Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses

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Willis, Adam P., and Charles W. Leffler. Endothelial NO and prostanoid involvement in newborn and juvenile pig pial arteriolar vasomotor responses. Am J Physiol Heart Circ Physiol 281: H2366–H2377, 2001.—Specific cerebrovascular dilatory responses in newborn piglets are entirely prostanoid dependent, but require both nitric oxide (NO) and prostanoids in juveniles. We examined endothelial dependency and mechanisms of NO- and prostanoid-mediated cerebrovascular responses in anesthetized newborn and juvenile pigs implanted with closed cranial windows. Light/dye endothelial injury inhibited newborn and juvenile hypercapnic and bradykinin (BK) responses and inhibited dilation to acetylcholine in juveniles. Iloprost and NO act permissively in restoring light/dye inhibited newborn and juvenile responses, respectively. Differences in sensitivity to iloprost and sodium nitroprusside were not observed. Juvenile (not newborn) hypercapnic and BK cerebrovascular responses were sensitive to soluble guanylyl cyclase inhibition. Pial arteriolar diameter and cortical production of prostacyclin, cAMP, and cGMP in response to BK were measured under control conditions, after treatment with indomethacin and/or NO-nitro-l-arginine methyl ester (l-NAME). Indomethacin inhibited BK responses in newborns. Juvenile responses were inhibited by l-NAME, and mildly by indomethacin. Cortical 6-keto-PGF₁α, cAMP, and cGMP increased in response to BK in both age groups. Newborn cerebrovascular responses are largely NO independent, but NO becomes more important with maturation.

The vascular endothelium is an integral component in many cerebral, pulmonary, and systemic vascular responses, by providing critical dilatory and/or constrictor signals to the underlying vascular smooth muscle. Communication between the vascular endothelium and smooth muscle can occur as an endothelial release of prostanoids and NO. Therefore, endothelial removal can modify or inhibit vascular responses. Functional removal of the endothelium from newborn piglet pial arterioles by using an in vivo light/dye technique that temporarily produces endothelial injury has been shown to prevent dilations to hypercapnia, histamine (19, 21), and hypotension (8), while having no effect on dilatory responses to isoproterenol or constrictor responses to hypocapnia and hypertension. Similarly, acetylcholine and BK fail to dilate mouse pial arterioles (32) or rat cremaster muscle arterioles (14) after light/dye endothelial injury, suggesting acetylcholine and BK dilatory responses are endothelially dependent in different species and circulatory beds. Whether endothelial dependency of cerebrovascular responses are altered with development in the pig remains to be investigated.

Recent data provides new insight into the mechanisms by which NO and prostanoids mediate cerebrovascular responses. Studies of newborn piglets support a permissive mechanism of action of prostacyclin (PGI₂) in cerebral dilatory responses to hypercapnia and histamine, and of thromboxane A₂ in constriction to acetylcholine. Inhibition of newborn piglet hypercapnic and histamine dilatory responses after either light/dye endothelial injury or treatment with indomethacin (19, 20, 39, 41) could be reversed with the addition of a subdilator concentration of the PGI₂ analog iloprost. Similarly, indomethacin-inhibited constricitions of newborn piglet pial arterioles in response to acetylcholine could be reversed with thromboxane receptor ago-
nists (2). In a previous study, we (41) were unable to demonstrate a permissive mechanism of action for either NO or prostanoids in juvenile pig hypercapnic- and histamine-induced cerebrovascular dilations that were attenuated after treatment with L-NNA or indo- methacin. However, in adult rats, hypercapnic cerebral dilation of pial arterioles appears to involve a permissive role for NO (12, 13, 26), whereas dilation to acetylcarnine is a result of a classical mechanism of action of NO. Therefore, the mechanistic route of action (whether conventional or permissive) may be stimulus, species, or age dependent (12).

Therefore, we hypothesize that the predominant endothelium-derived relaxing factor (EDRF) influence in the cerebral vasculature is altered during maturation. Experiments were designed to achieve three goals. The first goal was to examine the hypothesis that mechanisms involved in BK-induced dilations involve prostanoids in newborn piglet and NO in the juvenile pig cerebral circulation. The second goal was to address the hypothesis that cerebrovascular responses to BK, hypercapnia, and acetylcholine are endothelium dependent in newborns and juveniles using a light/dye model of endothelial injury. The third goal was to determine whether the mechanism of action of prostanoids and/or NO in BK, hypercapnia, and acetylcholine responses are permissive or classical in nature.

