Stress Worsens Endothelial Function and Ischemic Stroke via Glucocorticoids

Mustafa Balkaya, MSc*; Vincent Prinz, MD*; Florian Custodis, MD; Karen Gertz, MD; Golo Kronenberg, MD; Jan Kroeber, MS; Klaus Fink, MD; Ralph Plehm, MSc; Peter Gass, MD; Ulrich Laufs, MD; Matthias Endres, MD

Epidemiological studies have identified psychosocial distress as an important independent risk factor for stroke. In addition, evidence from experimental studies suggests that both chronic stress and elevated glucocorticoid (GC) levels negatively influence stroke outcome. However, the underlying pathomechanisms remain incompletely understood. Results from clinical studies indicate that stress may impair endothelial function, which is a key risk factor for both increased risk and poor outcome of cerebrovascular diseases and stroke. We tested whether chronic stress induces endothelial dysfunction and increases stroke vulnerability in healthy mice. Mifepristone, a GC receptor antagonist widely used in in vivo models, was used to probe whether the observed effects of stress were mediated by GC receptor signaling. It should be noted, however, that mifepristone also binds to the progesterone receptor.

Materials and Methods

**Animals and Drug Administration**

All experimental procedures conformed to institutional guidelines and were approved by an official committee (LaGeSo, Berlin, Germany). Wild-type male 129/SV mice (Forschungseinrichtungen für Experimentelle Medizin, Berlin, Germany) aged 6 to 8 weeks were housed in standard mouse cages in groups of 5 to 6 mice per cage at 22 to 23°C with a standard light-dark cycle (7 AM to 7 PM). Animals were randomized to experimental groups. Experimenters were blind to experimental assignment at the time of testing and only had access to tail marks. Mifepristone (Sigma) was dissolved in absolute ethanol and diluted in sesame oil (1:10 vol/vol). Thirty minutes before initiation of the stress procedure (vide infra), each animal received a 25 mg/kg-1 intraperitoneal injection of mifepristone or vehicle. During exposure to rat, animals received a second injection of mifepristone at 7 PM. Additional groups, which were not subjected to stress, received either vehicle or mifepristone and served as controls.

**Results**

Chronic stress caused a significant increase in heart rate, impaired endothelium-dependent vasorelaxation, increased superoxide production, and reduced aortic and brain endothelial nitric oxide synthase levels. Animals exposed to chronic stress showed major increases in ischemic lesion size. These deleterious effects of stress were completely reversed by treatment with mifepristone.

**Conclusions**

Chronic stress increases stroke vulnerability likely through endothelial dysfunction, which can be reversed by a glucocorticoid receptor antagonist. (Stroke. 2011;42:3258-3264.)
Chronic Stress Procedure
The stress procedure was carried out as described and applied in the following order: Days 1 to 7, rat exposure; Days 8 to 10, restraint stress; Days 11 to 14, tail suspension; Days 15 to 21, rat exposure; Days 22 to 25, restraint stress; and Days 26 to 28, tail suspension.6

Exposure to Rat
At the beginning of the dark phase, 2 mice were placed inside a cage with diameters of 16×14×22 cm, which was then placed inside a rat cage with diameters of 33×19×55 cm. A rat was introduced into the rat cage for 15 hours (11:30 PM to 2:30 PM).

Restraint Stress
Animals were placed inside the restraining syringe (internal diameter 30 mm) for 2.5 hours during the dark phase (7:30 PM to 10:00 PM).

Tail Suspension
Mice were suspended by the tail approximately 80 cm above the ground for 6 minutes/day. The procedure started at 7:30 PM.

