Perivascular Blood Attenuates Noradrenergic but Not Cholinergic Effects on Piglet Pial Arterioles

David W. Busija, PhD, and Charles W. Leffler, PhD

We examined the chronic and acute effects of perivascular blood on cerebrovascular responses to norepinephrine and acetylcholine in 35 piglets. In the chronic experiment, fresh autologous blood (n = 15) or cerebrospinal fluid (n = 14, control) was placed under the dura mater over the parietal cortex, and the piglets were allowed to recover from anesthesia. One to 4 days later, a closed cranial window was placed over the parietal cortex and baseline pial arteriolar responses and responses to topical application of the neurotransmitters norepinephrine (10^{-4} and 10^{-5} M) and acetylcholine (10^{-4} M) were determined. We also sampled cerebrospinal fluid from under the window during baseline conditions and during application of the neurotransmitters, and we measured the concentrations of prostanoids (6-ketoprostaglandin F_1 alpha, thromboxane B_2, prostaglandin F_2 alpha, and prostaglandin E_2) via radioimmunoassay. Pial arterioles in the chronic control group (n = 13) constricted by 20 ± 2% (mean ± SEM) in response to 10^{-4} M norepinephrine and by 28 ± 2% in response to 10^{-4} M acetylcholine. In the chronic blood group (n = 14), pial arterioles did not constrict significantly in response to 10^{-4} M norepinephrine but constricted normally (23 ± 4%) in response to 10^{-4} M acetylcholine. In the acute experiment, six other piglets had blood placed on the brain surface for 30 minutes and then removed; pial arterioles constricted by 21 ± 1% in response to 10^{-4} M norepinephrine (n = 5) and by 28 ± 4% in response to 10^{-4} M acetylcholine (n = 3). Prostaglandin E_2 concentrations in the cerebrospinal fluid increased similarly for both chronic groups during application of 10^{-4} M norepinephrine, and the concentrations of all prostanoids increased in the cerebrospinal fluid during application of 10^{-4} M acetylcholine. There were no significant differences in prostanoid levels between the two chronic groups under any condition. We conclude that prolonged contact of the pial arterioles with extravascular blood selectively attenuates cerebrovascular constriction in piglets. (Stroke 1990;21:441–446)
ulii in the cerebral circulation in vivo. In addition, topical application of these substances increases the production of prostanoids by cerebral tissues and/or vessels. Consequently, we also determined whether chronic placement of blood altered the production of prostanoids.

Materials and Methods

The animal protocols used were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee, Memphis.

In our model of ICH, fresh autologous blood is placed in the subarachnoid space so that it comes into direct contact with pial blood vessels. We anesthetized 29 piglets with halothane and nitrous oxide. Using aseptic procedures, we made a small burr hole in the skull over the frontal cortex. A 20-gauge Teflon catheter (Angiocath, Deseret Co., Sandy, Utah) was used to pierce the dura at an angle to the surface sufficient to prevent penetration into the brain. Following removal of the needle, the tip of the catheter was advanced 2 cm posteriorly under the dura to the parietal cortex and 2 ml of either fresh sterile nonheparinized blood (removed via direct puncture of the precava) (n=15) or sterile artificial cerebrospinal fluid (CSF) (see below for composition) (n=14, control) was injected over 1–2 minutes. The catheter was removed, the burr hole was filled with sterile bone wax, and the scalp was sutured. Piglets in these two chronic groups were treated with gentamicin and benzathine penicillin after surgery. Following surgery, the piglets exhibited no apparent behavior abnormalities.

The acute effects of blood on cerebrovascular dynamics were assessed in six other piglets. Following exposure of the brain surface as part of the procedure for implanting the cranial window (see below), 1 ml of fresh nonheparinized autologous blood was placed over the parietal cortex surface and allowed to sit for 30 minutes. The clot was then removed without touching the brain, and the brain surface was flushed gently with artificial CSF.

In the acute experiment, the cranial window was put into place after the clot was removed. In the chronic experiment, the cranial window was placed over the parietal cortex at the site of the injection of CSF (n=10) or blood (n=15) or on the side opposite the CSF injection (n=4). Since responses were similar (see “Results”), all 14 piglets that received CSF were combined; two, five, four, and three piglets were studied 1, 2, 3, and 4 days after CSF injection, respectively. In the chronic ICH group, two, six, three, and four piglets were studied 1, 2, 3, and 4 days after the injection of blood, respectively. The effects of dilator stimuli on pial arteriolar responses in the acute and chronic experiments have been described in preliminary form.