**METHODS**

The animal protocols used were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. Thirty-two female juvenile (3–4 mo of age, 27.6 ± 1.0 kg) and thirty-nine newborn piglets of either sex (1–3 days old, 2.2 ± 0.1 kg) were used in this set of experiments. Animals were anesthetized with ketamine and acepromazine, and anesthesia was maintained with α-chloralose (50 mg/kg initially, supplemented with 2–5 mg/kg·h⁻¹). Catheters were placed in the femoral artery and vein to permit the sampling of blood gas and pH, to monitor arterial pressure, and to administer drugs and anesthesia. Animals underwent a tracheotomy with an endotracheal tube inserted and were mechanically ventilated with room air. Core temperature was monitored with a rectal probe, and maintained between 37 and 38°C.

**Cranial window placement.** After instrumentation, the scalp was surgically removed, and a 2-cm-diameter hole was cut in the skull over the parietal cortex. The dura was cut and the space under the window was filled with artificial cerebrospinal fluid (aCSF) composed of (in mg/ml) 220 KCl, 1,132 MgCl₂, 221 CaCl₂, 7,710 NaCl, 401 urea, 665 dextrose, and 2,066 NaHCO₃. aCSF was warmed in a water bath to 37–38°C and bubbled with CO₂, O₂, and N₂ (pH 7.33, Po₂ 46 mmHg, and Po₃ 43 mmHg).

In each preparation, two or three pial arterioles (~40–120 μm) were measured via a dissecting microscope with a mounted video camera and an inline micrometer (model VPA-1000; For-A Corp.; Los Angeles, CA).

**Experimental design.** After we completed an initial observation period of 20 min, serial measurements of pial arteriolar diameter were recorded at 1, 3, and 5 min to obtain baseline values. With each measurement recorded, mean arterial pressure (MAP) was also recorded. At the end of 5 min, an arterial blood gas sample was also drawn. All tested responses lasted 5 min with serial measurements of vessel diameter and MAP taken at 1, 3, and 5 min. At the end of each tested response, arterial blood gas was drawn and the window was gently flushed with fresh aCSF to obtain peri-arachnoid CSF samples for determination of cyclic nucleotides and prostanoids, and to remove the previous stimulus to allow vessels to return to baseline diameters.

**NO and prostanoid dependency in BK-induced pial dilations.** To determine the individual and combined involvement of NO and prostanoids in BK-induced dilations, newborn and juvenile pigs were divided into two groups regarding the order of inhibitor administration: group A NOS inhibitor L-NAME and cyclooxygenase inhibitor (BK + indomethacin + L-NAME) and group B (BK + L-NAME + indomethacin). In group A, pial arteriolar responses to ascending concentrations of topical BK (10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M) were initially recorded. After the control responses to BK were recorded, and after vessels had returned to baseline diameters, the COX inhibitor indomethacin trihydrate was administered (10 mg/kg iv; gift from MerckSharp & Dohme Research Laboratories). Twenty minutes after indomethacin treatment, the dose response curve was rerun and pial arteriolar responses were recorded. Once vessels returned to pre-BK diameters, the NOS inhibitor L-NAME (Sigma) was administered (30–40 mg/kg ia). Forty minutes after L-NAME administration, pial arteriolar responses to BK were once again recorded. In group B, the order of addition of inhibitors was reversed. Dilations of pial arterioles to isoproterenol (topical 10⁻⁶ M; Sigma) were recorded at the beginning and end of the experiment to ensure stability of the preparation throughout the experiment. Preparations that demonstrated a significant decline in response to isoproterenol from beginning to end were discarded.

**cAMP assays.** cAMP was measured in CSF samples with use of radioimmunoassay (RIA) procedures, as described previously (30). All unknowns were assayed in duplicate. CSF samples were acetylated with 2:1 triethylamine-acetic anhydride immediately before assay to increase the sensitivity of the method (analysis range 2–128 fmol cAMP).

**cGMP assays.** cGMP was measured in CSF samples with use of a commercially available enzyme-linked immunosor- bant assay purchased from Stratagene. All unknowns were assayed in duplicate. CSF samples were acetylated with 2:1 triethylamine-acetic anhydride immediately before assay to increase the sensitivity of the method (analysis range 0.5–50 fmol cGMP).

**Prostanoid assays.** Concentrations of 6-keto-PGF₁α (the stable hydrolysis product of prostacyclin) were measured in CSF samples with use of RIA procedures, as described previously (27). All unknowns were assayed in duplicate.

**Dilution correction.** Because of possible differences in the volume of space beneath cranial windows between newborns and juveniles that would affect aCSF prostanoid and cyclic nucleotide measurements, we determined the percent recovery of a radiolabeled tracer. Briefly, the space underneath the window was flushed with aCSF containing radiolabeled tracer, and a sample was collected to determine the total counts per minute (Tᵥₑᵣₒᵣ). At the end of the stimulus period (5 min), a sample to be used for prostanoid, cAMP, and cGMP measurements...
determination was collected but the aCSF used to flush contained no tracer. A small aliquot was then counted to determine the recovered cpm (Rcpm). The percent recovery was then calculated as %Recovery = (Rcpm/Tcpm) × 100.

