Mild to Moderate Hypothermia Prevents Microvascular Basal Lamina Antigen Loss in Experimental Focal Cerebral Ischemia

Gerhard F. Hamann, MD; Dorothe Burggraf, PhD; Helge K. Martens, MD; Martin Liebetrau, MD; Gabriele Jäger; Nathalie Wunderlich; Michael DeGeorgia, MD; Derk W. Krieger, MD

Background and Purpose—Microvascular basal lamina damage occurs after cerebral ischemia and is important for the development of hemorrhage. The aim of this study was to determine whether hypothermia could maintain microvascular integrity in ischemic stroke.

Methods—Using the suture model, we subjected 12 rats to 3 hours of focal ischemia and 24 hours of reperfusion. Six rats received postischemic normothermia (37°C) and 6 received hypothermia (32°C to 34°C) for the reperfusion period; a group of 6 sham-operated animals without ischemia was used as control. Collagen type IV and hemoglobin were measured by Western blot analysis, matrix metalloproteinase (MMP)-2 and MMP-9 by gelatin zymography, and urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) by plasminogen-casein zymography.

Results—Hypothermia reduced basal lamina collagen type IV loss: 87±16% (hypothermia) versus 43±4% (normothermia) in basal ganglia and 74±16% versus 64±24% in cortex; hypothermia reduced hemorrhage from 431±65% (normothermia) to 241±28% (basal ganglia) (P<0.05). Hypothermia also reduced MMP-2, MMP-9, uPA, and tPA activity: 310±86% versus 1019±22%; tPA activity: 61±17% versus 111±13%; cortex: MMP-2: 53±6% versus 116±1%; MMP-9: 16±4% versus 123±3%; uPA: 180±27% versus 176±10%; tPA: 91±15% versus 101±8%; each difference: P<0.001 (nonischemic control side=100%).

Conclusions—Hypothermia maintains microvascular integrity and reduces hemorrhage and the activities of MMP-2, MMP-9, uPA, and tPA. (Stroke. 2004;35:764-769.)

Key Words: basement membrane ■ cerebral ischemia ■ hypothermia ■ metalloproteinases ■ microcirculation ■ plasminogen activators ■ tissue plasminogen activator

Interendothelial tight junctions, the basal lamina, and perivascular astrocytes constitute the blood-brain barrier. After disruption of the endothelium in focal cerebral ischemia, the basal lamina prevents extravasation of cellular blood elements. Loss of basal lamina integrity results in hemorrhage. The components of the basal lamina (type IV collagen, laminins, and fibronectin) were shown to be degraded in a baboon stroke model. The mechanisms involved are not entirely understood. Noncellular proteolytic systems, eg, matrix metalloproteinases (MMPs) and the plasminogen-plasmin system, hydrolyze the basal lamina. MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) are known to degrade type IV collagen and laminin, and both have been reported to be increased in experimental cerebral ischemia.

Treatment with intravenous recombinant tissue-type plasminogen activator (tPA) within 3 hours after stroke onset improves clinical outcome but carries the risk of hemorrhage. Clinical trials with mild to moderate hypothermia in acute stroke patients are under way, but its benefit has not yet been proven. Hypothermia limits ischemic damage by decreasing metabolism, suppressing blood-brain barrier breakdown, and reducing free radical formation and inflammation. In animal stroke models, hypothermia was also shown to decrease infarct size.

The aim of this study was to evaluate the effect of postischemic mild to moderate hypothermia on the microvascular basal lamina during focal cerebral ischemia.

Materials and Methods

Methods
All experimental procedures were approved by the government of Upper Bavaria (211-2531/48/98) and were in accordance with animal protection guidelines. For additional details, see the Appendix, which is available online at http://stroke.ahajournals.org.
Experimental Groups
All experiments used male Wistar rats (weight, 250 to 300 g) (Charles River Laboratories, Sulzfeld, Germany) (a total of 18 rats: 12 for ischemia/reperfusion, 6 for sham-operated controls). A period of 3 hours of ischemia was followed by 24 hours of reperfusion. Six of these animals were kept at a body temperature of 37°C as the normothermic group, and the remaining 6 were kept at a body temperature of 32°C to 34°C for the reperfusion period as the hypothermic group.

Preparation Protocol
For details, see Hamann et al.17 The suture model was used.18 At the end of the reperfusion period the brains were removed immediately, and the skull base was inspected for hemorrhage.

