Cholinergic Mechanisms in the Cerebral Circulation of the Newborn Piglet: Effect of Inhibitors of Arachidonic Acid Metabolism

L. Craig Wagerle and David W. Busija

The cerebrovascular response to cholinergic stimulation and the effect of inhibition of the lipoxygenase and cyclooxygenase pathways on the response to acetylcholine (ACh) was investigated in newborn piglets. Responsiveness of pial arterioles to ACh, methacholine, and nicotine was studied using a closed cranial window. Pial arteriolar diameter was measured using intravital microscopy. Pial arteriolar responses to ACh, 10^-7 - 10^-4 M, applied to the cortical surface, were variable and dose-dependent. At low concentration, 10^-7 M, 45% of the arterioles increased diameter by 9±1%, 19% responded by decreasing diameter by 9±1%, and 35% did not respond. The response to high concentration, 10^-4 M, was a profound decrease in diameter, 28±3%, in 78% of the arterioles studied. These effects were abolished by atropine (0.5 mg/kg i.v.). Muscarinic agonist, methacholine, 10^-5 - 10^-3 M, also resulted in a decrease in cerebral vascular diameter, while nicotine, 10^-4 - 10^-4 M, had no effect. In six animals, administration of cyclooxygenase inhibitor, indomethacin (5 mg/kg i.v.), blocked the response to 10^-4 M ACh but did not affect the response to 10^-7 M ACh. In five animals, administration of lipoxygenase inhibitor, nordihydroguaiaretic acid (2 mg/kg i.v.) augmented the vasoconstrictor response at both ACh concentrations. The data suggest that, in the newborn piglet, vasoactive prostanoids released following cholinergic activation of a muscarinic-type receptor mediate vasoconstriction in cerebral arterioles. (Circulation Research 1989;64:1030-1036)

Biochemical and histochemical evidence establish the association of cholinergic nerves with cerebral vessels. However, little is known about responsiveness of the neonatal cerebral circulation, in vivo, to cholinergic activation. We have shown that, in the neonatal period, other neural stimuli have important effects on the cerebral circulation. In addition, cerebrovascular responses to norepinephrine and histamine are modulated by cyclooxygenase products. More recently, we have presented indirect evidence that prostanoids may modulate the response of cerebral vessels to acetylcholine (ACh) in newborn piglets. Specifically, topical application of ACh to the cortical surface caused a dramatic increase in levels of prostanoids in the cerebrospinal fluid covering pial arterioles.

The functional role and mechanism of action of cholinergic nerves in the developing cerebral circulation is not well understood nor has the role of prostanoids in the cerebral arteriolar response to ACh been investigated. The objectives of the present study were to characterize the response, in vivo, of newborn pig cerebral arterioles to cholinergic agonists, and to test the hypothesis that prostanoids are involved in the cerebrovascular response to exogenous ACh.

Materials and Methods

Animal Preparation

Experiments were carried out on 57 newborn piglets 2–7 days of age. Piglets were anesthetized with sodium pentobarbital (30 mg/kg i.p., 5 mg/kg/hr maintenance). Following tracheostomy, polyethylene catheters were placed into a femoral artery and vein to monitor arterial blood pressure and for fluid administration. A continuous intravenous drip (12 ml/hr) of 5% glucose was started and continued throughout the experiment. Skeletal muscle paralysis was induced with 0.5 mg pancuronium bromide given intravenously. The piglets were mechanically ventilated and
end expiratory CO₂ continuously monitored with an infrared CO₂ analyzer (Beckman Instruments, Fullerton, California). End-expired CO₂ was maintained between 30 and 35 mm Hg and PaO₂ was maintained between 80 and 100 mm Hg. Animals were wrapped in a warming blanket, and core body temperature was continuously monitored and maintained at 38° C. Arterial blood pressure was measured with a Statham P23Db pressure transducer (Gould Instruments, Cleveland, Ohio) connected to the femoral arterial catheter. Arterial blood samples were collected and analyzed for pH, Pco₂, and Po₂ at appropriate times during the experimental protocols. Blood gas tensions and pH were measured with conventional electrodes (Radiometer America, Westlake, Ohio) and hemoglobin concentration and saturation were measured with a co-oximeter (Instrumentation Laboratories, Lexington, Massachusetts). Hematocrit was measured by micromethod.