Telemetric Recordings of Blood Pressure and Heart Rate
TA-PAC20 transmitters (DSI, St Paul, MI) were implanted into the left femoral artery. After a recovery period of 10 days, heart rate and blood pressure were recorded for 20 seconds every 5 minutes over 32 consecutive days and averaged as presented in Figure 1 and the Supplemental Figure 1 (http://stroke.ahajournals.org). Data were stored and analyzed using Dataquest ART software 3.0.7

Measurement of Corticosterone Plasma Levels and Adrenal Gland Weight
Corticosterone concentrations were measured using a commercially available radioimmunoassay (MP Biomedicals Inc, Europe, Belgium; sensitivity 3 ng/mL; interassay variance 6.9%). After euthanasia, adrenal glands of the animals were carefully dissected and weighed.8

Preparation of Aortic Rings and Measurement of Endothelium Function
The preparation and the protocol of the tension recording of aortic rings were performed as previously described.9

Endothelial Nitric Oxide Synthase mRNA TaqMan Polymerase Chain Reaction Measurement
Endothelial nitric oxide synthase (eNOS) reverse transcription–polymerase chain reaction was performed as described.9

Western Blot Analysis
Immunoblotting for eNOS (BD 610296) and β-actin (sc1615-R), to control for equal protein loading, using an enhanced chemiluminescence kit (Amersham) was performed as described.9

Measurement of Vascular Superoxide Production
Superoxide release in intact aortic segments was determined by L-012 chemiluminescence as described.9 Superoxide (SO) release is expressed as relative chemiluminescence per milligram aortic tissue.

Measurement of Lipid Peroxidation
Brain tissue was 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, Gibbstown, NJ) and expressed as nanomoles per milligram of protein as described.9

Cerebral Ischemia
Mice were anesthetized by 1.0% (vol/vol) isoflurane in 69% N2O and 30% O2. Focal cerebral ischemia was induced by 30 minutes filamentous middle cerebral artery occlusion and reperfusion as described previously.10 Regional cerebral blood flow was measured by means of a flexible probe and laser-Doppler monitoring (Perimed) in a subset of animals. Independent of group assignment, regional cerebral blood flow dropped to <15% of baseline at filament insertion (ie, reduction in regional cerebral blood flow in percent of baseline: naïve vehicle: 89.8±1.7; stress vehicle: 89.6±1.4; naïve mife: 90.4±2; naïve stress: 87.6±0.6; n=5–7 per group). Rectal temperature was controlled and kept constant at 36.5±0.5°C. After 72 hours reperfusion, animals were euthanized by a pentobarbital overdose and brains quickly removed from the skull and snap-frozen in isopentane on dry ice for cryostat sectioning. Direct and indirect lesion areas were quantified by computer-assisted volumetry on 20-μm hematoxylin-stained sections as described.10

Measurement of Cerebral Blood Flow
Cerebral blood flow was quantified using the 14C-iodoantipyrine technique under etomidate anesthesia as described in detail previously.10

Data Analysis
Data are presented as means±SEM. For statistical comparisons, 2-way analysis of variance followed by Tukey post hoc test was applied as applicable. P<0.05 was considered statistically significant.

Results
Effects of Stress and Mifepristone Treatment on Physiological and Hypothalamic–Pituitary–Adrenal Axis Parameters
Mice were either subjected to chronic stress (ie, by repeated exposure to rat, tail suspension, and restraint stress) or were left undisturbed in their home cages (naïve) for a total of 28 days. In a 2×2 design, mice were treated with either vehicle or mifepristone. Physiological parameters were measured by continuous telemetric recordings until Day 32.

Figure 1 shows the acute effects of the 3 different stress paradigms on heart rate in vehicle-treated animals. Although there were marked (and also repeated) effects on heart rate directly linked to all 3 stressors, there were only subtle effects on blood pressure (not shown). Similarly, in mifepristone-treated mice, all 3 stressors conferred immediate heart rate increases with no apparent effects on blood pressure (Supplemental Figure IA–C). When chronic effects on these physiological parameters were averaged over 28 days, there was a significant increase in heart rate induced by stress, which was only modestly blunted in mifepristone animals (Table). In contrast, stress had no relevant effect on systolic or diastolic blood pressure, whereas mifepristone conferred a modest (but significant) reduction of systolic (but less so of diastolic) blood pressure (Table). In accordance with these results, chronic stress conferred a significant increase in adrenal gland weight and significantly increased corticosterone plasma levels. Mifepristone treatment increased both adrenal gland weight as well as corticosterone levels independent of stress (Figure 2A–B).