Animal Experiments
Mild to moderate hypothermia (32°C) was induced 30 minutes before reperfusion by applying active external cooling. Both the temperature control and the method of inducing hypothermia were adapted from Yanamoto et al.19 The body temperature was measured by a small thermistor in the right temporal muscle. The hypothermic rats were kept in a refrigerated cage at 4°C. Cage and body temperatures were continuously monitored; body temperature was kept at 32°C by feedback. The normothermic animals were also continuously monitored, and a heating pad was adjusted to maintain normothermia.

Control animals were sham-operated by advancing the thread only 12 mm toward the intracranial part of the internal carotid artery so that it did not occlude the middle cerebral artery.

Preparation of Cryostat Sections
Cryostat sections of 10-μm thickness were taken from regions 0 to 1 mm behind the bregma20 and stored at −80°C.

Protein Isolation and Western Blot for Collagen Type IV and Hemoglobin
For details, see Hamann et al.17 The antibodies used were goat anti-collagen type IV at a dilution of 1:500 (Southern Biotechnology) and a polyclonal rabbit anti-hemoglobin antibody at a dilution of 1:200 (DPC Biermann).

Gelatin Zymography for MMPs
For details, see Burggraf et al.21 Molecular standards and recombinant human MMP-2 and MMP-9 standards (Sigma) were used to calibrate molecular weights.

Plasminogen-Dependent Casein Zymography
Gel zymography was adapted from the procedure described.22 Transparent zones of lysis at 64 and 46 kD correspond to tPA and urokinase-type plasminogen activator (uPA), respectively. Molecular standards were used to calibrate molecular weights.

Analysis of Blotting Results
The bands of the Western blot and zymography were scanned and analyzed with an optical analysis program (TINA, version 2.08, Raytest Isotopenmessgeräte GmbH) by optical densitometry. To allow comparison over multiple samples run in different gels, the amount of the proteins in the ischemic side was normalized by dividing it by the nonischemic side. For details, see Hamann et al.17

Immunohistochemistry
The presence and volume of brain infarction were determined by microtubule-associated protein 2 (MAP-2) staining; for details, see Hamann et al.17 and Kloss et al.23

Statistical Analysis
Data were expressed as mean±SEM. All analyses were done by ratios of the ischemic to the nonischemic sides. Comparisons between the experimental groups were made with the Mann-Whitney U test (level of significance of 5%). MMP-2, MMP-9, uPA, and tPA analyses were performed with an ANOVA test (Kruskal-Wallis analysis).

Results
Reduced Infarct Volume
Rats undergoing hypothermia had significantly lower infarct volumes: 153±42 mm³ after hypothermia versus 192±43 mm³ in normothermic animals (Table 1).

Protection of Basal Lamina
The Western blot analysis revealed a significant loss of collagen type IV. In the normothermic group, collagen type IV was reduced to 64±4% in the cortex and to 43±4% in the basal ganglia compared with the nonischemic control side. The breakdown of collagen was significantly less in the hypothermic animals. Collagen was reduced to 74±16% in the cortex and to 87±16% in the basal ganglia (Figure 1). In the sham-operated animals, no difference of the collagen type

<table>
<thead>
<tr>
<th>Table 1. Infarct Volume</th>
<th>Infarct Volume, mm³</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>152.5</td>
</tr>
<tr>
<td>Normothermia</td>
<td>191.9</td>
</tr>
</tbody>
</table>

Infarct volume in rats (3 h ischemia/24 h reperfusion) undergoing hypothermia compared with that in normothermic controls.

![Figure 1. A, Western blot analysis of collagen type IV in brain sections after ischemia/reperfusion at normothermia (lanes 1 to 4) and hypothermia (lanes 5 to 8). Protein extracts (20 µg) from ischemic cortex (lanes 1 and 5), nonischemic cortex (lanes 2 and 6), ischemic basal ganglia (lanes 3 and 7), and nonischemic basal ganglia region (lanes 4 and 8) were analyzed with a rabbit anti-collagen type IV antibody 1:500 (Southern Biotechnology) followed by a peroxidase stain. B, Reduction of collagen type IV content in control animals (normotherm), animals treated with hypothermia (hypotherm), and sham-operated animals (no ischemia). Data are mean values of 6 animal experiments. *P<0.05 (ANOVA), significance of ratio of ischemic to nonischemic groups for comparison between control and hypothermia groups.](image-url)
IV content of both hemispheres could be seen (Table 2). The reduced loss of collagen type IV was significant (cortex and basal ganglia) (P<0.05).

**Reduction of Hemoglobin Extravasation**
Table 2 shows the hemoglobin data of the normothermic and the hypothermic groups. Ischemia led to the extravasation of hemoglobin (431±65% basal ganglia, 197±25% cortex), which was dramatically reduced by hypothermia (to 241±28% and 163±10%, respectively; P<0.05).

