Compensatory role of NO in cerebral circulation of piglets chronically treated with indomethacin

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Zhang, Yifan, and C. W. Leffler. Compensatory role of NO in cerebral circulation of piglets chronically treated with indomethacin. Am J Physiol Regulatory Integrative Comp Physiol 282: R400–R410, 2002; 10.1152/ajpregu.00256.2001.—We hypothesize that inhibitory effects exist between prostanoids and nitric oxide (NO) in their contributions to cerebral circulation. Piglets (1–4 days old) were divided into three chronically treated (6–8 days) groups: control piglets, piglets treated with indomethacin (75 mg/day), and piglets treated with Nω-nitro-L-arginine methyl ester (L-NAME, 100 mg·kg−1·day−1). Pial arterioles dilated in response to hypercapnia similarly among the three groups (41 ± 4, 40 ± 6, and 45 ± 11%). Cerebrospinal fluid cAMP increased in control piglets, while cGMP increased in indomethacin-treated piglets. L-NAME, but not 7-nitroindazole, inhibited the response to hypercapnia only in indomethacin-treated piglets (40 ± 6 vs. 17 ± 5%). Topical sodium nitroprusside or iloprost restored dilation in response to hypercapnia. Similar results were obtained when the dilator was bradykinin. Pial arterioles of control and L-NAME-treated piglets constricted in response to ACh (−24 ± 3%). However, those of indomethacin-treated piglets dilated in response to ACh (15 ± 2%). This dilation was inhibited by L-NAME. NO synthase activity, but not endothelial NO synthase expression, increased after chronic indomethacin treatment. These data suggest that chronic inhibition of cyclooxygenase can increase the contribution of NO to cerebrovascular circulatory control in piglets.

Cerebral blood flow is well regulated. Prostanoids (PGs) and nitric oxide (NO) are among many vasomotor factors that contribute to the precise control of cerebral circulation. All the cells in the brain can produce PGs. The quantities and types of PGs, which can be constrictors or dilators, are cell type and age specific (16). NO is a potent vasodilator that can produce relaxation of vessels in vivo and in vitro (13, 21). NO is released in the conversion of L-arginine to L-citrulline via NO synthase (NOS). NO diffuses very easily from the endothelium and neurons into the adjacent smooth muscle cells, activates soluble guanylyl cyclase, and increases the intracellular level of cGMP. Mechanisms of cerebrovascular circulatory control can change with development. Pearce et al. (27) found that the transition from fetal to newborn life is associated with a decreased water content in the arteries, an increase in wall thickness, and an increase in maximum contractile tension and stiffness. Hayashi and colleagues (9) reported a decrease in norepinephrine- and acetylcholine (ACh)-induced contractions during maturation. NO has been generally accepted as a major regulator of the adult cerebral circulation. However, PGs appear to be the dominant endothelium-derived relaxing factor (EDRF) in the regulation of cerebral circulation in newborn animals of several species, including humans (16). However, with maturation, the contribution of NO becomes more important. For example, Willis and Leffler (37) found that as newborn piglets matured to juvenile pigs, NO emerges as a significant EDRF in mediating the cerebral vasodilatory response to hypercapnia, but PGs still contributed to this response, albeit to a lesser extent. Zuckerman et al. (39) found that NO and PGs contributed to the pial arteriolar response to ACh in juvenile pigs, while only PG-associated vasoconstriction to ACh was found in newborn piglets.

Progressively accumulating evidence shows that, in addition to producing dose-dependent responses directly, PGs and NO can have permissive functions in the regulation of cerebral circulation. By “permissive,” we mean that presence of the mediator, not its increasing concentrations, is necessary to allow the dose-dependent response to an alternative stimuli to occur. In the cerebral vasculature, the permissive mechanism was first described in regard to the vascular response to ACh (3). Topically applied ACh causes constriction of pial arterioles in the piglet and, at the same time, a dose-dependent increase of PGs in the cerebrospinal fluid (CSF). Because the predominant PGs produced are dilators, one might expect an augmentation of the constriction after inhibition of PG production. However, indomethacin totally eliminates the constrictive responses of pial arterioles. The thromboxane receptor agonist U-46619, at a constant subconstrictor concentration, restored the dose-dependent constriction in response to ACh. Furthermore, in intact piglets, the thromboxane receptor antagonist SQ-29548 totally...
blocks the ACh-induced vasoconstriction. These results indicate that activation of the thromboxane receptor is necessary and sufficient for the response to ACh (3).