Effects of Stress and Mifepristone Treatment on Endothelium Function
After termination of the chronic stress procedure, animals were euthanized and their aortas and brains harvested. Chronic stress significantly downregulated eNOS mRNA expression in the aorta to 75%±21% of vehicle-treated controls (P<0.05). Similarly, eNOS mRNA levels in brain were reduced to 72%±8%...
of vehicle-treated controls ($P<0.05$). In addition, eNOS protein expression in the brain determined by Western blotting was significantly reduced in stress animals. These effects of stress on eNOS mRNA expression and protein levels were completely abrogated by mifepristone. SO release as assessed by L012 chemiluminescence was also significantly increased by exposure to chronic stress ($P<0.05$). Furthermore, stressed animals displayed a significant upregulation of lipid hydroperoxides compared with nonstressed animals ($P<0.05$). Again, mifepristone treatment completely reversed these effects (Figures 3A–B and 4A–C).

Finally, endothelium-dependent vasorelaxation of isolated aortic rings by carbachol was significantly impaired in the stress group ($P<0.05$), which was completely reversed by mifepristone (Figure 3C). In contrast, there were no differences among groups regarding endothelium-independent vasorelaxation induced by glyceroltrinitrate or vasoconstriction induced by phenylephrine and KCl (not shown).

Table. Average Heart Rate and Systolic/Diastolic Blood Pressures During the Stress Procedure

<table>
<thead>
<tr>
<th></th>
<th>Naive Vehicle</th>
<th>Stress Vehicle</th>
<th>Naive Mife</th>
<th>Stress Mife</th>
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<tbody>
<tr>
<td><strong>Morning</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate,</td>
<td>487±3</td>
<td>532±4.3*</td>
<td>471±4.8†</td>
<td>511±3.6‡</td>
</tr>
<tr>
<td>beats/min</td>
<td></td>
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<tr>
<td>Systolic pressure, mm Hg</td>
<td>129.6±0.5</td>
<td>132.5±0.6*</td>
<td>120.5±0.6†</td>
<td>127.2±0.5‡</td>
</tr>
<tr>
<td>Diastolic pressure, mm Hg</td>
<td>95.1±0.3</td>
<td>96.5±0.5*</td>
<td>92.8±0.8†</td>
<td>97.3±0.4</td>
</tr>
<tr>
<td><strong>Evening</strong></td>
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</tr>
<tr>
<td>Heart rate,</td>
<td>549±5.4</td>
<td>579±5.3*</td>
<td>543±5.8</td>
<td>562±5.9‡</td>
</tr>
<tr>
<td>beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic pressure, mm Hg</td>
<td>137.8±0.7</td>
<td>139.6±0.8</td>
<td>130.5±0.7†</td>
<td>135.1±0.7‡</td>
</tr>
<tr>
<td>Diastolic pressure, mm Hg</td>
<td>101.5±0.5</td>
<td>101.1±0.7</td>
<td>100.5±0.6</td>
<td>103±0.6‡</td>
</tr>
</tbody>
</table>

*P<0.05 for stress vs naïve.
†P<0.05 naïve/mifepristone vs naïve.
‡P<0.05 stress mifepristone vs stress vehicle.