For implantation of the cranial window, the 35 piglets were initially anesthetized with 33 mg/kg i.m. ketamine hydrochloride and 3.3 mg/kg i.m. acepromazine, followed by α-chloralose (50 mg/kg i.v. initially, then 5–10 mg/kg/hr). Catheters were placed into a femoral artery to record blood pressure and to sample blood gases and pH and into a femoral vein to inject drugs and fluids. The piglets were intubated and ventilated with air. Body temperature was maintained at 37–38°C using a water-circulating rubber heating pad. After the scalp was removed, a 2-cm-diameter hole was made in the skull over the parietal cortex. The dura and arachnoid membranes were cut without touching the brain, and all cut edges were reflected over the bone. In the chronic ICH group, the clot was removed with forceps without touching the brain surface, and the brain was flushed gently with CSF. A stainless steel and glass cranial window was placed in the hole and cemented to the skull with dental acrylic. Through needles incorporated into the sides of the windows, the space under the window was filled with artificial CSF of the following composition: 220 mg KCl, 132 mg MgCl₂, 221 mg CaCl₂, 7,710 mg NaCl, 402 mg urea, 665 mg dextrose, 2,066 mg NaHCO₃; pH=7.33, Pco₂=46 mm Hg, and Po₂=43 mm Hg. The volume of fluid directly under the window was approximately 500 μl and was continuous with the subarachnoid space. Pial arterioles were observed with a Wild trinocular stereomicroscope (Rockleigh, New Jersey). Diameter of the erythrocyte column (internal diameter minus plasma stream) was measured with a television camera mounted on the microscope, a video monitor, and a video microcalorimeter (model VPA-100, For-A-Corp., Boston, Massachusetts).

In each piglet, the cranial window was flushed with artificial CSF several times. The baseline diameter of one pial arteriole in each rat was measured while CSF containing no drug was infused under the window and blood gases and arterial blood pressure were within normal limits. The piglets were then exposed to two different neural stimuli (10⁻⁶ M and 10⁻⁴ M topical norepinephrine and 10⁻⁴ M topical acetylcholine in CSF), and maximal responses (pial diameter after 2–4 minutes) were recorded. Approximately 300 μl CSF was sampled during the baseline period and during the application of 10⁻⁴ M norepinephrine or acetylcholine.

Concentrations of 6-ketoprostaglandin F₁α (the hydrolysis product of prostacyclin), thromboxane B₂ (the hydrolysis product of thromboxane), prostaglandin F₂α (PGF₂α), and prostaglandin E₂ (PGE₂) in the CSF sampled from under the window were determined by radioimmunoassays. Antibodies to the prostanoids were produced in rabbits immunized with prostanoids coupled to thyroglobulin using the mixed anhydride method. Cross-reactivities of our antibodies with other known, biologically relevant prostanoids were all <1% and nonexistent to norepinephrine and acetylcholine. Prostanoid concentrations for tubes containing only CSF were below detectable values. The radioimmunoassays were performed in gelatin (Tris) buffer using the appropriate tritiated prostanoid. Following incubation for 24 hours at 4°C, the free fraction was separated from...
TABLE 1. Effects of Perivascular Blood on Pial Arteriolar Responses in Piglets

<table>
<thead>
<tr>
<th>Response</th>
<th>Norepinephrine</th>
<th>Acetylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>$10^{-4}$ M</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>166±6</td>
<td>166±8</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>62±2</td>
<td>61±2</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>151±7</td>
<td>154±8</td>
</tr>
<tr>
<td>MABP (mm Hg)</td>
<td>59±3</td>
<td>58±3</td>
</tr>
<tr>
<td>n</td>
<td>13</td>
<td>13</td>
</tr>
</tbody>
</table>

Values are mean±SEM. MABP, mean arterial blood pressure.
*p<0.05 different from baseline by paired t test.

the fraction bound to antibody using dextran-coated charcoal. Concentrations were calculated by a computer, with second-order regression of tracer bound to antibody vs. unlabeled prostanoid by the least-squares method. All unknowns were assayed at three dilutions, with parallelism between the unknown dilution curve and the standard curve required before the result was used. The sample dilutions that we used allowed analysis of prostanoid concentrations between 100 and 50,000 pg/ml.

