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_{1alpha}, thromboxane B_{2}, prostaglandin F_{2alpha}, 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)

Cerebral blood vessels receive extensive innervation from both branches of the autonomic nervous system.1-4 Stimulation of autonomic nerves and topical application of norepinephrine or acetylcholine can affect cerebrovascular tone.5-7 The extent of innervation and the vascular responses to autonomic neurotransmitters can be altered by cerebral insults. For example, the presence of perivascular blood, which is a consequence of intracranial hemorrhage (ICH), has been reported to decrease the norepinephrine and acetylcholinesterase contents of blood vessels8,9 and to alter vascular responses to amines but not to acetylcholine.10-13 Recently, we found that cerebrovascular dilator responses are selectively affected by chronic placement of perivascular blood in newborn pigs.14 Thus, pial arteriolar dilation in response to arterial hypercapnia or hemorrhagic hypotension is eliminated, while dilation in response to topical isoproterenol is intact. However, it is not known how responses to norepinephrine and acetylcholine are affected in newborns. Following a different cerebral insult in piglets (namely, cerebral ischemia followed by reperfusion), vascular responsiveness to acetylcholine but not norepinephrine was altered.15,16

We examined the effects of perivascular blood on the cerebrovascular responses to norepinephrine and acetylcholine in newborn pigs. We tested the hypothesis that pial arteriolar constrictor responses to these two autonomic neurotransmitters are reduced due to the chronic presence of perivascular blood. ICH, which results in blood around cerebral blood vessels, is a common and serious problem of neonates. In the piglet pial circulation, both neurotransmitters are present in cerebral vessels17 and are constrictor stim-
ulii in the cerebral circulation in vivo.\textsuperscript{18–24} In addition, topical application of these substances increases the production of prostanoids by cerebral tissues and/or vessels.\textsuperscript{21–24} 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 benzathene 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 chronic 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.\textsuperscript{14}

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 $\alpha$-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.\textsuperscript{18} 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 MgCl$_2$, 221 mg CaCl$_2$, 7,710 mg NaCl, 402 mg urea, 665 mg dextrose, 2,066 mg NaHCO$_3$; pH=7.33, $P_{CO_2}$=46 mm Hg, and $P_{O_2}$=43 mm Hg. The volume of fluid directly under the window was approximately 500 $\mu$L 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 microscaler (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 acetylcysteine in CSF), and maximal responses (pial diameter after 2–4 minutes) were recorded. Approximately 300 $\mu$L CSF was sampled during the baseline period and during the application of $10^{-4}$ M norepinephrine or acetylcysteine.

Concentrations of 6-ketoprostaglandin F$_{1 \alpha}$ (the hydrolysis product of prostacyclin), thromboxane B$_2$ (the hydrolysis product of thromboxane), prostaglandin F$_{2 \alpha}$ (PGF$_{2 \alpha}$), and prostaglandin E$_2$ (PGE$_2$) in the CSF sampled from under the window were determined by radioimmunoassay.\textsuperscript{22,23} 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 acetylcysteine. 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>Neopinephrine</th>
<th>Acetycholine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>$10^{-4}$ M</td>
</tr>
<tr>
<td>Diameter ($\mu$m)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</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 ($\mu$m)</td>
<td></td>
<td>151±7</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 $t$-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, $P_{aco_2}$ was 31±1 and 33±1 mm Hg, and $P_{o_2}$ 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 ($r^2$ 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/)

**FIGURE 1.** 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 the presence 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 al11 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,20 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 (pg/ml)</th>
<th>n</th>
<th>Concentration (pg/ml)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Ketoprostaglandin F₁α</td>
<td>609±133</td>
<td>712±160</td>
<td>12</td>
<td>585±108</td>
<td>11</td>
</tr>
<tr>
<td>Thromboxane B₁</td>
<td>&lt;100</td>
<td>&lt;100</td>
<td>11</td>
<td>&lt;100</td>
<td>10</td>
</tr>
<tr>
<td>Prostaglandin F₂α</td>
<td>980±234</td>
<td>1,243±232</td>
<td>12</td>
<td>776±91</td>
<td>11</td>
</tr>
<tr>
<td>Prostaglandin F₃α</td>
<td>607±91</td>
<td>1,255±308*</td>
<td>12</td>
<td>925±167</td>
<td>11</td>
</tr>
<tr>
<td>Blood</td>
<td>447±87</td>
<td>702±160*</td>
<td>14</td>
<td>515±70</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>14</td>
</tr>
<tr>
<td>Prostaglandin F₁α</td>
<td>1,136±151</td>
<td>1,370±214</td>
<td>14</td>
<td>792±106</td>
<td>14</td>
</tr>
<tr>
<td>Prostaglandin F₃α</td>
<td>962±226</td>
<td>1,565±261*</td>
<td>14</td>
<td>1,047±156</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 prostanooids could be augmented in the presence of norepinephrine, thereby counteracting the constriction response. However, concentrations of prostanooids in the CSF were similar in both chronic groups. The concentrations of prostanooids 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 prostanooid 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 report 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 dilation 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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