Similarly, the role of prostacyclin in dilation of newborn pig cerebral arterioles to hypercapnia and histamine can be permissive (17, 18). In adult rats, NO might also play a permissive role in the regulation of cerebral circulation. NO appears to be permissive in hypercapnia-induced cerebral vasodilation (11), but it is conventional in the pial arteriolar response to ACh (10).

The underlying mechanisms of the permissive actions of PGs and NO may be different. Surprisingly, the prostacyclin receptor is coupled to phospholipase C as well as adenylyl cyclase, and it is the phospholipase C pathway that seems to account for the permissive function of prostacyclin in the piglet (26, 30). A minimal necessary cellular level of cGMP has been suggested to be necessary for the permissive contribution of NO in the adult rat (38).

Numerous potential mechanisms exist for interactions between PGs and NO. One is that NO, or a related chemical species, can stimulate or inhibit cyclooxygenase (COX) by binding the heme moiety of the enzyme (32). NO can increase/decrease the half-life of COX-2 by affecting the autoinactivation of COX (7, 32). NO can also react with superoxide to produce peroxynitrite, and the following peroxidation of lipid might affect the liberation of arachidonic acid from the cell membrane (6). The effects of PGs on NOS are poorly understood, yet most data attribute the effect of PGs on NO to cAMP (20). cAMP may affect the mRNA stability of NOS or its transcriptional activation (14). Others have suggested that the downregulation of tumor necrosis factor-α by cAMP is involved (31).

Because PGs and NO can interact and, in the newborn, where PGs are higher, the role of NO appears to be small, we hypothesize that inhibition of COX in the newborn will increase the role of NO in cerebrovascular circulatory control.

METHODS

The animal protocols were reviewed and approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center.

Primary Culture of Endothelial Cells

Brains were extracted from newborn pigs anesthetized with ketamine and acepromazine. After homogenization of the cortex with a loose-fitting pestle in culture medium 199, 60- to 300-μm-diameter microvessels were obtained by series filtration. To isolate the endothelial cells, the microvessels were treated with collagenase-dispase (1 mg/ml) for 2 h at 37°C and then subjected to Percoll density gradient centrifugation. The cells were plated onto Matrigel-coated 12-well cell culture plates and maintained in Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum, 30 μg/ml endothelial cell growth supplement, 1 U/ml heparin, and antibiotic-antimycotic mixture. Cells were grown in a 5% CO2-air incubator at 37°C for 5–7 days until they reached confluence.

All experiments using cultured endothelial cells were performed on confluent quiescent cells. To achieve quiescence, cells were exposed to serum-depleted medium (1% fetal bovine serum, 0% endothelial cell growth supplement) for 15–24 h before the experiments.

Western Blot Analysis for Endothelial NOS Protein

Freshly collected microvessels or cultured endothelial cells were suspended in Laemmli sample buffer (0.06% bromophenol blue, 2.5% SDS, 0.125% Tris-HCl, 10% glycerol), and β-mercaptoethanol was added to a final concentration of 5%. The samples were dispersed with sonication (3 times for 10 s with 10-s intermissions) using an ultrasonic cell processor (Sonica and Material, Danbury, CT). The samples were boiled for 10 min and then centrifuged for 10 min at 5,000 g to clarify the cell lysate. Total protein concentration of the samples was determined by the method described earlier (25).

The cell lysate was subjected to electrophoresis on 7.5% polyacrylamide gel. Proteins (60–80 μg) were loaded onto each lane and run overnight (30–40 V). Human lysate (Transduction Laboratories, Lexington, KY) was used as a positive control, and high-range biotinylated SDS-PAGE standards (Bio-Rad, Hercules, CA) were used as molecular weight markers. After electrophoresis, the proteins were transferred to nitrocellulose membranes in Towbin buffer [25 mM Tris, 192 mM glycine, 15% methanol (vol/vol), 0.05% SDS] by the wet transfer method (800 mA, 1.5 h). Nonspecific binding sites were blocked by incubation of membranes with 5% bovine serum albumin in Tris-buffered saline [10 mM Tris-HCl, 0.9% NaCl, pH 7.4, 0.05% Tween 20 (vol/vol)] for 1 h. For endothelial NOS (eNOS) detection, the membrane was incubated with polyclonal eNOS antibody (Transduction Laboratories; 1:2,000 dilution) for 1 h at room temperature. After the membrane was washed three times for 15 min each with Tris-buffered saline, the membrane was incubated with horseradish peroxidase-conjugated donkey anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 h at room temperature. Streptavidin-biotin-horseradish peroxidase complex was added to the second antibody incubation medium to detect biotinylated standards. The blot was then developed with a chemiluminescence detection system (Du Pont, Boston, MA), and the protein band was detected by autoradiography. For actin detection, the membrane was incubated with monoclonal actin antibody (Chemicon, Temecula, CA; 1:5,000 dilution) at the first antibody incubation step with subsequent treatment as for eNOS detection.