All values are presented as mean±SEM. We compared the pial arteriolar diameter responses to each neural stimulus with baseline diameter using the paired t test and Bonferroni's correction for $t_{	ext{corr}}$ value with norepinephrine. We compared baseline prostanoid concentrations with those during applications of $10^{-4}$ M acetylcholine or $10^{-4}$ M norepinephrine using the paired t test; when prostanoid concentrations were below the detection limit of our assay, a value of 100 pg/ml was assigned to allow statistical analysis. For the chronic experiment, we compared percentage change from baseline in the control and ICH groups using the unpaired t test; an arcsine transformation was used to normalize the percentage change values. We compared differences in prostanoid concentrations between the two chronic groups under all conditions using the unpaired t test. We also used regression analysis to determine whether vascular responses varied with the number of days following the placement of CSF or blood. An $\alpha$ level of $p<0.05$ was used in all statistical tests.

Results

Visual examination of the brain surface by both the unaided eye and intravital microscopy revealed blood around the cerebral vessels, often as a frank clot and always as erythrocytes trapped in the pia-arachnoid. Under the microscope following hematoxylin and eosin staining, the erythrocytes appeared to be spherical and intact. The brain surface was not softened or distorted, and the behavior of the piglets was normal.

In the chronic control group, pial arterioles constricted in response to topical application of $10^{-4}$ M norepinephrine and $10^{-4}$ M acetylcholine (Table 1, Figure 1). For the four piglets in this group that received CSF on the contralateral side, pial arterioles constricted by 23±1% in response to $10^{-4}$ M norepinephrine and by 31±4% in response to $10^{-4}$ M acetylcholine; these values are very close to the overall values ($n=13$) of 20±2% and 28±2%, respectively. In contrast, significant pial arteriolar constriction was observed only in response to acetylcholine in the chronic ICH group (Table 1, Figure 1); there was a significant difference from control in the response to $10^{-4}$ M norepinephrine. Arterial pH was 7.58±0.04 and 7.58±0.02, $\text{Paco}_2$ was 31±1 and 33±1 mm Hg, and $\text{Pao}_2$ was 89±3 and 86±2 mm Hg for the chronic control ($n=13$) and the chronic ICH ($n=15$) groups, respectively. Regression analysis indicated that pial arteriolar responses were independent of days since placement of CSF or blood ($p^2$ of 0.03 and 0.08 for norepinephrine and 0.001 and 0.03 for acetylcholine in the chronic control and chronic ICH groups, respectively; no correlation coefficient was significantly different from 0).

The concentrations of prostanoids in the CSF from both chronic groups are given in Table 2. Application of $10^{-4}$ M acetylcholine increased the concentrations of all four prostanoids in both groups, whereas $10^{-4}$ M norepinephrine increased PGE$_2$ concentrations in both groups and 6-keto-PGF$_{1\alpha}$ concentrations only in the chronic ICH group.

![Figure 1](http://stroke.ahajournals.org/) Bar graph of percent changes in pial arteriolar diameter in response to constrictor stimuli in control (shaded bars) and blood (filled bars) groups of piglets. Values are mean±SEM. Sample sizes are in parentheses. NOR, norepinephrine; ACH, acetylcholine.
For the acute experiment, baseline arteriolar diameter before acetylcholine was 209±34 μm (mean arterial blood pressure [MABP]=56±6 mm Hg); diameter decreased to 154±3 μm during application of 10^{-4} M acetylcholine (MABP=54±3 mm Hg) (change in diameter of 28±4%; p<0.05, n=3). In addition, baseline diameter before norepinephrine was 167±13 μm (MABP=59±6 mm Hg); during 10^{-6} M norepinephrine it declined to 163±15 μm (MABP=57±5 mm Hg) (difference not significant) and during 10^{-4} M norepinephrine to 133±17 μm (MABP=56±5 mm Hg) (change in diameter of 21±1%; p<0.05 different from baseline, n=5). Arterial blood pH was 7.56±0.03, Paco2 was 33±1 mm Hg, and PaO2 was 80±3 mm Hg.