Percent recoveries were determined for all control and BK samples, and were used to correct raw values from all assays.

**Endothelial dependency of newborn and juvenile cerebrovascular responses.** To determine whether newborn and juvenile cerebrovascular responses to BK, hypercapnia, and acetylcholine are endothelium dependent, pial arteriolar responses to isoproterenol (10^-6 M topically), BK (10^-6 M topically), hypercapnia (PaCO2, 60 mmHg), and acetylcholine (10^-5 M topically) were recorded before and after production of endothelial injury in vivo using the light/dye technique (see Production of endothelial injury). Hypercapnia was achieved by changing the ventilation source from room air to a tank containing 10% CO2-21% O2-balance N2, and by ventilating for 5 min.

**Production of endothelial injury.** Endothelial damage in vivo was produced as described previously in newborn pigs (19). Briefly, intravenously injected sodium fluorescein (160 mg/kg in 8 ml/kg volume; Sigma), was activated with appropriately filtered light from a mercury arc lamp to produce microvascular endothelial injury. The mercury arc lamp was focused to produce uniform illumination of the surface under the cranial window. After light/dye injury, the cranial window was repeatedly flushed during a 30-min period in darkness, at which time the experiment was resumed. After injury, measurements of pial arterioles were made using a halogen source at low intensity that was turned off between measurements to avoid further damage to vessels.

Light/dye treatment has previously been shown (21) to cause ultrastructural changes in pial vascular endothelial cells. After light/dye treatment, vascular endothelium displayed more numerous surface pits, vacuolar cytoplasmic inclusions, and some mitochondrial damage. Tight junctions remained intact, and no evidence of endothelial sloughing was observed. In addition, no detectable damage to vascular smooth muscle was observed.

**Dye-only control experiments.** In some experiments, after the control responses were recorded, sodium fluorescein was injected alone, without activation with the mercury lamp, and the responses were retested. These experiments were conducted to determine any effects dye alone would have on the endothelium. Previous experiments (23) have shown that dye alone can impair dilatory function of the cerebral circulation.

**Permissive mechanism of action of NO and prostanoids.** The mechanism of action of prostanoids and NO in BK, hypercapnia, and acetylcholine cerebrovascular responses were also examined. If newborn and juvenile cerebrovascular responses were inhibited after endothelial injury in vivo, either the prostacyclin analog iloprost (usually 10^-5 M), or the NO donor sodium nitroprusside (usually 10^-6 M) was administered at sublilator concentrations to the brain surface and the responses were rerecorded. When lost vasodilation was recovered in the presence of iloprost or sodium nitroprusside, the agonist was removed and responses were retested in the absence of the agonist.

**BK receptor subtype specificity.** BK purportedly causes dilation via the B2 receptor subtype. We tested whether the specific B2 receptor antagonist HOE-140 would prevent BK-induced dilations of newborn and juvenile pial arterioles. After recording of BK, isoproterenol, and hypercapnic responses in the presence of dye alone, HOE-140 (10^-7 – 2 × 10^-7 M, Sigma) was flushed beneath the cranial window. One hour later, the response to BK was tested. To ensure HOE-140 had no nonspecific effects, responses to isoproterenol and hypercapnia were also recorded in the presence of the inhibitor.

**Soluble guanylyl cyclase dependency of dilatory responses.** Pial arteriolar responses to hypercapnia, BK, and sodium nitroprusside were recorded in the absence, and after a 60-min incubation with the soluble guanylyl cyclase (SGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (10^-4–10^-3 M) to determine cGMP requirement in newborn and juvenile responses.

**Iloprost and sodium nitroprusside dose-response curves.** To determine whether age-dependent differences in NO and prostanoid permissive mechanism of action are due to differences in sensitivities of newborn and juvenile pial arterioles to NO and prostanooids, dose-response curves to iloprost (10^-8, 10^-7, 10^-6, and 10^-5 M), and sodium nitroprusside (10^-7, 10^-6, and 10^-5 M) were performed.

**Statistical analysis.** Data are expressed as means ± SE. Comparisons among populations used analysis of variance with repeated measures. Fisher’s protected least-significant differences test was used to determine differences between groups. Significant responses to stimuli (i.e., comparisons with zero change) used Student’s t-tests. P < 0.05 was considered significant.