Animal Groups and Treatments

Fifty-one newborn piglets (2.2 ± 0.3 kg) were randomly assigned to three groups simultaneously (typically 2–3 piglets per week, with 1 control and the others in indomethacin and/or L-NAME groups).

The control group consisted of 21 piglets (9 ± 2 days at the end of treatment): 11 were subjected to a sham procedure for oral indomethacin, and the other 10 were housed with indomethacin- and L-NAME-treated piglets for 6–8 days. The indomethacin group (n = 24, 11 ± 2 days at the end of treatment) was treated with indomethacin (75-mg sustained-release capsule po, twice a day, for 6–8 days). The L-NAME group (n = 6, 11 ± 3 days at the end of treatment) was treated with L-NAME (100 mg·kg⁻¹·day⁻¹ ip for 6–8 days). At the end of the treatment, animals were anesthetized with ketamine hydrochloride (33 mg/kg im) and acepromazine (3.3 mg/kg im). The anesthesia was maintained with α-chloralose during the experiment (30–40 mg/kg initially, supplemented with 7 mg·kg⁻¹·h⁻¹). A catheter was placed in the femoral...
artery to record systemic blood pressure and to sample arterial blood for the measurement of pH, PCO₂, and PO₂. A second catheter was placed in the femoral vein for anesthesia and experimental drug administration. Animals underwent a tracheotomy with insertion of an endotracheal tube and mechanically ventilated with room air to maintain arterial pH, PCO₂, and PO₂ within the normal range. The core temperature was monitored with a rectal probe and maintained at 37.5–38.5°C. The indomethacin-treated piglets were given indomethacin acutely (5 mg/kg iv) before the experiment to ensure complete block of COX during cranial window experiment. This dose was repeated every 90 min.

**Cranial Window Placement and Pial Arteriolar Monitoring**

After catheter placement and tracheotomy, the scalp was surgically removed, and a 2-cm-diameter hole was cut in the skull over the parietal cortex. Bone wax was applied to stop bleeding. The dura was gently cut and pulled over the cut bone edge. Care was taken to avoid contact with the brain surface. A stainless steel cranial window with a glass pane was placed in the hole and solidly cemented sequentially with bone wax, Super glue, and meditone. A dissecting microscope with a mounted video camera was used to observe pial arterioles (50–140 μm). Vessel diameter was measured with a video microscaler. The space under the window was filled with artificial CSF (aCSF; in mg/l: 220 KCl, 1,132 MgCl₂, 221 CaCl₂, 7,710 NaCl, 401 urea, 665 dextrose, 2,066 NaHCO₃). The aCSF was warmed in a water bath to 37°C and bubbled with 6% CO₂–6% O₂–88% N₂ to maintain approximate values of pH 7.33, 45 mmHg Pco₂, and 45 mmHg Po₂.

After an initial observation period of 30 min, 50- to 140-μm-diameter pial arterioles were measured and recorded. Mean arterial pressure and core temperature were recorded. At the end of each tested response, an arterial blood gas sample was drawn, and the area under the window was gently flushed with fresh aCSF to remove the previous stimulus and allow the vessels to recover to baseline diameters before the next measurement.

Dilations of pial arterioles in response to topical isoproterenol (10⁻⁶ M; Sigma Chemical, St. Louis, MO) were recorded at the beginning and end of each experiment to ensure consistent responsiveness of pial arterioles to stimuli and stability of the preparation throughout the experiment. Preparations that demonstrated >15% decline in response to isoproterenol from beginning to end were discarded.

**Pial Arteriolar Response to Hypercapnia**

Piglets were mechanically ventilated with room air plus supplemental CO₂ to achieve 55–65 mmHg PCO₂. Our goal was to determine the involvement of PGs and or NO in mediating the pial arteriolar dilation to hypercapnia in these three groups. In the control group, responses of the pial arterioles were recorded before and after the administration of L-NAME and indomethacin in the presence of a subdilatory dose of iloprost. In the indomethacin group, responses were recorded before and after topical administration of L-NAME and 7-nitroindazole (7-NI) and in the presence of subdilatory concentrations of iloprost (0.5 × 10⁻⁹ and 1 × 10⁻⁹ M), sodium nitroprusside (SNP, 0.5 × 10⁻⁷ and 1 × 10⁻⁷ M), or iloprost + SNP. In the L-NAME group, the pial arteriolar dilatory responses to hypercapnia were recorded before and after the application of indomethacin.