**Discussion**

Our new finding is that the prolonged presence of perivascular blood selectively inhibits constriction to autonomic neurotransmitters in the cerebral circulation of piglets. Thus, pial arteriolar constriction in response to norepinephrine was reduced, while that to acetylcholine was not affected. In contrast, acute exposure to blood did not affect the cerebrovascular responses to either norepinephrine or acetylcholine.

Perivascular blood in the central nervous system is a consequence of rupture of the cerebral blood vessels. The immediate result of vascular rupture is reduced blood flow to areas served by these vessels. Secondary, delayed effects of perivascular blood can involve altered responsiveness to vasoactive stimuli, vasospasm, increased blood–brain barrier permeability, and biochemical and anatomic changes of the vessel wall. Pertinent to our study, perivascular blood has been reported to reduce autonomic innervation of cerebral vessels and to alter responsiveness to norepinephrine and acetylcholine. Porcine cerebral vessels receive both cholinergic and noradrenergic innervations. Based on the density of cholinergic and noradrenergic innervations of the choroid plexus, which probably is a reliable indicator of more general cerebrovascular innervation, pigs have dual innervation as great as or greater than that of other species. Following accumulation of blood around the cerebral vessels, vascular norepinephrine content and catecholamine fluorescence decrease within 1 day and remain reduced for up to 30 days. Similarly, vascular acetylcholinesterase activity, widely used to estimate cholinergic innervation, is also reduced by perivascular blood. In addition, cerebrovascular levels of other neurotransmitters (such as vasoactive intestinal peptide and substance P) are also diminished under these conditions. In terms of vascular responsiveness, it has been reported that perivascular blood both increases and decreases constriction to norepinephrine. Lobato et al found that after the intracisternal injection of blood into cats, constrictor responses of the posterior communicating artery were increased at 3 days but returned to control at 7 days. In contrast, following rupture of the intracranial section of the internal carotid artery in dogs, the response of the middle cerebral artery to norepinephrine ipsilateral to the blood was less than that on the contralateral side at 1 and 7 days; responses were not different between the sides 2 hours after carotid artery rupture. Similarly, in our piglets, acute exposure (30 minutes) to perivascular blood did not alter the constrictor responses of pial arterioles to norepinephrine, while responses were reduced following chronic exposure to blood. The pial arterioles that we studied are branches of the middle cerebral artery, and the similarity in our results and those of Toda et al may indicate that regional differences in responsiveness to perivascular blood exist in the cerebral circulation. Biochemical studies indicate regional variations in the extent of sympathetic innervation in the cerebral circulation, and these variations may be related to the responsiveness to norepinephrine after the placement of blood. Specifically, diminution of the constrictor effects of norepinephrine because of chronic exposure to perivascular blood appears to be more pronounced in arteries with a greater degree of innervation.

**TABLE 2. Effects of Perivascular Blood on Concentrations of Prostaglandins in Cerebrospinal Fluid of Piglets**

<table>
<thead>
<tr>
<th>Prostaglandin</th>
<th>Baseline</th>
<th>Concentration</th>
<th>n</th>
<th>Baseline</th>
<th>Concentration</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Ketoprostaglandin F1α</td>
<td>609±133</td>
<td>712±160</td>
<td>12</td>
<td>585±108</td>
<td>3,224±490*</td>
<td>11</td>
</tr>
<tr>
<td>Thromboxane B2</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>11</td>
<td>&lt;100</td>
<td>461±101*</td>
<td>10</td>
</tr>
<tr>
<td>Prostaglandin F3α</td>
<td>980±234</td>
<td>1,243±232</td>
<td>12</td>
<td>776±91</td>
<td>8,571±1,739*</td>
<td>11</td>
</tr>
<tr>
<td>Prostaglandin E2</td>
<td>607±91</td>
<td>1,255±308*</td>
<td>12</td>
<td>925±167</td>
<td>31,746±6,635*</td>
<td>11</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Ketoprostaglandin F1α</td>
<td>447±87</td>
<td>702±160*</td>
<td>14</td>
<td>515±70</td>
<td>4,684±720*</td>
<td>14</td>
</tr>
<tr>
<td>Thromboxane B2</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>13</td>
<td>&lt;100</td>
<td>825±113*</td>
<td>14</td>
</tr>
<tr>
<td>Prostaglandin F3α</td>
<td>1,136±151</td>
<td>1,370±214</td>
<td>14</td>
<td>792±106</td>
<td>10,584±1,268*</td>
<td>14</td>
</tr>
<tr>
<td>Prostaglandin E2</td>
<td>962±226</td>
<td>1,565±261*</td>
<td>14</td>
<td>1,047±156</td>
<td>41,590±3,812*</td>
<td>14</td>
</tr>
</tbody>
</table>