**RESULTS**

Blood gases and pH were within normal physiological ranges, and are therefore not reported unless significant changes were observed. Larger and smaller vessel responses were similar in all experiments. Therefore, in each of the experiments, the vessel responses nearest to 60 μm are reported. This arteriolar size is also useful anatomically, because they are just upstream of penetrating arterioles in both newborn and juveniles. Initial pial arteriolar diameters were similar between newborns and juveniles (newborn: 70 ± 2 μm, n = 39; juvenile: 66 ± 2 μm, n = 32).

In experiments involving hypercapnic challenges, newborn (n = 69) and juvenile (n = 53) prehypercapnic and hypercapnic arterial PCO2 values were not different within or between age groups (prehypercapnic arterial PCO2:3 ± 0.6 M, newborns and juveniles, respectively; hypercapnic arterial PCO2:6 ± 1, and 69 ± 1 mmHg, newborns and juveniles, respectively).

**Pial arteriolar responses to topical BK (group A).** Newborn and juvenile pial arterioles dilated similarly in a dose-dependent fashion to topical BK (Fig. 1, A and B). After indomethacin treatment, baseline diameters from both newborns and juveniles were significantly reduced compared with preindomethacin baseline diameters. Indomethacin completely inhibited dilations of newborn pial arterioles to 10^-8–10^-6 M BK, and attenuated the dilation to 10^-5 M BK. Treatment with indomethacin + L-NAME tended to further reduce baseline diameters of newborn vessels (not significant), and further attenuated the response to 10^-5 M BK, with significant dilation still observed. Dilations of juvenile pial arterioles were reduced after indomethacin treatment, with significant dilation observed in response to 10^-6 and 10^-5 M BK. A significant reduction of baseline diameters was observed after treatment of juveniles with L-NAME, compared with pre-L-
NAME baseline diameters. Furthermore, NOS inhibition prevented BK-induced dilations of juvenile arterioles (10⁻⁸–10⁻⁶ M), and almost completely prevented dilation in response to 10⁻⁵ M BK. Indomethacin significantly elevated juvenile, but not newborn, MAP (Table 1), and L-NAME significantly elevated newborn MAP and further increased juvenile MAP (Table 1).

**Pial arteriolar responses to topical BK (group B).** Before inhibitor treatment, dilations of newborn and juvenile pial arterioles to BK of pigs in group B (Fig. 1, C and D) were the same as those in group A (Fig. 1, A and B). After NOS inhibition with L-NAME, BK-induced dilatory responses from newborn arterioles were mildly attenuated, with significant dilation still observed at concentrations of 10⁻⁶ and 10⁻⁵ M BK. However, after L-NAME treatment, juvenile pial arterioles failed to dilate, even when exposed to a concentration of 10⁻⁵ M BK. Treatment of newborns with indomethacin after L-NAME completely prevented BK-induced dilations of newborn vessels, except at the highest concentration. Furthermore, indomethacin, but not L-NAME, treatment significantly reduced baseline diameters of newborn pial arterioles. L-NAME treatment significantly increased newborn and juvenile MAP (Table 1), but subsequent treatment with indomethacin did not further elevate newborn MAP.

**Effects of BK on cortical 6-keto-PGF₁α, cAMP, and cGMP production.** Baseline concentrations of 6-keto-PGF₁α, cAMP, and cGMP were not different when comparing newborns to juveniles (Table 2). Figure 2 shows BK-induced fold changes in periarachnoid CSF

**Table 1. Baseline mean arterial pressures of newborn and juvenile pigs**

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Control</th>
<th>Indo</th>
<th>Indo + L-NAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>8</td>
<td>76 ± 6</td>
<td>78 ± 5</td>
<td>87 ± 3*</td>
</tr>
<tr>
<td>Juvenile</td>
<td>6</td>
<td>94 ± 5</td>
<td>113 ± 4*</td>
<td>139 ± 7*</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Control</th>
<th>L-NAME</th>
<th>L-NAME + Indo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborn</td>
<td>10</td>
<td>82 ± 4</td>
<td>101 ± 6*</td>
<td>95 ± 4*</td>
</tr>
<tr>
<td>Juvenile</td>
<td>6</td>
<td>98 ± 3</td>
<td>126 ± 11*</td>
<td></td>
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</tbody>
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Values are means ± SE; n, number of pigs. Control, no inhibitors; Indo, indomethacin (10 mg/kg); L-NAME, N⁵-nitro-L-arginine methyl ester (30–40 mg/kg). Blood pressure was measured during control. Mean arterial pressures were measured in millimeters per mercury. *P < 0.05, statistical significance compared with control value.