**Pial Arteriolar Response to Topical ACh**

Topical ACh (10⁻⁶ M; Sigma Chemical) was applied to the cranial window to determine the responses of pial arterioles to ACh and to investigate possibly different roles of PGs and NO in these responses in the three groups of piglets. In control piglets, responses were recorded before and after the application of L-NAME and indomethacin. In indomethacin-treated piglets, responses were recorded before and after L-NAME (10⁻³ M) administration and in the presence of U-46619 (0.5 × 10⁻⁸ M). In L-NAME-treated piglets, the responses before and after indomethacin administration were compared.

**Pial Arteriolar Response to Topical Bradykinin**

Bradykinin (BK, 10⁻⁷ M; Sigma Chemical) was applied topically. In control piglets, the responses to BK were recorded before and 1 h after application of topical L-NAME (10⁻³ M) or indomethacin (5 mg/kg iv) to investigate the respective contributions of PG and NO. In indomethacin-treated piglets, the responses before and after administration of L-NAME or 7-NI (25 mg/ml dissolved in mineral oil, 50 mg/kg ip) or in the presence of SNP (0.5 × 10⁻⁷ and 1 × 10⁻⁷ M) after L-NAME were recorded.

**cAMP and cGMP Assay**

CSF samples were carefully collected by flushing the closed window with aCSF. The cranial window has a volume of 500 μl, and only 300 μl were collected for the detection of cyclic nucleotides. cAMP levels in CSF were determined by radioimmunoassay. CSF samples were acetylated with 2.1 triethylamine-acetic anhydride immediately before the assay to increase the sensitivity. ¹²⁵I-labeled cAMP and rabbit anti-cAMP antibody were added to the samples and incubated overnight. Polyethylene glycol (25%) was added to precipitate antibody-bound cAMP. The cAMP concentration in the CSF was calculated from the standard curve.

The cGMP levels in the CSF samples were measured with an ELISA kit (Stratagene, La Jolla, CA). CSF samples were acetylated with 2.1 triethylamine-acetic anhydride immediately before they were applied to 96-well plates. After the samples were incubated with rabbit anti-cGMP on a shaker for 1 h at room temperature, horseradish peroxidase-conjugated GMP was added to each well and incubated at 4°C overnight.

After the plates were washed, tetramethylbenzidine-H₂O₂ substrate was added. After 10 min, phosphoric acid was added to stop the color-developing reaction. Concentrations of cGMP were calculated by measuring the absorption at 450 nm and constructing a standard curve.

**NOS Assay**

The activity of NOS, including eNOS, neuronal NOS (nNOS), and inducible NOS, was determined using an NOS assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, the fresh isolated microvessels were homogenized, and then the reaction mixture, including [³H]arginine, CaCl₂, and NADPH, was added. After the sample was incubated at room temperature for 60 min, resin was added to bind unreacted arginine. Samples were transferred to spin cups and centrifuged; the eluant was collected, mixed with scintillation fluid, and then counted.

**Statistical Analysis**

Values are means ± SE. Comparisons among populations were made by analysis of variance with repeated measures. Fisher’s protected least significant difference was used to determine differences between groups. Significant responses were made by analysis of variance with repeated measures. Fisher’s protected least significant difference was used to determine differences between groups.
to stimuli (i.e., comparisons with zero change) were determined by Student’s t-tests. P < 0.05 was considered significant.

RESULTS

eNOS Protein Levels in Cultured Endothelial Cells

Treatment with NS-398, indomethacin, arachidonic acid, or interleukin-1β for 24 h did not change the expression of eNOS protein in primary cultures of endothelial cells compared with the control (Fig. 1).

eNOS Protein Levels in Microvessels

The protein levels of eNOS did not differ significantly among microvessels from the three groups of piglets (Fig. 2). (We performed these experiments 4 times and, according to quantitative analysis using NIH Image 1.62, could detect no significant difference.)

NOS Activities in Microvessels

The NOS activity was higher in the microvessels from the indomethacin-treated piglets than from the control group. Furthermore, as expected, the NOS activity in microvessels from L-NAME-treated piglets was significantly reduced (Fig. 3).