Values are mean±SEM concentration (pg/ml); n, number of piglets for comparison. *p<0.05 different from baseline by paired t test.
The mechanism for the diminution of constriction in response to norepinephrine is unclear. We considered four possibilities. First, constrictor effects in general could be reduced. This explanation is unlikely because responses to acetylcholine were not affected. Second, production of dilator prostanoids could be augmented in the presence of norepinephrine, thereby counteracting the constriction response. However, concentrations of prostanoids in the CSF were similar in both chronic groups. The concentrations of prostanoids are lower than we had reported previously and represent inadvertent dilution during sampling because samples larger than normal were taken. Nevertheless, since similar procedures were used in both chronic groups and for all intra-animal samples, CSF prostanoid levels between groups are comparable. Third, the number or affinity of beta-adrenoceptors could increase, and thus augmented beta-adrenoceptor-mediated dilation could counteract alpha-adrenoceptor-mediated constriction. It has been shown by numerous investigators that sympathectomy increases the number of beta-adrenoceptors. Interestingly, subarachnoid hemorrhage reduces the amount of sympathetic innervation of cerebral blood vessels. However, we have shown that perivascular blood does not change dilator responses to isoproterenol. Fourth, the number or affinity of alpha-adrenoceptors could decrease. Tsukahara et al reported that the number and affinity of alpha-adrenoceptors in human cerebral arteries decreases following subarachnoid hemorrhage. This possibility is particularly intriguing because both postjunctional and extrajunctional receptors in piglet pial arterioles are of the alpha-adrenoceptor subtype, and activation of either subtype results in constriction. Validation of this possibility will require future radioligand binding studies.

In contrast to our norepinephrine findings, it is difficult to compare our acetylcholine results with those of other investigators. In the porcine cerebral circulation and dog basilar artery, the predominant response to exogenous acetylcholine is constriction, while the response in many other species is dilatation mediated by a nonprostanoid endothelium-derived relaxing factor. The responses of intact, healthy cerebral arteries from neonatal and adult humans are unknown, although the predominant response of human coronary arteries appears to be constriction. The chronic presence of perivascular blood does not reduce dilatation of dog or rabbit basilar arteries in response to acetylcholine. In our experiments, acetylcholine-mediated constriction was not altered by the acute or chronic presence of perivascular blood. Thus, acute exposure of pial arterioles to perivascular blood affects responses to neither norepinephrine nor acetylcholine, and exposure lasting 1–4 days inhibits constriction to the former but not the latter neurotransmitter.

Vasospasm is a serious problem associated with ICH and typically appears after 7–10 days. The precise mechanism mediating vasospasm is unclear. We examined cerebrovascular responses before vasospasm is expected to occur. There was a tendency for baseline arteriolar diameters to be smaller in the chronic ICH than in the chronic control group. However, we could not say much about this relation because considerable random variability occurs in vessel size among piglets. What we can say is that, paradoxically, constrictor responsiveness to norepinephrine is reduced due to the presence of perivascular blood. On the other hand, dilation responses to arteriolar hypercapnia and hypotension are also largely eliminated. Whether these changes in responsiveness are related to the later development of vasospasm is unclear.

In a previous study in piglets, we found that acute exposure to perivascular blood did not alter pial arteriolar dilation in response to arteriolar hypercapnia, hemorrhagic hypotension, or exogenous isoproterenol. However, chronic exposure to blood eliminated dilation in response to hypercapnia and hypotension, but not to isoproterenol. Thus, taken together with our present findings, it is clear that the prolonged presence of perivascular blood has selective effects on both dilator and constrictor responses of pial arterioles that depend on the specific stimuli examined. However, shortly after exposure to blood, normal responsiveness to dilators or constrictors (present study) is retained.

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References

KEY WORDS • acetylcholine • microcirculation • prostaglandins • pigs
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