**Table 2. Baseline periarachnoid CSF concentrations of 6-keto PGF₁α, cAMP, and cGMP from newborn and juvenile pigs**

<table>
<thead>
<tr>
<th></th>
<th>Newborns</th>
<th>Juveniles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborns</td>
<td>829 ± 121*</td>
<td>638 ± 77</td>
</tr>
<tr>
<td>Juveniles</td>
<td>1,404 ± 199*</td>
<td>1,950 ± 320*</td>
</tr>
</tbody>
</table>

Values are means ± SE (in fmol/ml). n, no. of pigs. CSF, cerebrospinal fluid; PGF₁α, prostaglandin F₁α. *Denotes Winsorization as per Dixon and Massey (6).
6-keto-PGF$_{1\alpha}$, cAMP, and cGMP collected from newborns and juveniles under control conditions (no inhibitors). Newborn and juvenile CSF concentrations of 6-keto-PGF$_{1\alpha}$, cAMP, and cGMP increased dose dependently in response to topical BK. Treatment with indomethacin prevented increases in cortical production of 6-keto-PGF$_{1\alpha}$ and cAMP (Fig. 3, A and C) in both age groups compared with control responses. Indomethacin treatment partially inhibited increases in cGMP levels in the newborn, but had no effect on juvenile cGMP responses to BK (Fig. 3, C and E). L-NAME, in addition to significantly attenuating BK-induced increases in cGMP (Fig. 4, A and B), also blocked elevations of 6-keto-PGF$_{1\alpha}$ in both newborns and juveniles (Fig. 4, C and D).

**Endothelial dependency of BK-, hypercapnia-, and acetylcholine-induced pial arteriolar dilations.** To ensure inhibition of responses after light/dye injury was not due to effects of dye treatment, dye-only control experiments were performed. Newborn isoproterenol, hypercapnia, BK, and acetylcholine responses after dye-only treatment were not different compared with control responses (Fig. 5A). Juvenile responses to isoproterenol and BK were unaffected by dye-only treatment. However, hypercapnic responses were significantly reduced compared with predye responses, although significant dilation was observed (Fig. 5B). BK responses appear to be mediated through activation of the B$_2$ receptor subtype because the selective B$_2$ antagonist HOE-140 nearly completely inhibited dilation of both newborn and juvenile pial vessels in response to BK. Furthermore, the effect of HOE-140 was selective for BK because dilations to isoproterenol and hypercapnia in the presence of the inhibitor were similar to pre-HOE-140 values (Fig. 5, A and B). Figure 6, A and B, shows dilations of newborn and juvenile pig pial arterioles, respectively, in response to topical isoproterenol, BK, hypercapnia, and acetylcholine before...
and after light/dye injury. Newborn and juvenile vessels dilated similarly to isoproterenol before and after endothelial injury, indicating vessel reactivity was maintained. However, compared with control responses, BK- and hypercapnia-induced dilations from both newborns and juveniles were inhibited after light/dye treatment, suggesting these responses are endothelium dependent in both age groups. Application of acetylcholine to the brain surface resulted in contractions of newborn vessels that were unaffected by light/dye treatment. However, juvenile pial arterioles responded to acetylcholine with initial constriction followed by prolonged dilation. After light/dye injury, the secondary dilation was inhibited, but the initial constriction remained (Fig. 6B).

**NO and prostanoid mechanism of action in cerebrovascular responses.** In newborn piglets, inhibition of dilations in response to BK, and hypercapnia were reversed in the presence of subdilator concentrations (10^-9 M) of the prostacyclin analog iloprost (Fig. 6A). After recovery of responses, iloprost was removed, and responses to hypercapnia and BK recorded. After removal of iloprost, newborn hypercapnic- and BK-induced dilations were not different from those recorded after light/dye. However, recovery of newborn responses after injury was not observed when sodium nitroprusside was substituted for iloprost (Fig. 7A), whereas subsequent addition of iloprost under the window again resulted in recovery of hypercapnia and BK responses. Furthermore, doubling the concentration of iloprost did not augment the degree of recovery of newborn responses (Fig. 7A). In contrast to newborns, reversal of light/dye-inhibited juvenile pial arteriolar dilatory responses to BK, hypercapnia, and acetylcholine was observed when sodium nitroprusside (10^-7 M) was present (Fig. 6B). When sodium nitroprusside was removed, juvenile responses to hypercapnia and BK were not significantly different from those observed.
after light/dye injury. Juvenile hypercapnic responses were not restored when iloprost was present at concentrations that were effective at restoring newborn responses. However, when the concentration of iloprost was increased, partial recovery of the hypercapnic response was observed (Fig. 7B). Dilations to BK were not reversed, even with the greater concentration of iloprost present (Fig. 7B).