**Concentration of MMPs**
Ischemia/reperfusion increased the MMP-2 concentration. Cortical areas in normothermic animals exhibited concentrations of 116±1% of the nonischemic control side, and the basal ganglia regions exhibited 115±5%. Sham-operated animals showed no change in the MMP activities (Table 3). Both MMP-2 and MMP-9 were significantly reduced by hypothermia (MMP-2 to 53±6% of the nonischemic side in cortical areas and to 71±20% in the basal ganglia, P<0.001; MMP-9 to 16±4% in cortical and 38±12% in basal ganglia regions, P<0.001) (Figure 2).

**Activity of Endogenous Plasminogen Activators**
The activity of tPA in ischemic cortical areas compared with the nonischemic side was 101±8% in normothermic animals; the respective value for the basal ganglia was 111±13%. Hypothermia reduced these activities to 91±15% in cortex and to 61±17% in basal ganglia (P<0.001). Animals with no ischemia showed no change in the activity of tPA. The uPA activity for the cortex was not significantly different between hypothermia (180±27%) and normothermia (176±10%), but there was a significant increase (P<0.0001) relative to sham-operated animals: 95±7% (cortex) and 101±8% (basal ganglia). In the basal ganglia there was a striking increase of uPA activity in normothermic controls (1019±22%); this was significantly reduced by hypothermia (310±86%; P<0.001) (Figure 3; Table 3).

**Discussion**
The main finding of this study is that postischemic mild to moderate hypothermia prevents microvascular basal lamina antigen loss and subsequent hemoglobin extravasation during focal ischemia. The degradation of the basal lamina probably begins very early in cerebral ischemia. A similar degree of microvascular damage in normothermic controls was reported previously. However, we have now shown for the first time that postischemic mild to moderate hypothermia after 3 hours of ischemia and 24 hours of reperfusion significantly reduces the loss of collagen type IV from the basal lamina of cerebral microvessels in the rat. Accordingly, infarct size was also reduced, as expected. It could also be shown that subsequent hemorrhage was significantly reduced. We did not examine the effects on edema in this study. The

### TABLE 2. Content of Collagen Type IV and Hemoglobin in the Cortex and the Basal Ganglia From Sham-Operated Animals and Animals With Ischemia/Reperfusion + Normothermia and + Hypothermia

<table>
<thead>
<tr>
<th></th>
<th>Collagen Type IV</th>
<th>Hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex P&lt;0.05</td>
<td>Basal Ganglia</td>
</tr>
<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>74 16</td>
<td>87 16</td>
</tr>
<tr>
<td>Normothermia</td>
<td>64 4</td>
<td>43 4</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>98 4</td>
<td>98 1</td>
</tr>
</tbody>
</table>

The average of 6 animal experiments was given. Numbers are percent compared with the nonischemic control side. The multicomparison analysis between the groups was done by ANOVA analysis.

### TABLE 3. MMP-2 and MMP-9 Expression and Enzymatic Activity of tPA and uPA in the Cortex and Basal Ganglia

<table>
<thead>
<tr>
<th></th>
<th>MMP-2</th>
<th>MMP-9</th>
<th>tPA</th>
<th>uPA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex P&lt;0.001</td>
<td>Basal Ganglia P&lt;0.001</td>
<td>Cortex P&lt;0.001</td>
<td>Basal Ganglia P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
<td>Mean SEM</td>
</tr>
<tr>
<td>Hypothermia</td>
<td>53 6</td>
<td>71 20</td>
<td>16 4</td>
<td>38 12</td>
</tr>
<tr>
<td>Normothermia</td>
<td>116 1</td>
<td>109 3</td>
<td>123 3</td>
<td>115 4</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>99 13</td>
<td>109 13</td>
<td>97 6</td>
<td>99 7</td>
</tr>
</tbody>
</table>

Active MMP-2 and MMP-9 expression and enzymatic activity of tPA and uPA in the cortex and the basal ganglia from sham-operated animals and animals with ischemia/reperfusion at 37°C and 32°C. Numbers are percent compared with the nonischemic control side.

*Difference between hypothermia and normothermia was not significant.
protective effect of hypothermia on the basal lamina may be explained by the reduction of the proteases MMP-2, MMP-9, tPA, and uPA.