**Effects of Chronic Stress and Mifepristone Treatment on Cerebral Blood Flow and Cerebral Lesion Size After Focal Cerebral Ischemia**

Additional groups of animals were exposed to 30 minutes middle cerebral artery occlusion after termination of the chronic stress paradigm. In naïve and stress animals, absolute...
cerebral blood flow was quantified during the middle cerebral artery occlusion procedure by $^{14}$C-iodoantipyrine autoradiography. In stress animals, the volume of tissue with severely reduced blood flow ($<10 \text{ mL/100 g/min}$) tended to be enlarged relative to naive animals (Figure 5A–B). Consequently, at 72 hours, vehicle-treated stress animals showed significantly enlarged (approximately 50%) lesions compared with controls ($P<0.05$). Mifepristone treatment reduced ischemic lesion sizes of stress mice to control levels, whereas mifepristone-treated naive animals displayed enlarged lesions relative to vehicle-treated control animals ($P<0.05$; Figure 5C–D).

Discussion

This study has the following major findings: (1) chronic stress induced a marked increase in heart rate associated with adrenal gland hypertrophy and elevated corticosterone levels in mice; (2) chronic stress impaired endothelium-dependent vasodilation and was associated with reduced levels of eNOS and increased reactive oxygen species and lipid hydroperoxide production in the vasculature and brain; (3) chronically stressed mice developed significantly larger ischemic lesion...
volumes when exposed to 30 minutes of middle cerebral artery occlusion/reperfusion; (4) the effects of chronic stress were mediated, at least in part, through activation of GC receptors, because the GC receptor-blocker mifepristone completely reversed the adverse effects of chronic stress on vascular function and stroke outcome.

We adapted a previously described model of chronic stress, which includes repeated exposure to restraint stress, tail suspension, and exposure to rat. Each of these stressors conferred immediate increases in heart rate. Consequently, heart rate averaged over 1 month of telemetric recordings was significantly increased by approximately 40 beats/min in stressed mice. In contrast, the different stressors only mildly affected acute blood pressure responses. Note that blood pressure measured telemetrically in awake, freely moving mice is higher than in anesthetized animals and also higher compared with tail-cuff measurements.11 Moreover, chronic stress led to increased hypothalamic–pituitary–adrenal system activity as demonstrated by elevated circulating corticosterone and adrenal hypertrophy. Exposure to stress has been characterized as an independent risk factor for stroke and dysregulation of the hypothalamic–pituitary–adrenal axis has been suggested to negatively influence stroke outcome.1,2

Other mechanisms apart from endothelial dysfunction by which GC excess may contribute to exacerbation of neuronal death may include calcium excitotoxicity, suppression of antiapoptotic gene expression, and altered neuroinflammation.3 The effects of GCs on stroke outcome are complex and may be beneficial or harmful depending on the time point and the duration of corticosteroid administration. For example, chronic GC treatment may increase stroke susceptibility and poststroke GC levels correlate with poor outcome and mortality.12 However, GC administration may also improve clinical outcome in a subset of patients with severe stroke and acute treatment with high doses of GCs reduces lesion size in experimental focal ischemia.13 This apparent paradox may relate to differential nontranscriptional versus transcriptional effects of the GC receptor.14,15

Although several studies addressed the immediate effects of acute stress on vascular disease and stroke outcome, relatively little is known with regard to chronic stress. In our study, end points (ie, vascular function, stroke outcome) were studied days after discontinuing the stress paradigm. Therefore, these end points do not reflect immediate responses to the acute stressors but rather prolonged vulnerability. To test whether these effects of chronic stress on vascular function...
were mediated by GC, we treated animals with mifepristone, a broad-spectrum GC receptor antagonist that was applied by daily intraperitoneal injections over 1 month. Interestingly, mifepristone-treated animals also responded with increases in heart rate to the acute stressors, and chronic heart rate values (and also blood pressure) of stressed mice were only modestly blunted by mifepristone treatment. As expected, mifepristone increased both adrenal gland weight as well as corticosterone levels (independent of stress exposure) due to impaired regulatory negative feedback of the hypothalamic–pituitary–adrenal axis.16