Pial Arteriolar Responses

Chronic treatment of piglets with L-NAME increased mean blood pressure (89 ± 17 mmHg) compared with the control group (65 ± 11 mmHg) and the indomethacin group (69 ± 9 mmHg). The blood gas and pH were not different among the groups. Initial diameters of pial arterioles were similar among the control, indomethacin, and L-NAME groups [control: small, 57 ± 7 μm (n = 21); large, 94 ± 9 μm (n = 18); indomethacin: small, 62 ± 8 μm (n = 25); large, 89 ± 7 μm (n = 24); L-NAME: small, 65 ± 6 (n = 6); large, 99 ± 8 μm (n = 8)]. Also, smaller and larger vessels responded similarly in all experiments. Therefore, only responses recorded from smaller (~60–70 μm) arterioles are reported below.

Response to hypercapnia. During control periods and hypercapnia challenges, the arterial PCO2 values were not significantly different among the three groups.

In the control, indomethacin, and L-NAME groups, the pial arterioles dilated to the same degree in response to hypercapnia (Fig. 4). In neither the control nor the L-NAME-treated groups did acute application of L-NAME affect the dilation. Conversely, in indomethacin-treated piglets, the application of L-NAME significantly decreased the dilatory response to hypercapnia.

The dilatory response to hypercapnia in control piglets, which was inhibited by indomethacin treatment, was restored by subdilatory concentrations of iloprost, a prostacyclin analog (Fig. 5), indicating that the role of PGs in this phenomenon might be permissive.

In indomethacin-treated piglets, L-NAME inhibited the dilation to hypercapnia. This inhibition was reversed by the application of the NO donor SNP in subdilatory concentrations (Fig. 6). Therefore, NO may play a permissive role in the response to hypercapnia. A doubling of the dosage of SNP did not have a greater effect, further indicating that the role of NO is a permissive one.

Iloprost also restored the dilatory response to hypercapnia inhibited by topical administration of L-NAME.
in the indomethacin-treated group (Fig. 6). Application of iloprost and SNP together did not further increase the previously inhibited dilatory effect by L-NAME.

In the control group, the cAMP level in CSF increased significantly in response to hypercapnia, while it remained nearly unchanged in the indomethacin-treated piglets (Fig. 7).

The basal cGMP level was higher in the indomethacin-treated piglets. In contrast to the control piglets, cGMP increased significantly in response to hypercapnia in the indomethacin-treated piglets, and this increase could be inhibited by the topical application of L-NAME (Fig. 7).

Response to ACh. In contrast to the constrictor response to ACh in control and L-NAME-treated piglets, the pial arterioles dilated in response to ACh in indomethacin-treated piglets. Similar to the response to hypercapnia, this dilation could be significantly inhibited by L-NAME administration (Fig. 8).

In indomethacin-treated piglets, the dilatory response to ACh, which was inhibited by topical administration of L-NAME (10^{-3} M), was reversed to constriction by topical U-46619 (0.5 \times 10^{-8} M; Fig. 9). In the control group, the presence of U-46619, a thromboxane receptor agonist, at this concentration had no effect on the already evident constrictor effect of ACh.

In the control and indomethacin-treated groups, the cAMP levels in CSF did not change significantly after topical application of ACh, although the cAMP levels were lower in the indomethacin-treated piglets than in the control group (Fig. 10A).

On the other hand, the basal cGMP level was higher in the indomethacin-treated piglets. Furthermore, cGMP increased significantly in response to ACh in this group (Fig. 10B).

Experimental Details

Hypercapnia was induced by inhalation of 5% CO2/95% O2 for 10 min in control group (Fig. 5A) and 10 min in indomethacin group (Fig. 5B), and SNP (0.5 \times 10^{-3} M) or indomethacin (5 mg/kg iv) or topical L-NAME (10^{-3} M) was applied.

Values are means ± SE. *Statistical significance compared with vehicle, P < 0.05. ‡Statistical significance compared with response after L-NAME in indomethacin group, P < 0.05.
Response to BK. In control piglets, inhibition of NOS with L-NAME did not decrease the dilation of pial arterioles in response to BK, while the administration of indomethacin nearly totally eliminated this dilatory response (Fig. 11). Conversely, in indomethacin-treated piglets, L-NAME significantly inhibited the dilatory response to BK.

In contrast to the dilatory response to hypercapnia, iloprost (0.5 × 10^{-9} and 1 × 10^{-9} M) alone could not restore the dilation to BK in indomethacin-treated piglets after L-NAME treatment (data not shown). A subdilatory concentration of SNP (0.5 × 10^{-7} and 1 × 10^{-7} M), however, did restore it (Fig. 12).

In the control group, cAMP increased significantly in response to BK. This increase could be largely inhibited by L-NAME treatment, while it was not further decreased by indomethacin. cAMP levels, significantly lower in CSF of indomethacin-treated piglets, remained flat in response to BK (Fig. 13A).