The piglets were equipped with a closed cranial window placed over the left parietal lobe as previously described for newborn piglets. The space under the window was filled with artificial cerebrospinal fluid (CSF) of the following millimolar composition: KCl 2.9, MgCl₂ 1.4, CaCl₂ 1.2, NaCl 132, NaHCO₃ 24.6, urea 6.7, and glucose 3.7. The CSF was warmed to 39° C and equilibrated with 6.6% CO₂, 6.0% O₂, with the balance N₂. We have previously demonstrated that injecting artificial CSF of this composition under the window has no effect on pial vascular caliber.

Pial arterioles ranging in diameter from 16 to 257 μm were visualized with a trinocular stereomicroscope (model M7S, Wild, Heerbrugg, Switzerland). One to four arterioles were studied in each animal. Vascular intraluminal width was measured using a television camera (model WV3030, Panasonic, Yokohama, Japan) mounted on the microscope, a video monitor (model CT1901A, NEC, Tokyo, Japan), and a video micro-scaler (model VPA-1000, For-A-Corp, Los Angeles, California). The video images were recorded on a video cassette recorder (model DL-HF900, Sony, Tokyo, Japan). The magnification of the total system, optical and video together, was ×105 to 520 with a resolution of 3 μm at the highest magnification. The magnification used in this study ranged from ×300 to ×520.

Acetylcholine chloride, nicotine ditartrate, methacholine chloride, atropine sulfate, bradykinin, nordihydroguaiaretic acid (NDGA), superoxide dismutase (SOD) from bovine blood (3200 U/mg prot) and catalase (CAT) from bovine liver (34,000 U/mg prot) were obtained from Sigma Chemical Co, St. Louis, Missouri. Sodium indomethacin trihydrate was a gift from Mr. William Henckler, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey.

**Experimental Protocols**

**Effect of acetylcholine, methacholine, nicotine, and bradykinin on pial arteriolar caliber.** In 27 piglets, the response of pial arterioles to ACh was determined. Control measurements of pial arteriolar diameter were made after flushing the cortical surface with CSF containing no drug. Then ACh in doses of 10⁻⁸-10⁻⁴ M in CSF was flushed under the window onto the cortical surface in a cumulative manner. Each ACh concentration was left on the surface for 4–5 minutes and was then replaced with the next concentration. In five additional animals, the response to ACh was examined following administration of muscarinic receptor antagonist atropine (0.5 mg/kg i.v.). In four piglets, we also examined the pial arteriolar response to nicotine (10⁻⁶–10⁻³ M) or methacholine (10⁻⁶–10⁻⁴ M).

The vasodilatory response to ACh, in several species, has been shown to be dependent on an intact endothelium. Since the vasodilatory effect of ACh in the present study was inconsistent, we also examined the response to bradykinin, which has been shown to be an endothelial-dependent vasodilator in some circulations including the cerebral circulation of mice and dogs. In four animals, following baseline measurements during injection of normal CSF, CSF containing 10⁻⁶ M bradykinin was flushed onto the pial surface and pial diameter measured.

**Effect of superoxide dismutase plus catalase on the pial arteriolar response to acetylcholine.** Previous studies in adult animals suggest oxygen free radicals may damage endothelium and impair the response of cerebral arterioles to ACh. This effect was partially reversed by treatment with the oxygen free radical scavenging system, superoxide dismutase plus catalase (SOD+CAT). Therefore, to determine the possible influence of superanion radicals released into the extracellular space, six piglets were pretreated with SOD+CAT. In these animals, SOD (40 U/ml)+CAT (40 U/ml) in CSF was placed over the cortical surface under the window immediately after opening the dura and during implantation of the window. The response to ACh was then determined as described above in the presence of SOD+CAT.