SGC dependency of dilatory responses. Effects of ODQ on newborn and juvenile pial arteriolar responses to hypercapnia, BK, and sodium nitroprusside are shown in Fig. 8. Newborn responses to sodium nitroprusside, but not hypercapnia and BK, were inhibited by SGC inhibition with ODQ (Fig. 8A). After treatment with ODQ, juvenile responses to BK and sodium nitroprusside were completely inhibited, and hypercapnic responses were significantly attenuated (Fig. 8B).

Prostanoid and NO sensitivity of newborn and juvenile pial arterioles. Newborn and juvenile pial arterioles dilated in a dose-dependent fashion to both the prostacyclin analog iloprost (Fig. 9A) and the NO donor sodium nitroprusside (Fig. 9B). Dose-response curves recorded for iloprost and sodium nitroprusside were found to be similar between newborns and juveniles.

DISCUSSION

New findings of this study are the following. First, cerebral arteriolar dilatory responses to topical BK were similar in magnitude in newborns and juveniles.
Second, newborn and juvenile cerebrovascular responses to BK and hypercapnia are endothelium dependent. Dilations of juvenile vessels to acetylcholine were also endothelium dependent, whereas contractions of newborn vessels in response to acetylcholine are endothelially independent. Third, the mechanisms involved in endothelial-dependent dilator responses in newborns and juveniles are not the same. Specifically, newborn pial arteriolar responses to BK involve both COX and NOS systems, but are more prostanoid dependent, whereas juvenile responses also requiring contributions from both pathways are more strongly influenced by NO. Fourth, newborn BK and hypercapnic responses involve a permissive mechanism of action from endothelially derived prostanoids, but not NO, whereas juvenile dilatory responses to BK, hypercapnia, and acetylcholine can occur via a permissive action involving NO. Fifth, pial arteriolar responses to iloprost and sodium nitroprusside were not different between newborns and juveniles. Sixth, BK dose-dependently increases newborn and juvenile cortical production of prostacyclin and cAMP, and these increases are inhibited by indomethacin. BK dose-dependently increases cGMP that is inhibited by L-NAME in both age groups. Seventh, elevations of 6-keto-PGF1α in both newborns and juveniles were inhibited after NOS inhibition, suggesting a modulatory role for NO in regulating COX activity. Finally, juvenile (but not newborn) hypercapnic- and BK-induced pial dilatory responses appear to be mediated by activation of SGC.

The results from this study are consistent with our overall hypothesis that NO is less important in mediating dilator responses in the immature piglet cerebral vasculature, but becomes a significant component in mechanisms governing cerebrovascular regulation of the juvenile pig.
BK-induced dilations appear to be endothelial dependent (11, 32), and are mediated via endothelial B₂ receptors (11, 25, 35). In the present study, newborn and juvenile pig pial arteriolar dilations to BK were inhibited when the specific B₂ receptor antagonist HOE-140 was used, whereas hypercapnic and isoproterenol responses were unaffected. The signaling mechanisms that govern dilatory responses to BK downstream of the B₂ receptor may involve reactive oxygen species (ROS), NO, or prostanoids.

COX-derived ROS have been proposed to mediate cerebrovascular BK effects, presumably through activation of calcium-dependent potassium channels (34). COX-derived ROS are potent dilators of the cerebral circulation (17, 18), and ROS have been shown to increase after BK exposure (15). In specific cases, dilation to BK can be inhibited with indomethacin (present study, 9, 16), ROS scavengers, and tetraethylammonium (34). Kontos et al. (16) and Sobey et al. (34), in studies on cats and rats, respectively, reported complete inhibition of BK-induced pial dilations with ROS scavengers, suggesting that ROS alone mediate this response. However, we report incomplete inhibition of BK-induced pial arteriolar responses with indomethacin. In fact, NOS inhibition was much more effective compared with indomethacin at inhibiting juvenile responses to BK. Partial inhibition of newborn responses was observed after L-NAME, though L-NAME as an inhibitor was not as effective when compared with indomethacin in this age group. Although we did not investigate the role of COX-derived ROS, our results suggest that ROS are not the sole mediators of pig pial arteriolar BK-induced dilations. Leffler et al. (22) reported that inhibition of ROS does not alter prostanoid-dependent, hypercapnia-induced cerebral vasodilations in newborn pigs, providing further evidence that COX-derived ROS, although released in response to certain stimuli and capable of producing dilation, do not necessarily mediate cerebrovascular responses on their release.