The basal level of cGMP was higher in the indomethacin-treated piglets than in the control group. Only in indomethacin-treated piglets did the level of cGMP increase significantly in response to BK. This increase was inhibited by the topical application of L-NAME (Fig. 13B).

Effect of 7-NI. In piglets chronically treated with indomethacin, the application of 7-NI, a specific nNOS inhibitor, did not, in contrast to L-NAME, inhibit the dilatory response of pial arterioles to BK (Fig. 14) or hypercapnia (Fig. 15).

**DISCUSSION**

The present study demonstrates overall that chronic treatment of newborn pigs with the COX inhibitor indomethacin causes transformation of the major dilator in cerebrovascular circulation from PG to NO. First, chronic treatment with indomethacin or L-NAME does not affect eNOS expression in cerebral microvessels, even though both substances affect NOS activity (increase or decrease, respectively). Second, chronic treatment of piglets with indomethacin or L-NAME does not change the pial arteriolar dilatory responses to hypercapnia or BK. Third, although the dilatory responses remain, chronic COX inhibition results in a change in the underlying mechanism from a...
PG-dependent to an NO-dependent mechanism, accompanied by an increased role of NO in the dilatory responses. Fourth, PGs and NO can serve permissive functions in indomethacin-treated piglets. Fifth, chronic treatment with indomethacin results in dilatory responses to ACh along with increases in cGMP, which are not normally present in piglets. Sixth, the increased contribution of NO after chronic treatment with indomethacin cannot be inhibited by 7-NI, suggesting that the source is not nNOS.

PGs, particularly prostacyclin, are dominant EDRFs in the cerebral circulation of newborns of several species, including humans (16). Contributions of PGs to dilator responses to numerous stimuli, including hypercapnia, histamine, and hypotension, have been demonstrated (16). This PG dominance, however, appears to diminish with age, and the role of NO becomes more predominant in adult animals (10, 34, 37, 39).

Although it is recognized that there is “cross talk” between products of the NOS and COX pathways, results are inconsistent with respect to whether the cross talk is stimulatory or inhibitory (5). Nitroglycerin, an NO donor, stimulates PG synthesis in endothelial cells (19), while NO inhibits the production of PGs in chondrocytes and LPS-stimulated macrophages (2). NO may exert divergent effects on constitutive COX-1 and inducible COX-2 (5). We hypothesized that there can be inhibitory effects between the COX and the NOS pathway and that chronically inhibiting one of these systems can enhance the role of the other.

It appears that when one system is inhibited, compensatory mechanisms are rapidly recruited to restore normal response. For example, Irikura et al. (12) demonstrated that, in nNOS knockout mice, alternative mediators could replace the function of NO. In dogs, a compensatory role of PGs in the regulation of coronary circulation was implied after sustained inhibition of NOS (29). Son and Zuckerman (34), using the same animal model used in the present experiment, demonstrated that chronic inhibition of COX did not change the cerebral vasodilator response of piglets to hypercapnia but that the underlying mechanism appeared to be different, with a role for NO appearing. The present study confirms and extends the findings of Son and Zuckerman. We demonstrate that by chronically inhibiting COX, the enzyme required to produce PGs, the pial arteriolar responses to hypercapnia and BK seem...
to shift from requiring PGs to requiring NO, and the classic NO-dependent dilation to ACh became evident.

Chronic inhibition of NOS with L-NAME decreased the NOS activity in the piglets without affecting eNOS expression. However, chronic NOS inhibition was without effect on pial arteriolar responses to hypercapnia, BK, or ACh. This result was not entirely unexpected, because acute L-NAME treatment does not alter pial arteriolar response to these stimuli, suggesting a minimal role of endogenous NO in the intact newborn pig cerebral circulation. Because PGs are playing a permissive role (see below) in the regulation of cerebral circulation in response to the stimuli, even if COX expression actually were enhanced, no change in dilator response to hypercapnia or BK or constriction to ACh would be expected. Whether chronic inhibition of NOS in the adult pig would augment the role of PGs in cerebrovascular control remains to be determined.

It has been shown that the role of prostacyclin underlying the dilatory response to hypercapnia can be permissive (16), which means that prostacyclin, instead of causing the relaxation of smooth muscle cells by directly increasing cAMP, can allow hypercapnia to enhance cAMP to produce dilation. In the present experiments, application of iloprost, a prostacyclin analog, restored not only the indomethacin-inhibited dilatory response in the control piglets, but also the L-NAME-inhibited dilation in the indomethacin-treated piglets. We suggest that the downstream mechanism responsible for the permissive effect of prostacyclin was not lost during chronic treatment. This restoration could not be further enhanced by doubling the dose of iloprost or by administering iloprost + SNP, which further supports the concept that their actions are permissive and alternative.