Interendothelial tight junctions, the basal lamina, and perivascular astrocytes are jointly referred to as the blood-brain barrier. The endothelial barrier regulates substrate transfer. The basal lamina provides a structural barrier to extravasation of cellular blood elements and anchors endothelial cells and astrocytes. Intact microvascular basal lamina and integrin-mediated matrix adhesion are essential for cellular function. During cerebral ischemia, the functional and structural integrity of the endothelial barrier rapidly deteriorates. The only barrier that protects the brain from protein-rich fluids and cellular blood elements in this situation is the basal lamina, which is embedded in the extracellular matrix and consists of a sheet of collagen type IV and a net of laminin interconnected by entactin. Proteolytic enzymes from both the blood and brain tissue instantly start digesting the basal lamina. The brain parenchyma is then exposed to the blood.

Microvascular basal lamina changes under ischemic conditions involve the plasminogen-plasmin system, various MMPs, and leukocyte activation. Our data suggest that mild to moderate hypothermia is accompanied by reduced activity and concentration of proteinases, including MMP-2, MMP-9, tPA, and uPA. Several studies have demonstrated the critical role of protease degradation after cerebral ischemia.

Endothelial cells produce endogenous tPA to prevent wall thrombosis. Compelling uPA activation is seen in cerebral ischemia. A balanced fibrinolytic activity is essential for microvascular function. Besides its thrombolytic activity, which provides microvascular patency, plasmin hydrolyzes extracellular matrix proteins and activates MMP-9. The balance between proteases and their inhibitors determines whether there is breakdown or buildup of the extracellular matrix. At least 2 MMPs (MMP-2 and MMP-9) are involved in these processes. MMP-2 and MMP-9 activities were increased in neutrophils, endothelial cells, and macrophages in a permanent stroke model. MMP-2 is constitutively expressed and activated by a membrane-type metalloproteinase. Activation of MMP-2 in turn activates pro-MMP-9 to MMP-9. Activated MMP-9 appears as early as 3 hours after transient focal cerebral ischemia. Rosenberg et al have shown increases of pro-MMP-9 after transient ischemia. In the present study both MMP-2 and MMP-9 were significantly decreased by posts ischemic hypothermia.

Figure 2. A, Representative zymogram presenting the gelatinolytic activities in protein extracts collected from ischemic (lanes 1 and 5) and nonischemic (lanes 2 and 6) cortex and ischemic (lanes 3 and 7) and nonischemic (lanes 4 and 8) basal ganglia of a normothermic animal (lanes 1 to 4) and a hypothermic animal (lanes 5 to 8). In addition, protein extracts from sham-operated animals are shown: from ipsilateral cortex (lane 9) and basal ganglia (lane 11) as opposed to contralateral cortex (lane 10) and basal ganglia (lane 12). The lytic zones of pro-MMP-2 (72 kDa) and pro-MMP-9 (96 kDa) activity are marked. B, Ratio of ischemic to contralateral area is shown for normothermic (normotherm), hypothermic (hypotherm), and sham-operated animals. Data are mean values of 6 animal experiments. The difference between the groups is significant (**P<0.001, ANOVA).
gel activity reflects the overall MMP concentration rather than an in vivo activity since the SDS from the gel also activates inactive pro-MMPs. One can speculate that the reduction in proteolytic activities below 100% on the ischemic side of hypothermic animals reflects an earlier activity (by the regularly available proteolytic systems, as in nonischemic brain tissue), which is used up after longer reperfusion. The later, more severe, and pronounced proteolytic activation (as seen in normothermic controls) is inhibited by hypothermia. The reduction in MMP activity may also at least partly reflect decreased ischemic injury after hypothermia.

This study also revealed that endogenous plasminogen activation is significantly reduced by hypothermia. In particular, the strong activation of uPA, which is thought to play a key role in the brain after ischemia,28 was reduced to approximately 20% of the level of the normothermic controls. The main change of uPA is seen in basal ganglia in contrast to the cortex. This may reflect the occurrence of hemorrhagic complications, which are known to show a preference for the basal ganglia.

Like others, we also found the most robust effects in uPA and MMP-9 expression.28,37 One could argue that the cutback in ischemic uPA upregulation reduces secondary MMP activation and prevents collagen IV loss in the basal lamina.

Although most protective mechanisms of hypothermia remain elusive, there is evidence that hypothermia can reduce the consequences of cerebral ischemia in experimental15 and clinical settings.38,39 Our results demonstrate that mild to moderate hypothermia protects the basal lamina, in addition to reducing the infarct size, as was expected. Therefore, hypothermia may reduce hemorrhage after thrombolysis for acute ischemic stroke. Hypothermia could influence the delicate risk/benefit ratio of thrombolysis and allow more aggressive or delayed restoration of blood flow at a reduced hemorrhagic risk.

Acknowledgments
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