preparations and Tension Recording
After excision of the descending aorta, the vessel was immersed in Tyrode solution containing, in mmol/L, NaCl 118.0, CaCl₂ 2.5, KCl 4.73, MgCl₂ 1.2, KH₂PO₄ 1.2, NaHCO₃ 25.0, Na EDTA 0.026, D(+)glucose 5.5, pH 7.4. Adventitial tissue was carefully removed. Three-millimeter rings were mounted in organ baths filled with the above-described buffer (37°C; continuously aerated with 95% O₂ and 5% CO₂) and were attached to a force transducer, and isometric tension was recorded. The vessel segments were gradually stretched over 60 minutes to a resting tension of 10 mN, which was maintained throughout the experiment, and were allowed to equilibrate for another 30 minutes. Drugs were added in increasing concentrations to obtain cumulative concentration-response curves: KCl (20 and 40 mmol/L), phenylephrine (1 nmol/L to 10 µmol/L), carbachol (1 nmol/L to 100 µmol/L), and glyceroltrinitrate (1 nmol/L to 10 µmol/L). The drug concentration was increased when vasoconstriction or vasorelaxation was completed. Drugs were washed out before the next substance was added. The relaxing effect of carbachol was abolished by adding L-NAME (1 µmol/L).

Vascular and brain gene expression
RNA from aortic and brain (block of brain from bregma 0 to bregma 3.14) homogenates were isolated with RNA-clean. 1 µg of the isolated total RNA was reverse transcribed using random primers and MMLV reverse transcriptase for 60 min at 42°C and 10 min at 75°C. Aortic and brain mRNA expressions of endothelial NO synthase (eNOS) and angiotensin II
type 1 (AT1) receptor were quantified with the TaqMan PCR system (Abi Prism 7700 Sequence Detection System, PE Biosystems) using TaqMan probes Mn00435204_m1Nos3 and Mn00616371_m1 provided by Applied Biosystems. Real-time RT-PCR using SYBR Green (Applied Biosystems, Darmstadt, Germany) was performed for mmMCP-1 (forward 5'-TGCCTAAGGTCTTCAGCAC-3', reverse 5'-AAAATGGATCCACACCTTGCG-3'), mmICAM-1 (forward 5'-ttccagtcaccatatccaaag-3', reverse 5'-ttcagaggcaggaaacagg-3') and mmVCAM-1 (forward 5'-ttctaccttgctgctgtgac-3', reverse 5'-acccacctgggtcttctt-3'). Expression was normalized to 18S ribosomal RNA.

**Immunofluorescence analysis**

Immunofluorescence studies were performed on paraffin-embedded 5 µm brain sections (block of brain from bregma 0 to -2.12) applying a monoclonal antibody against CD31 (PECAM-1, CD31, Santa Cruz). An anti-goat antibody coupled peroxidase (Sigma, Germany) was used as a secondary antibody. The signal was enhanced by thymidine-streptavidin amplification method (Perkin Elmer, USA and Dianova, Germany). Nuclei were counterstained with 4',6 diamidino-2-phenylindole (DAPI Calbiochem, Germany) as described previously.

**Cultured brain endothelial cells**

Brain endothelial cells (bEnd.3, ATCC, CRL-2299) were cultured and grown to confluence using DMEM (ATCC-30-2002). Cellular viability was determined by cell count, morphology, and trypan blue exclusion. Cellular mRNA expression of endothelial NO synthase (eNOS) was quantified with the TaqMan PCR system (Abi Prism 7700 Sequence Detection System, PE Biosystems) using TaqMan probe Mn00435204_m1Nos3. Expression was standardized to 18S ribosomal RNA. For 18S the primers were forward 5'-TCAACACGGGAAACCTCAC-3', reverse 5'-ACCAGACAAATCGCTCCAC-3'.

**Cerebral Ischemia and Measurement of Physiological Parameters**

After 6 weeks of treatment including 4 weeks of stress procedures mice were anesthetized with 1.0 volume percent isoflurane in 69% nitrous oxide (N₂O) and 30% oxygen (O₂) and subjected to left middle cerebral artery occlusion (MCAo) for 30 minutes followed by reperfusion (72h) as described previously. Regional cerebral blood flow (CBF) measured using laser Doppler-flowmetry (Perimed, Järfälla, Sweden) fell to less than 20% during ischemia and returned to approximately 80% to 100% within 5 minutes after reperfusion in all groups (p>0.05). Core temperature was maintained at 36.5±0.5°C. In randomly assigned animals, the left femoral artery was cannulated for arterial blood pressure monitoring and blood withdrawal. Arterial blood samples were analyzed for pH, arterial oxygen pressures, and partial pressure of carbon dioxide as described. After 72 hours, animals were euthanized by a pentobarbital overdose, and brains were quickly removed from the skull and snap-frozen in isopentane on dry ice for cryostat sectioning. Direct and indirect lesion volumes were quantified by computer assisted volumetry on 20 µm hematoxylin-stained cryostat sections as described previously.
Supplemental References