Both NO and prostanoids are involved as mediators of BK-induced pial arteriolar dilations. Indomethacin significantly inhibited newborn responses, but was less effective at inhibiting juvenile dilations to BK, suggesting that prostanoids contribute significantly to newborn, but not juvenile, cerebrovascular dilations to BK. Inhibition of NOS, in addition to COX, completely prevented juvenile responses to lower concentrations of BK, attenuated the response at the highest dose, but
only slightly attenuated dilations of newborn pial arterioles to the highest concentration. When the order of inhibitors was reversed, a similar profile was observed regarding prostanoid and NO involvement. Specifically, L-NAME mildly attenuated newborn responses to BK, but completely abolished juvenile responses, even at a BK concentration of $10^{-5}$ M, suggesting that NO is the more important mediator of juvenile, but not newborn, BK-induced cerebrovascular dilations. Furthermore, in newborns, only COX inhibition resulted in a reduction of baseline diameters, whereas in juveniles, a decrease in baseline diameter was observed after either NOS or COX inhibition.

Developmental changes in NOS and COX expression and/or activity could explain our age-related dichotomy in results. When comparing expression and activity of endothelial NOS and COX in cultured vascular endothelial cells isolated from newborn and adult pig cortex, Parfenova et al. (29) found no differences in COX expression and activity between these groups, but they did find higher endothelial NOS expression and activity in adult cells. In contrast with data from the newborn piglet cerebral circulation, Van Bel et al. (38) report a more significant role for NO in regulating ovine fetal pulmonary blood flow and resistance when compared with newborns, suggesting NO is downregulated in the pulmonary circulation with maturation. Although there are conflicting data when comparing the expression of COX and endothelial NOS enzymes with respect to age, in vivo data from our laboratory support results from Parfenova et al. (27–30) insofar as we observed little, if any, contribution from NO in mediating hypercapnic-, histamine-, and BK-induced dilations of the newborn piglet cerebral vasculature (present study and Ref. 41), but have shown markedly reduced juvenile hypercapnic, acetylcholine, and BK dilatory responses after NOS inhibition (41, 42, and the present study).

It is generally accepted that NO produces dilation via activating SGC, resulting in cGMP elevation in vascular smooth muscle. Newborn pial arterioles dilate in response to NO donors (4 and present study) or compounds that mimic elevated cGMP levels (8-bromo-cGMP) (30), suggesting the machinery necessary to respond to NO is present in the neonatal vasculature. Furthermore, newborn piglet periarachnoid cGMP, in addition to cAMP, is elevated in vivo after hypercapnia (30), BK (present study), and iloprost (1), suggesting that cGMP may be involved, possibly through activation of SGC by NO. However, we have repeatedly demonstrated that in newborn piglets, pial arterial dilations in response to histamine, elevated arterial $P_{CO_2}$, and BK are predominantly NO-independent, even though cGMP levels may be increased (41, 42). In contrast to newborns, juvenile cerebrovascular dilations to BK and sodium nitroprusside were inhibited, and the hypercapnic response was significantly attenuated after treatment with ODQ, strongly implicating a role for the NO/SGC pathway in juvenile responses. Consistent with studies by Armstead et al. (3) and Castro et al. (4), we report increased cGMP levels in CSF collected from both newborns and juveniles in response to BK that were inhibited by L-NAME, suggesting that BK stimulates the NOS/cGMP pathway in both newborns and juveniles. However, BK-stimulated increases in cGMP via NO release may be functionally significant only in juveniles, as juvenile, but not newborn, BK-induced dilations were inhibited by L-NAME.

We also report data suggesting that NOS and COX pathways interact, rather than acting independently of one another. BK-induced increases in 6-keto-PGF$_{1a}$ were attenuated after L-NAME treatment in both newborns and juveniles, suggesting that NO may positively modulate COX activity. The majority of information concerning a modulatory role of NO on COX has been obtained in vitro. These studies examined NO derived from inducible NOS, rather than constitutive NOS, and its effects on COX activity. Using macrophages and perfused rat lung, Swierkosz et al. (36) showed that release of both NO$_2^-$ (NO breakdown product) and 6-keto-PGF$_{1a}$ are increased by lipopolysaccharides, and were attenuated by cyclohexamide or dexamethasone. Perkins and Kniss (31) reported downregulation of COX-2 activity and PGE$_2$ production in macrophages after NO inhibition. Constitutive NOS inhibition has been shown to attenuate BK-induced PGE$_2$ production in the normal and hydronephrotic rabbit kidney, suggesting that NO may regulate COX activity in normal as well as inflamed tissue (33). In support of a negative modulatory role of NOS on COX activity, Doni et al. (7) have shown that BK-stimulated increases in 6-keto-PGF$_{1a}$ from cultured bovine aortic endothelial cells can be inhibited by preexposure to NO. Depending on the experimental model, NO may negatively or positively modulate COX activity. Data concerning a modulatory effect of COX products on NOS activity are controversial, with little in vivo data available (5).