**Effect of indomethacin and NDGA on the pial arteriolar response to acetylcholine.** To investigate the involvement of the lipoxygenase and cyclooxygenase pathways in the cerebrovascular response to ACh, we examined the pial arteriolar response to ACh before and following either cyclooxygenase inhibition with indomethacin (5 mg/kg i.v.; n=6) or lipoxygenase inhibition with NDGA (2 mg/kg i.v.; n=5). In this series of experiments, we elected to use only two concentrations of ACh, 10⁻⁷ and 10⁻⁴ M because, based on the dose response curves for ACh (see "Results," Figure 1), these two concentrations produced the most extreme differences in vessel response suggesting different mechanisms may be involved. Of the entire ACh dose range, 10⁻⁷ M ACh most frequently resulted in an increased arteriolar diameter, while 10⁻⁴ M ACh elicited the most consistent decrease in diameter. In each animal, after determination of the response to each ACh concentration, ACh was washed off of the cortical...
surface; we allowed a recovery period of at least 45 minutes. Indomethacin or NDGA was then administered, and, after 20 minutes, the response to ACh was determined for a second time. Time control experiments in which no drug was administered showed that the response to ACh was reproducible.

Statistical Analysis

All values are presented as mean±SEM. One to four arterioles (mean±SD, 1.8±0.9) were studied in each animal. For statistical analysis, however, all arteries within an animal were averaged so that in the analysis of variance, each animal carried equal weight. Comparisons among treatments were made with two-way analysis of variance with repeated measures followed by pairwise t test with Bonferroni correction when appropriate. Analysis of pial diameters were made following square root transformation and percent changes were analyzed following arcsine transformation. Differences were considered significant when p<0.05.

Results

Effect of Acetylcholine, Methacholine, Nicotine, and Bradykinin on Pial Arteriolar Diameter

Arterial PCO2, PO2, pH, and mean arterial blood pressure during application of cholinergic agonists are presented in Table 1. Application of ACh to the cortical surface invoked alterations in pial arteriolar diameter (Figure 1). At low doses, 10^{-4}-10^{-6} M ACh, 35-45% of the arterioles did not respond, in 39-45% of the vessels diameter increased, and in 15-19% diameter decreased. Due to the variability of the response, no statistically significant changes could be shown. An increase in vascular caliber was observed most frequently at an ACh concentration of 10^{-7} M where 45% of the vessels showed a 9±1% increase in diameter. At higher concentrations, >10^{-5} M, the response was predominantly vasoconstriction where 10^{-4} M ACh resulted in a 28±4% decrease in diameter in 78% of the vessels.

The response to increasing ACh concentrations for all arterioles combined is presented in Figure 2. When the vascular responses were averaged, ACh resulted in no significant effect on pial arteriolar diameter until ACh concentrations were increased to 10^{-3} and 10^{-4} M where pial diameter was decreased. The decrease in diameter observed at high concentrations of ACh was transient, where the peak effect was noted within 1 minute and by 4 minutes, the arterioles had returned to near baseline. The values presented represent the maximum change in vessel diameter. The effect of ACh was abolished in animals pretreated with muscarinic receptor antagonist atropine (Figure 2). In animals pretreated with SOD+CAT, ACh also caused constriction that was not significantly different from nontreated animals.

Methacholine, a muscarinic receptor agonist that is not metabolized by acetylcholine esterase, also caused a decrease in pial arteriolar diameter while nicotine was without effect (Figure 3). Exogenous bradykinin applied to the pial surface (10^{-6} M) caused a significant 56±7% increase in pial arteriolar diameter from 144±26 to 215±30 μm. Arterial blood gases and blood pressure were not altered by bradykinin.

Table 1. Arterial Blood Gases and Mean Arterial Blood Pressure During Cortical Suffusion With Vasoactive Drugs

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PaCO2 (mm Hg)</th>
<th>PaO2 (mm Hg)</th>
<th>MABP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>7.44±0.03</td>
<td>34±1</td>
<td>96±3</td>
<td>72±3</td>
</tr>
<tr>
<td>Acetylcholine + atropine</td>
<td>7.45±0.02</td>
<td>36±2</td>
<td>100±6</td>
<td>71±3</td>
</tr>
<tr>
<td>Nicotine and methacholine</td>
<td>7.44±0.02</td>
<td>32±2</td>
<td>102±3</td>
<td>71±3</td>
</tr>
<tr>
<td>Acetylcholine + SOD &amp; CAT</td>
<td>7.37±0.02</td>
<td>34±3</td>
<td>77±7</td>
<td>68±7</td>
</tr>
</tbody>
</table>

Data are mean±SEM. MABP, mean arterial blood pressure; SOD & CAT, superoxide dismutase and catalase; n, number of animals.

