Evidence for Platelet-Activating Factor as a Novel Mediator in Experimental Stroke in Rabbits

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Platelet-activating factor is a potent mediator of inflammation, which has untoward effects on cerebrovascular and neural elements. While several investigators have reported attenuation of ischemic damage after treatment with antagonists of platelet-activating factor, no study has proved endogenous production of platelet-activating factor in ischemia of the central nervous system. We hypothesized that endogenous production of platelet-activating factor participates in the early pathologic manifestations of deteriorating stroke. In 12 rabbits, we found tissue levels of platelet-activating factor measured by the release of serotonin from washed platelets to be elevated by approximately 20-fold in spinal cord injured by 25 minutes of ischemia and 2 hours of reperfusion (2.80 ± 0.98 ng/g) compared with that in normal spinal cord (0.15 ± 0.06 ng/g, p < 0.01). Given during ischemia to seven rabbits, 10 mg/kg i.p. of a highly selective and potent antagonist of platelet-activating factor (BN 50739) accentuated the early postischemic hyperemia and prevented the delayed hypoperfusion measured by on-line laser-Doppler flowmetry (−35 ± 7% of baseline [n = 7] without versus 33 ± 14% with treatment, p < 0.01) and the edema formation measured as the increase in tissue water content (4.4 ± 0.7% without [n = 6] versus 2.1 ± 0.6% with [n = 7] treatment, p < 0.05) after 2 hours of reperfusion. This neurochemical and pharmacologic evidence emphasizes a new perspective of ischemia-induced phospholipid degradation and suggests an important role for platelet-activating factor in the early manifestations of stroke.

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Materials and Methods

We employed the well-described rabbit spinal cord model of deteriorating stroke\textsuperscript{13-15} during on-line monitoring of the spinal cord microcirculation with laser-Doppler flowmetry through a laminectomy at L-5. Recordings from two independent laser-Doppler probes were used to estimate average blood flow with excellent accuracy, which we have previously validated in this model.\textsuperscript{16} After a stabilization period of at least 30 minutes, spinal cord ischemia was induced in 35 tracheotomized and artificially ventilated, pentobarbital-anesthetized, male New Zealand White rabbits (for details see Reference 16) by inflating a balloon-tipped Swan-Ganz catheter (4F, American Edwards, Irvine, Calif.) advanced via the right femoral artery to occlude the abdominal aorta directly below the renal arteries (confirmed postmortem) and to monitor proximal mean arterial blood pressure. Another catheter (PE-60) was positioned via the left femoral artery to observe mean arterial blood pressure distal to the occlusion. The physiologic variables \( \text{Paco}_2 \), \( \text{PaO}_2 \), arterial pH, and rectal temperature were monitored periodically and were maintained within the normal ranges as described previously in this ischemia model.\textsuperscript{13} The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Resources, National Research Council, DHEW publ. No. (NIH) 85-23.

Spinal cord blood flow and proximal and distal mean arterial blood pressures were monitored during 25 minutes of ischemia and 2 hours of reperfusion in seven untreated rabbits. Another seven rabbits were treated with 10 mg/kg i.p. BN 50739, a potent and selective PAF antagonist,\textsuperscript{17} 10 minutes after the onset of ischemia. We have observed that this dosage of BN 50739 virtually blocks PAF-induced platelet aggregation for up to 2 hours (78±15% inhibition at 2 hours\textsuperscript{18}). Five more rabbits received only the vehicle (dimethyl sulfoxide with saline) of BN 50739. Seven sham-operated control rabbits not subjected to ischemia were monitored during the same period to reveal possible spontaneous or inadvertent alterations in microcirculation. At the end of the monitoring period in these 26 rabbits, tissue samples were excised promptly (<3 minutes after death) from the lumbar and normal (upper thoracic) zones of the spinal cord for the measurement of water content.\textsuperscript{19} In the 12 nontreated (the seven untreated plus the five vehicle-treated) rabbits subjected to spinal cord stroke, similarly obtained tissue samples excised <1 minute after death were homogenized rapidly in chloroform-methanol-saline. PAF was extracted from these samples using the Bligh-Dyer method\textsuperscript{20} followed by thin-layer chromatography isolation.\textsuperscript{21} The quantitative PAF bioassay was based on tritium-labeled serotonin release from washed rabbit platelets\textsuperscript{22} with a sensitivity of approximately 100 pg/g tissue. The PAF bioassay was performed by an investigator (T-L.Y.) not aware of the experimental groups.