It is noteworthy that, in the indomethacin-treated piglets, SNP, an NO donor, also can restore the dilatory response of pial arterioles to hypercapnia at a subdilatory concentration. Similar to the case with iloprost, doubling the dosage did not further dilate the arterioles. It has been suggested that NO might be the final mediator of smooth muscle relaxation or can allow another dilatory mediator to act (10). Hence, it appears that whether the actions of dilatory regulators are conventional or permissive depends on the nature, and possibly the strength, of the stimuli as well as the maturity of the animal.

Chronic indomethacin treatment changes the pial arteriolar response to ACh from constriction to dilation. The dilation is inhibited by topical application of L-NAME. Zuckerman et al. (39) demonstrated that the constriction in response to ACh in piglets was PG dependent, while the biphasic (constriction-dilation) response to ACh in juvenile pigs was dependent on PGs for the initial constriction, as in the newborn, but dependent on NO for the prolonged dilation (39). We report that the pial arteriolar response to ACh after indomethacin treatment is more adultlike, showing only dilation and relying solely on NO. Of course, the

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**Fig. 11.** Effect of topical bradykinin (10^{-7} M) on pial arterioles in control (A, n = 9) and indomethacin-treated piglets (B, n = 8). Responses are shown with topical vehicle, L-NAME (10^{-3} M), or L-NAME (10^{-3} M) + indomethacin (5 mg/kg iv). Values are means ± SE. *Statistical significance compared with vehicle, P < 0.05. ‡Statistical significance compared with control group, P < 0.05.

**Fig. 12.** Effect of topical bradykinin (10^{-7} M) on pial arterioles in control (A, n = 9) and indomethacin-treated piglets (B, n = 8). Responses are shown with topical vehicle, L-NAME (10^{-3} M), L-NAME (10^{-3} M) + indomethacin (5 mg/kg iv), SNP (0.5 × 10^{-7} M), and SNP (1 × 10^{-7} M). Values are means ± SE. *Statistical significance compared with vehicle, P < 0.05. †Statistical significance compared with response after L-NAME treatment in indomethacin group, P < 0.05. ‡Statistical significance compared with control group, P < 0.05.
lack of initial constriction is the same as with the acute treatment with indomethacin.

The role of PGs in the newborn's pial arteriolar constriction to ACh is also permissive (3). In the present study, we found that a subconstrictor concentration of U-46619, a thromboxane receptor agonist, can restore the initial constriction to ACh in chronically indomethacin-treated piglets just as in the control piglets. Therefore, the permissive mechanism for constriction to ACh remains in the chronically indomethacin-treated piglet.

A variety of mediators, including oxygen radicals, NO, and endothelium-derived hyperpolarizing factors, have been reported to mediate dilation of blood vessels to BK (15, 23). We found that, in control and indomethacin-treated piglets, pial arterioles dilate in response to BK. The dilator response of control piglets was only mildly inhibited by L-NAME but was nearly abolished by indomethacin. Although NO does not appear to be an important mediator in control piglets, it is much more important for the pial arteriolar response to BK in the indomethacin-treated piglets. The inhibition by L-NAME of the dilator response to BK can be reversed by a subdilatory concentration of SNP, suggesting that NO may play a permissive role here.

It is generally assumed that the vasodilator effects of PGs are mediated by stimulation of adenylyl cyclase, while the dilator effects of NO are mediated by guanylyl cyclase. In the present study, we found that the basal level of cAMP in CSF decreased after chronic indomethacin treatment, while that of cGMP increased. In contrast to the control piglets, the level of cGMP, instead of cAMP, increased in the indomethacin-treated piglets along with the pial arteriolar dilatory responses to hypercapnia and BK. The increase in cGMP could be inhibited by topical application of L-NAME, suggesting an increased activity of NOS after

![Graph A](image1.png)

**Fig. 13.** A: effects of topical bradykinin on cortical production of cAMP. Values are means ± SE (n = 6 piglets). *Statistical significance compared with vehicle, P < 0.05. †Statistical significance compared with bradykinin alone (P < 0.05). ‡Statistical significance compared with control group, P < 0.05.

B: effects of topical bradykinin on cortical production of cGMP. Values are means ± SE (n = 6 piglets). *Statistical significance compared with vehicle, P < 0.05. †Statistical significance compared with cGMP production in response to bradykinin in indomethacin group, P < 0.05. ‡Statistical significance compared with control group, P < 0.05.