FIGURE 1. Variability of the cerebral arteriolar response to cortical suffusion of acetylcholine in newborn piglets. Arteriolar response was classified as either no response (<5% change in diameter), constriction (>5% decrease in diameter) or dilation (>5% increase in diameter). Data are from a total of 27 animals. n, total number of arteries studied at each acetylcholine concentration.
Effect of Indomethacin and Nordihydroguaiaretic Acid on the Response to Acetylcholine

Arterial blood gases and mean arterial blood pressure during topical application of ACh before and after administration of indomethacin or NDGA are presented in Table 2. The effect of indomethacin and NDGA on the pial vascular response to exogenous ACh are shown in Figure 4. Indomethacin had no effect on the pial vascular response to $10^{-7}$ M ACh but nearly abolished the vasoconstrictory response to $10^{-4}$ M ACh where the $18\pm6\%$ decrease in diameter was significantly reduced to $3\pm2\%$. Following administration of NDGA, the vasoconstrictory response to ACh was significantly enhanced at both $10^{-7}$ M (3±3% increase to 6±3% decrease in diameter) and $10^{-4}$ M ACh (25±8% decrease to 39±4% decrease in diameter).

Discussion

The new findings of the present study can be summarized as follows: 1) In the newborn pig, exogenous ACh causes inconsistent changes in pial diameter at low dose and a profound decrease in diameter at high dose. 2) The decrease in diameter was blocked by indomethacin and potentiated by NDGA suggesting a role for cyclooxygenase products. 3) The response was blocked by muscarinic antagonist atropine and mimicked by another muscarinic receptor agonist methacholine, while nicotine had no effect on vascular diameter. 4) SOD+CAT did not alter the cholinergic response indicating that oxygen free radicals did not interfere with the cholinergic vasodilatory mechanism. These data are the first in vivo demonstration that the cholinergic effect on cerebral vessels can be mediated by arachidonic acid products.

There is convincing evidence in adult animals that cholinergic mechanisms are important to the regulation of cerebrovascular tone. Cerebral blood vessels have been shown to contain ACh and possess muscarinic receptors, and the presence of cholinergic innervation has been demonstrated by a high-affinity choline uptake system and high choline acetyltransferase activity. Cerebral arteries show higher levels of ACh and high-affinity choline accumulation than noncerebral arteries. Physiological evidence further supports cholinergic regulation since cerebral vessels dilate and cerebral blood flow increases in response to exogenous cholinergic agonists. These effects are blocked by atropine. There is good evidence for cholinergic transmission in cerebral arteries in vitro where stimulation evoked release of ACh as well as atropine sensitive vasodilator responses could be demonstrated during transmural electrical stimulation.

We have shown that application of exogenous ACh to the cortical surface of the newborn piglet is associated with release of substantial quantities of both vasodilator and vasoconstrictor prostanooids, i.e., prostaglandin (PG)E$_2$, PGF$_{2\alpha}$, 6-keto-PGF$_{1\alpha}$ (the hydrolysis product of prostacyclin), thromboxane B$_2$ (the hydrolysis product of thromboxane A$_2$), and PGD$_2$ into the cortical CSF. Detectable increases were noted at physiologically relevant concentrations of $10^{-6}$ M ACh. The source of the prostaglandins is not easily identified in this study since neurons, glial cells, as well as vascular smooth muscle and endothelial cells possess cholinergic receptors. The fact that PGF$_{2\alpha}$ and thromboxane B$_2$ are derived from cerebral vessels while PGE$_2$, PGD$_2$, and PGF$_{2\alpha}$ are produced by neural tissue suggest that both neural and vascular tissues are involved.
Released arachidonic acid is normally metabolized by cyclooxygenase and/or lipoxygenase; therefore, likely mediators of the decrease in diameter following ACh could be products of the lipoxygenase pathway, leukotrienes C₄, D₄, or E₄, which cause vasoconstriction in newborn pig cerebral arteries. Alternatively, products of the cyclooxygenase pathway that cause vasoconstriction in cerebral arteries, PGF₂α or thromboxane A₂, may mediate the decrease in diameter following ACh. Since the vascular response to ACh was blocked by the cyclooxygenase inhibitor, indomethacin but not by lipoxygenase inhibitor, NDGA, it would appear that in the newborn piglet, activation of the muscarinic receptor is associated with release of arachidonic acid and that a cyclooxygenase product mediates vasoconstriction in the cerebral arterioles. This is in apparent contrast to the effect of adrenergic agonist norepinephrine where prostanoids are also released into cortical CSF, but the vasocostrictor response was potentiated by indomethacin treatment.