We undertook a separate study to show that the isolated substance had the biologic properties of PAF. In five untreated rabbits subjected to spinal cord stroke, tissue samples from the injured zone obtained <5 minutes after death were assayed for PAF in the absence and presence of 2\times10^{-6} M BN 50739.\textsuperscript{16} In another four untreated rabbits the tissue samples were incubated for 20 minutes at 37°C\textsuperscript{23} with 1.5 \( \mu \)g phospholipase C instead of BN 50739.

All data are presented as mean±standard error of the mean for the indicated number of rabbits. Continuous blood flow recordings were digitized by using a digitizing tablet (Model 2210, Numonics, Montgomeryville, Calif.) with a program written for an IBM-PC (Sigma-Scan version 3.90, Jandel Scientific, Corte Madera, Calif.). Blood flow and edema data of the groups were compared using Kruskal-Wallis analysis of variance followed by the Mann-Whitney \( U \) test. We used Wilcoxon's paired test to compare PAF levels in normal and injured zones of the spinal cord of the same rabbit. Significant differences were considered to be indicated by \( p<0.05 \).

Results

In the 12 nontreated rabbits, the PAF bioassay suggested an approximately 20-fold increase in \( [\text{H}] \)serotonin release by samples from the injured zone compared with those from the normal zone of the same rabbit (2.80±0.98 and 0.15±0.06 ng/g, respectively; \( p<0.01 \) [Figure 1A]). Postmortem PAF metabolism did not seem to play a role since the PAF levels in samples from the injured zone obtained <5 minutes after death (Figure 1B) were similar to those obtained <1 minute after death. Serotonin release by samples from the injured zone of untreated rabbits was completely blocked by both BN 50739\textsuperscript{17} and phospholipase C (Figure 1B), which supports the specificity of this bioassay to measure the concentration of PAF.

There were no significant differences in the depth of ischemia among the untreated, BN 50739-treated, and vehicle-treated groups. Blood flow in the sham-operated control group showed no changes, proving the specificity of the ischemia-induced blood flow patterns. Among the seven untreated rabbits, hyperemia following reperfusion was observed in four (blood flow: 23%, 44%, 184%, and 202%, average percentage change over the first 15 minutes) while the circulation failed to recover in the other three (blood flow: -23%, -30%, and -48% percentage change), indicating early reperfusion failure. During the first 15 minutes of reperfusion, blood flow was not correlated with mean arterial blood pressure (data not shown). Furthermore, since no differences in mean arterial blood pressure existed among groups (even during the early reperfusion), laser-Doppler flowmetry probably reflects changes in blood flow at the level of the microcirculation. Treatment with BN 50739 effectively blocked the delayed...
hypoperfusion ($p<0.01$; Figure 2), but the vehicle-treated group showed no improvement and in fact suffered a $41\pm5\%$ ($p<0.05$) decrease in blood flow after 2 hours of reperfusion (data not shown). To characterize the variable early reperfusion patterns, we analyzed the area under the continuous laser-Doppler flowmetry curves during the initial 15 minutes of reperfusion, which is sensitive to both the degree and duration of early reperfusion hyperemia/failure (Figure 3). Area was increased by treatment with BN 50739, which confirmed the facilitation of initial reperfusion by the PAF antagonist.

In the sham-operated control group, a difference in the water content of the injured and normal zones of the spinal cord was expected because the lumbar sections contain more gray matter than the thoracic sections. Treatment with BN 50739 blocked postischemic edema formation since the difference between zones in the antagonist-treated group was significantly less than in the vehicle-treated group but not significantly greater than in the sham-operated control group (Table 1). Edema data from one untreated rabbit could not be obtained because of technical difficulties.

**Discussion**

Our experiments suggest that the concentration of PAF was increased by approximately 20-fold after stroke in spinal cord tissue. Since a highly selective PAF receptor antagonist prevented the simultaneous postischemic microcirculatory hypoperfusion and edema formation (Figure 2, Table 1), PAF could play a pivotal role in the early deterioration in stroke. Inhibition of the early and delayed postischemic hypoperfusion by the PAF antagonist is in agreement with our previous observation demonstrating perturbation of the rabbit spinal cord microcirculation by exogenous PAF. During postischemic hypoperfusion following forebrain ischemia in gerbils and in a new model of laser-induced focal penumbral microcirculatory failure in rats, similar beneficial effects of PAF antagonism were demonstrated, but not in association with PAF production. However, PAF levels comparable to our results have been detected in rat brain injured by chemically induced convulsions. To date, no study has indicated PAF production in ischemic CNS tissue.