Stressed animals developed a robust vascular phenotype characterized by impaired endothelium-dependent vasodilation, reduced eNOS mRNA and protein levels in the aorta and brain, and an excessive increase in SO production in the aorta and in lipid hydroperoxides in the brain. Concomitant treatment with GC receptor antagonist mifepristone completely reversed this phenotype, normalized eNOS levels, reduced stress-induced SO production and hydroperoxides, and restored endothelial function. There is evidence that endothelium function in 129/SV mice is impaired compared with other mouse strains.11 The impact of stress on top of this, however, appears quite clear. Taken together, these results indicate that the effects of chronic stress on endothelium-dependent vasodilation were, at least in part, mediated through the GC receptor. The protective effects of mifepristone were apparent despite the fact that it only modestly blunted the immediate (and repeated) effects of stress on heart rate (Supplemental Figure I). Interestingly, a suppressive GC response element was identified in the eNOS promoter region, which may account for decreased eNOS expression induced by chronic stress (and its reversal by mifepristone).17 In preliminary experiments, we did not observe differences in eNOS phosphorylation and Akt phosphorylation in either the aorta or brain induced by stress or after mifepristone treatment (not shown).

Chronic stress resulted in enlarged ischemic brain lesions, which was reversed by mifepristone treatment.3 These results indicate that (1) endothelial dysfunction may contribute to stroke vulnerability induced by chronic stress; and (2) that this effect is mediated through activation of GC receptors. In the context of stroke, several studies have reported beneficial effects of the corticosteroid synthesis inhibitor metyrapone or of mifepristone when administered in the presence or absence of stress or exogenous GC administration.18,19 However, in most of these studies, drugs were administered acutely before or during cerebral ischemia and/or stress exposure. For example, the treatment of mice with a GC receptor antagonist immediately before stress also prevented the negative effects of stress on ischemic outcome (although the effects of exogenous corticosterone mimicked the effects of stress on infarct size).19 In contrast, in our study, ischemia experiments were performed after termination of the chronic stress exposure (see previously) and mifepristone was administered chronically during the stress paradigm. Surprisingly, mifepristone treatment increased cerebral lesion sizes in naïve mice (compared with vehicle treatment), which may at least in part be mediated by reduced GC signaling. Similarly, Risedal and colleagues reported an increase in stroke lesion size with metyrapone treatment in unstressed animals.19 Glucocorticoid actions before, during, and after the termination of stress are highly complex.20 It is possible that blocking the effects of GCs in the absence of a full-scale stress response may have different effects on compensatory mechanisms from the ones observed in the presence of chronic stress. Further research is needed to fully understand the mechanisms behind chronic GC blockage-induced lesion exacerbation.

**Conclusions**

Our study links chronic stress and activation of the hypothalamic–pituitary–adrenal axis to endothelial dysfunction conferring increased vulnerability to brain ischemia. Downregulation of eNOS as well as increased SO production clearly demonstrate stress-induced endothelial pathology. The reversal of endothelial dysfunction (along with the associated changes in eNOS mRNA, eNOS protein expression, and SO production) by mifepristone treatment indicates that most of the deleterious effects of chronic stress are mediated through GC signaling. We here provide a reliable model for studying the relation between stress and stroke outcome. Our findings contribute to the understanding of the biological mechanisms underlying adverse health effects of chronic stress.

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**Disclosures**

None.

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Supplemental Data

Supplementary Figure 1: Telemetric recordings of heart rate in response to different stress protocols (stressed vs. naïve mifepristone-treated animals). The duration of the stressor is highlighted in grey: (A) rat exposure, (B) restraint, (C) tail suspension; n = 4-5 animals per group.
Figure Sup. 1

A. Exposure to Rat

B. Restraint

C. Tail Suspension
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Abstract

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Abstract

Background and objectives: Mice exposed to chronic stress have an increased risk of aortic aneurysm development. This study aimed to investigate the effects of chronic stress on aortic aneurysm development in mice. Methods: Mice were exposed to chronic stress for 12 weeks and the development of aortic aneurysms was assessed. Results: Chronic stress exposure increased the incidence of aortic aneurysms in mice. Conclusion: These findings suggest that chronic stress may contribute to the development of aortic aneurysms in mice.