Two explanations for the potentiation of ACh mediated decrease in vessel diameter caused by NDGA may be considered. Since NDGA at this dose inhibits endothelium-derived relaxing factor (EDRF)-mediated relaxations, assuming the piglet cerebral arteries produce EDRF in response to ACh stimulation, then its inhibition would appear as enhanced constriction. The fact that indomethacin blocked the decrease in diameter to high-dose ACh but did not unmask a vasodilatory response to low-dose ACh suggests that EDRF is not produced by piglet cerebral arterioles. We speculate that the potentiation of the decrease in diameter following NDGA might be explained by an increased availability of arachidonic acid as substrate to the cyclooxygenase pathway and enhanced prostanoid production.

Muscarnic receptor-mediated release of eicosanoids is most likely associated with direct activation of phospholipase C³⁰,³¹ or indirect activation of phospholipase A₂.³²,³³ Alterations in membrane phospholipid metabolism, i.e., hydrolysis of phosphatidylinositol 4,5-bisphosphate, release of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, and increased intracellular free Ca²⁺ concentration following activation of muscarinic receptors is known to occur in several tissues³¹,³⁴ including vascular smooth muscle.³⁵,³⁶ Subsequent release of arachidonic acid might occur either from further breakdown of diacylglycerol via diacylglycerol lipase³⁷-³⁹ or via Ca²⁺ activation of membrane phospholipase A₂.

The exact nature of the metabolism of phospholipids and the products produced following muscarinic stimulation no doubt varies with tissue type and species and most likely will play a role in the nature of the cholinergic response. In both rabbits⁴⁰ and dogs,⁴¹ for example, pulmonary arteries constrict to cholinergic agonists, but prostanoids were implicated only in the rabbit as indomethacin blocked the response only in rabbits but potentiated the response in dogs. In isolated saphenous vein, the constriction to ACh and Ca²⁺ ionophore A23187 appears to be mediated by a lipoxygenase product⁴² as the response could be blocked by NDGA but was not altered by indomethacin. It has been demonstrated that eicosanoids are important in vascular control processes in the cerebral circulation of the newborn pig. The present study suggests that arachidonic acid metabolism and the release of prostanoids is involved in the contractile response of cerebral arteries to muscarinic activation in this species.

TABLE 2. Effect of Indomethacin and Nordihydroguaiaretic Acid on Arterial Blood Gases and Mean Arterial Blood Pressure During Cortical Suffusion of Acetycholine

<table>
<thead>
<tr>
<th></th>
<th>pH</th>
<th>PaCO₂ (mm Hg)</th>
<th>PaO₂ (mm Hg)</th>
<th>MABP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.42±0.04</td>
<td>32±2</td>
<td>95±7</td>
<td>64±6</td>
</tr>
<tr>
<td>Indomethacin (n=6)</td>
<td>7.38±0.04</td>
<td>33±1</td>
<td>106±11</td>
<td>76±3</td>
</tr>
<tr>
<td>Control</td>
<td>7.42±0.03</td>
<td>35±1</td>
<td>104±5</td>
<td>71±3</td>
</tr>
<tr>
<td>NDGA (n=5)</td>
<td>7.32±0.05</td>
<td>35±1</td>
<td>101±7</td>
<td>69±4</td>
</tr>
</tbody>
</table>

Data are mean±SEM. MABP, mean arterial blood pressure; NDGA, nordihydroguaiaretic acid (2 mg/kg i.v. or 10⁻⁴ M applied to the cortical surface). Indomethacin (5 mg/kg i.v.).