![Graph B](image2.png)

**Fig. 14.** Effect of topical bradykinin ($10^{-7}$ M) on pial arterioles in control (A, n = 9) and indomethacin-treated piglets (B, n = 8). Responses were recorded with topical vehicle under the cranial window after L-NAME ($10^{-3}$ M) or 7-nitroindazole (7-NI, 50 mg/kg ip). Values are means ± SE. *Statistical significance compared with vehicle, P < 0.05. †Statistical significance compared with response after L-NAME treatment in indomethacin group, P < 0.05. ‡Statistical significance compared with control group, P < 0.05.

![Graph C](image3.png)

**Fig. 15.** Effect of hypercapnia on pial arterioles in control (A, n = 9) and indomethacin-treated piglets (B, n = 8). Responses were recorded with topical vehicle under the cranial window after L-NAME ($10^{-3}$ M) or 7-NI (50 mg/kg ip). Values are means ± SE. *Statistical significance compared with vehicle, P < 0.05. †Statistical significance compared with response after L-NAME treatment in indomethacin group, P < 0.05. ‡Statistical significance compared with response after L-NAME treatment in indomethacin group, P < 0.05.
the indomethacin treatment. Furthermore, in indomethacin-treated, but not in control, piglets, cGMP was increased by the administration of ACh. This increase, similarly, was eliminated by L-NAME.

The data from the l-arginine-to-l-citrulline conversion experiment clearly demonstrated that NOS activity increased in microvessels of indomethacin-treated piglets and decreased in microvessels of L-NAME-treated piglets. However, we could not detect any changes in eNOS protein levels after treating cultured endothelial cells from newborns with the PG precursor arachidonic acid, the COX-2 inhibitor NS-398, indomethacin, or interleukin-1β. In addition, contrary to our anticipation from vascular responses, the eNOS protein levels also were not different among the microvessels from the three chronic piglet groups.

We considered the possibility that the other constitutive isoform of NOS, nNOS, could contribute to the compensatory enhancement of the role of NO in the indomethacin-treated piglets. In fact, selective nNOS inhibitors reduce the cerebral vasodilator response to hypercapnia in rats (37). Some studies also suggest that nNOS-derived NO acts permissively in mediating this hypercapnic response (24). However, in the present study, 7-NI, the specific nNOS inhibitor, did not affect the pial arteriolar dilatory response to hypercapnia or BK in the indomethacin-treated piglets, suggesting that nNOS probably is not involved. It is unlikely that the dose of 7-NI was insufficient, because it was the same as that used in the adult rat studies cited above (36). In this study, nNOS protein was undetectable in the cerebral microvessels of all three groups using Western blot (data not shown).

Although chronic indomethacin treatment makes the responses of piglets appear more adultlike, significant differences remain between indomethacin-treated piglets and juvenile pigs. First, in contrast to juvenile pigs (37), iloprost restored the dilatory response to hypercapnia that had been inhibited by L-NAME in indomethacin-treated piglets. Second, SNP reversed the decreased dilatory response to hypercapnia and BK caused by L-NAME in indomethacin-treated piglets, but not in juvenile pigs (37). Third, we could not detect any increase in eNOS protein levels in the microvessels of indomethacin-treated piglets, while in juvenile pigs, eNOS expression is much higher than in newborns (25). Finally, in juvenile pigs, the full responses of pial arterioles to hypercapnia and BK require PGs and NO (39).

The mechanism by which the role of NO increases after chronic treatment with indomethacin is not known. We do know that the level of PGs in CSF decreases with the maturation of the animal, so it is possible that the higher level of PGs in the newborn is having sustained inhibitory effects on the NO system. When the PG concentration decreases with maturation, this inhibitory effect decreases, enhancing the activity of the NO system.

In conclusion, the results from this study support the hypothesis that chronically inhibiting COX causes an increased influence of NO in the newborn cerebrovascular circulation. Hence, the inhibition of COX did not change the dilatory response of pial arterioles to hypercapnia or BK but did produce a dilation in response to ACh, which is not present in the untreated newborn pigs. All these dilations can be blocked by L-NAME in chronically indomethacin-treated piglets, suggesting that an enhanced role of NO underlies these effects. Along with the dilatory responses to hypercapnia and BK, CSF cAMP increased in control piglets, while cGMP increased in indomethacin-treated piglets. The NOS activity increased in indomethacin-treated piglets and decreased in L-NAME-treated piglets. On the other hand, eNOS protein levels did not change in the microvessels, and nNOS seems not to be involved, suggesting that the increase or decrease in activity of NOS was independent of protein level. The mechanism responsible for enhancement of the NO system needs further investigation.

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