Ischemia of the nervous tissue immediately activates phospholipases A$_2$ and C, which hydrolyze phospholipids from excitable membranes. Reperfusion enhances the metabolism of free fatty acids along the arachidonic acid cascade, which has been the focus of much of the research in the area of stroke. A significant source of arachidonate is alkylacylglycerol-3-phosphorylcholine, which is also the precursor of PAF. Therefore, this molecule could be the origin of both eicosanoids and PAF in ischemia-reperfusion. As the alternate metabolic pathway to the arachidonate cascade, conversion of this molecule by phospholipase A$_2$ could lead to the accumulation of lysophospholipids in general and PAF in particular.

Edema is frequently the immediate cause of death and the common denominator in vascular and traumatic accidents of the CNS. Therapeutic efforts aimed at removing intravascular blood clots in stroke patients have been described as precipitating massive, fatal edema formation during reperfusion after thrombolysis with tissue plasminogen activator. The sudden increase in available oxygen has been widely viewed as amplifying the state termed "reperfusion injury" and leading to enhanced lipid peroxidation. Therefore, efforts focused solely on the restoration of blood flow could be of limited value if the...
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Controversy still exists in the literature about the effect of early reperfusion hyperemia on the postischemic recovery of CNS tissue. In our experiments, increased hyperemia brought about by treatment with the PAF antagonist was associated with reduced postischemic edema after 2 hours of reperfusion. On the other hand, several of our untreated rabbits suffered from incomplete restoration of the circulation, which was not the case in the BN 50739–treated animals. Increased extravasation of labeled plasma protein following 25 minutes of ischemia occurs after 30 minutes of reperfusion in this stroke model. Therefore, postischemic edema after 2 hours of reperfusion can derive in part from leakage of fluid through the endothelium. It is possible that early impairment of reperfusion causes or reflects exacerbated damage to the endothelium. Interestingly, recent evidence links increased endothelial PAF

TABLE 1. Difference in Water Content Between Injured Lumbar and Normal Upper Thoracic Zones of Spinal Cord in Rabbits After 2 Hours of Reperfusion

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Difference in water content (%) vs. Control vs. Vehicle</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>0.99±0.23</td>
<td>...</td>
</tr>
<tr>
<td>Untreated</td>
<td>6</td>
<td>4.39±0.68</td>
<td>0.0031</td>
</tr>
<tr>
<td>BN 50739-treated</td>
<td>7</td>
<td>2.08±0.62</td>
<td>NS 0.027</td>
</tr>
<tr>
<td>Vehicle-treated</td>
<td>5</td>
<td>4.31±0.41</td>
<td>0.0048</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Probability values according to Kruskal-Wallis analysis of variance.
production with perturbation by oxygen radicals. This corresponds to our observation of facilitation of reperfusion by BN 50739 treatment. Arresting the early lack of reflow, which is also postulated to harbor local infarction, may therefore be a beneficial mechanism of PAF antagonism at the level of the endothelium.

PAF can be envisaged to play a critical role in the interaction between the multiple components suggested to be responsible for microcirculatory failure. Increased PAF production in the injured spinal cord may be the result of interrelated in situ and blood-borne factors. Several pathways of positive feedback for PAF production could be evoked by the blood–endothelium interaction. Local action of phospholipases could eventually liberate PAF from excitable membranes. Thrombin stimulates endothelial PAF production, which has further been observed to enhance the adherence of neutrophils and to activate macrophages. In stroke, local activation of the blood coagulation cascade might engage this mechanism to attract inflammatory cells. Activated macrophages can release toxic cytokines such as tumor necrosis factor and interleukin-1. PAF can impair the endothelial cytoskeleton to cause detachment from the basal membrane. As significant sources of blood-borne PAF, neutrophils could penetrate the broken BBB, potentiate PAF production in the injured area, and lead to an aggravated acute inflammatory response. Besides proinflammatory and procoagulant effects at nanomolar concentrations, PAF has been described to possess direct neurotoxicity and even general membrane detergent effects at micromolar concentrations. Indeed, the diversity of PAF effects described to possess direct neurotoxicity and even general membrane detergent effects may be useful in combating neuroinjury following CNS ischemia or trauma.

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