**Figure 4.** Effect of cyclooxygenase (indomethacin, 5 mg/kg i.v.) or lipoxygenase (NDGA, nordihydroguaiaretic acid, 2 mg/kg i.v.) inhibition on the cerebral arteriolar response to acetylcholine in newborn piglets. Data are presented as percent of control diameter for each group. Control diameters were; indomethacin group: 120±17 and 110±17 μm for baseline and following indomethacin, respectively, 11 arterioles, six animals; NDGA group: 129±12 and 139±15 μm for baseline and following NDGA, respectively, seven arterioles, five animals. Data are mean±SEM. Significantly different from control, p<0.05.
Similar studies in adults of other species have clearly demonstrated that ACh in concentrations of $10^{-7}$ to $10^{-4}$ M cause vasodilation of cerebral arteries. In the present study, no consistent, statistically significant, vasodilatory response to exogenous ACh could be demonstrated at any concentration. An increase in pial diameter was most frequently observed at an ACh concentration of $10^{-5}$ M, however, at this concentration 55% of the vessels either did not respond or constricted. When those vessels that dilated (i.e., increased diameter >5%) to $10^{-7}$ M ACh were analyzed separately, the increase in diameter was 9±1%, which is of similar magnitude to that reported using comparable methods in adult animals of other species. However, the inconsistency of the vasodilatory response suggests that the cholinergic vasodilatory mechanism in newborn piglets may not be functional.

We considered the possibility that our experimental preparation was traumatized, which was perhaps associated with production of oxygen free radicals. Recent studies in adult cats and mice have shown that traumatized cerebral arteries lose their responsiveness to the endothelium-dependent vasodilators ACh and bradykinin. These effects were attributed to oxygen free radical generation and could be prevented by the free radical scavenging system SOD+CAT. Our experimental evidence indicates that this is unlikely in the present study since the response to ACh in the presence of SOD+CAT was vasoconstriction and no vasodilatory component was observed. In addition, we have demonstrated an intact vasodilatory response to bradykinin, which has been shown to be endothelium dependent in cerebral arteries of mice and dogs, as well as coronary arteries and aorta of pig. We have also previously shown pial arterioles in newborn pigs to be responsive to other vasodilatory stimuli such as adenosine and CO$_2$. These data suggest that endothelial function was intact and that oxygen free radicals did not impair the cholinergic vasodilatory mechanism in these vessels.

It is possible that dilatory effects of ACh were masked by the more potent vasoconstrictory component. Our data cannot rule out this possibility, however, indomethacin blocked the vasoconstrictory response to a high concentration of ACh yet did not reveal a vasodilatory response at the low concentration (Figure 4). Therefore, if the vasoconstrictory component masked the vasodilatory mechanism, then one must reason that both vasodilatory and vasoconstrictory responses are mediated by a cyclooxygenase product. The latter seems unlikely since current evidence indicates that EDRF is not a product of the cyclooxygenase pathway.

Finally, it is possible that the cholinergic mechanism in the cerebral circulation of the newborn piglet is immature. Isolated cerebral arteries from premature and newborn monkeys showed marked contraction to ACh ($10^{-7}$ to $10^{-4}$ M) in vitro while arteries from adults showed little contraction, suggesting developmental differences in that species. In isolated cerebral arteries from older pigs (4–5 months), however, the response to low concentrations of ACh ($<3 \times 10^{-6}$ M) was predominantly constriction (61% of the vessels) while 25% dilated. The similarly inconsistent response to $10^{-6}$ M ACh observed in newborn piglets of the present study (Figure 1) to that of Lee et al suggests little developmental change in pigs over the first five postnatal months. Comparisons between in vitro and in vivo studies, however, may be misleading. Consequently, it remains uncertain whether the cholinergic response observed in the present study is representative of immature cholinergic mechanism in the piglet cerebral circulation or simply represents an unusual vascular cholinergic function in this species as previously suggested.

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KEY WORDS: cerebrovascular • newborn • prostanoids • lipooxygenase • cyclooxygenase • acetylcholine • muscarinic receptor
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