We suggest prostanoids are important mediators of newborn cerebral dilatory responses to BK. Paradoxically, we still observed dilation to BK in newborns treated with L-NAME that prevented elevations in CSF 6-keto-PGF$_{1a}$. How can this be so if prostanoids are the primary mediator of BK-induced dilations in the cerebral circulation of the newborn piglet? We (19, 20, 41) have previously demonstrated that newborn piglet cerebral dilatory responses to hypercapnia and histamine are prostanoid dependent, and the mechanism of action of prostanoids is permissive. That is, only a very low concentration of prostanoid is required for dilation to occur, much lower than is required to produce dilation directly. Thus elevation of prostacyclin is not necessary, only some minimal presence. Although L-NAME prevented increases in prostacyclin, baseline levels were present. If sufficient prostanoid is available (we have used iloprost at a concentration as low as $10^{-12}$ M), and if prostanoids act permissively to produce responses to BK, one would not expect increased production to be necessary to allow dilation. Indomethacin can completely block newborn BK responses because it not only inhibits COX activity but also prevents prostacyclin binding to the IP prostanooid receptor (28).
Light/dye endothelial damage provides a means to elucidate endothelial dependency of vascular responses in vivo. Results from the present study indicate the following: 1) newborn pial arteriolar dilatory responses to hypercapnia and BK, but not acetylcholine-induced constrictions, are endothelium dependent, and 2) juvenile pial arteriolar dilatory responses to hypercapnia, BK, and acetylcholine are endothelium dependent; however, constriction to acetylcholine is endothelium independent. Light/dye endothelial injury prevented cerebrovascular dilations of both newborns and juveniles in response to hypercapnia and BK and, in juveniles, blocked dilation in response to acetylcholine, whereas the constrictor response was left unaffected. Isoproterenol responses were unaltered by light/dye injury in both age groups, indicating damage to vascular smooth muscle did not occur or was negligible. Inhibition of cerebrovascular responses after light/dye treatment was not due to effects of dye treatment. Addition of subdilator concentrations of iloprost beneath the cranial window restored newborn responses to hypercapnia and BK that were inhibited by light/dye endothelial injury, similarly to when the responses were blocked with indomethacin (19, 20, 41). In contrast with newborns, juvenile hypercapnic, BK, and acetylcholine dilations inhibited by light/dye injury were restored when sodium nitroprusside was present. Furthermore, dilation to hypercapnia, but not BK, could be partially restored with iloprost, but only at concentrations that were fourfold higher than concentrations required to restore newborn responses. However, in newborns, when sodium nitroprusside was substituted for iloprost, restoration of light/dye inhibited responses was not observed, even at concentrations four times greater than those that were effective at restoring juvenile responses. Previously, we were unable to demonstrate a permissive role for NO or prostanoids in juvenile pial cerebrovascular responses to hypercapnia and histamine. 1-NNA- and indomethacin-inhibited responses could not be restored when sodium nitroprusside or iloprost, respectively, were present (41). The reason for the discrepancy between the present study and our previously published results is not readily apparent, but may be age related. In the previous study, the juvenile pigs were slightly older than those used in this study, as seen when comparing pig weights (previous, 52.2 ± 1.3 kg, and present, 27.6 ± 0.9 kg). A permissive mechanism of action for NO may exhibit a biphasic response in these animals, such that as the pig matures from neonate to juvenile, NO becomes a permissive mediator, and during development from juvenile to near adult, NO begins to act in a more classical or direct fashion. Pig pulmonary artery responses to acetylcholine have been shown to exhibit a biphasic response pattern. Acetylcholine-induced constriction was reported to initially increase postnatally compared with neonates, but began to decrease with development to adulthood (24).

The ability of iloprost to restore newborn (but not juvenile) responses and sodium nitroprusside to restore juvenile (but not newborn) responses that were inhibited after endothelial injury suggests the possibility that differences in sensitivity of the cerebral circulation to prostanoids and NO could occur during development. However, when dose-response curves were compared, no differences between newborns and juveniles in dilator responses to either iloprost or sodium nitroprusside were detected. These results suggest that the abilities of NO and prostanoids to act permissively causing dilations of juvenile and newborn pial arterioles, respectively, are not related to age-dependent sensitivity differences to prostanoids and NO.

In conclusion, these results are consistent with the hypothesis that the predominant EDRF influence is altered during maturation from neonate to juvenile. Prostanoids are the predominant EDRF mediating neonatal cerebrovascular responses, but become less important in the juvenile, where NO emerges as a significant EDRF signal. Furthermore, we provide evidence for a permissive mechanism of action of prostanoids and NO mediating newborn and juvenile endothelially dependent cerebrovascular responses, respectively. Finally, we also show that endothelially derived COX and endothelial NOS products may not act independently of each other, but rather, significant cross talk may exist between these two pathways, resulting in a complex and dynamic cerebrovascular regulatory system.

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