Developmental Regulation of Endothelial Nitric Oxide Synthase in Cerebral Vessels of Newborn Pig by Prostaglandin E$_2$\textsuperscript{1}

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**ABSTRACT**

We investigated whether prostaglandins regulate endothelial nitric oxide synthase (eNOS) in the pig cerebral vasculature during the neonatal period. Prostaglandins, eNOS mRNA, eNOS protein, and NO production were higher in cerebral microvessels of newborn (1 day old) than in those of adult (6- to 8-month-old) pigs. The treatment of isolated cerebral microvessels of newborn animals with ibuprofen for 24 h reduced eNOS mRNA and nitrite production to levels in the adult; this effect of ibuprofen was prevented by concurrent treatment with prostaglandin (PG)E$_2$ analog 16,16-dimethyl-PGE$_2$, nonspecific PGE$_2$ receptor agonist sulprostone, and prostaglandin EP$_3$ receptor agonists M&B 28,767 and carbaprostacyclin, PGD$_2$, and EP$_1$ receptor agonist 17-phenyl trinor PGE$_2$. Correspondingly, 16,16-dimethyl-PGE$_2$ and M&B 28,767 increased eNOS mRNA expression of adult microvessels to values in the newborn. Data similar to those with isolated cerebral vessels were obtained through histochemical analysis (NADPH-diaphorase positivity) of brain from newborn animals treated in vivo with ibuprofen in combination or not with sulprostone. Furthermore, substance P-induced NO-mediated cerebral vasorelaxation was decreased to adult values through the treatment of newborn pigs with ibuprofen; this effect was prevented by concomitant treatment with sulprostone. It is concluded that PG$_E_2$ regulates eNOS in newborn pig cerebral microvessels via EP$_3$ receptors; this may be physiologically required during normal neurovascular development.

Endothelial nitric oxide synthase (eNOS) plays an important role in the control of cerebral blood flow, metabolism, and, in turn, neuronal activity (Prado et al., 1992; Lo et al., 1996; Ohashi et al., 1998). Mice with a disrupted eNOS gene are hypertensive and exhibit a reduction in cerebral blood flow (Huang et al., 1995), which could favor cerebral ischemia (Prado et al., 1992; Lo et al., 1996). NOS activity is developmentally regulated in cerebral vasculature (Northington et al., 1996, 1997). Increased cerebrovascular levels of NOS activity during the perinatal period could have functional significance during brain development (Bredt and Snyder, 1994; Northington et al., 1996, 1997; Estrada and DeFelipe, 1998). However, the mechanisms regulating the ontogeny of cerebrovascular NOS are not known. A role for prostaglandins in regulating inducible NOS expression in macrophages has been proposed (Gaillard et al., 1992; Raddassi et al., 1993; Kuchiwa et al., 1994; Aeberhard et al., 1995), but this type of regulation has not been reported for constitutive NOS, specifically eNOS, and especially not during development.

There is some evidence that the ontogeny of cerebral prostaglandin synthesis parallels that of NOS (Leffler et al., 1993; Peri et al., 1995; Busija, 1997). Based on evidence presented and because brain microvessels (<150 μm) are major regulators of cerebral circulation (Baumbach and Heistad, 1985), we hypothesize that high levels of prostaglandins regulate eNOS expression and activity in cerebral microvessels. For this purpose, we characterized the expression of eNOS in cerebral microvessels of newborn and adult pigs and determined whether increased prostaglandin levels in the former modulate eNOS expression, activity, and function. Our findings reveal that prostaglandin (PG)E$_2$ positively regulates eNOS expression in the cerebral microvessels of the newborn and that this effect is mediated via the EP$_3$ subtype of PG$_E_2$ receptors.

**ABBREVIATIONS:** eNOS, endothelial nitric oxide synthase; ASA, acetylsalicylic acid; PMSF, phenylmethylsulfonyl fluoride; L-NMMA, L-nitro- monomethyl arginine; PG, prostaglandin.
Materials and Methods

Chemicals. M&B 28,767 was a gift from Rhone-Poulenc Rorer (Dagenham Essex, UK). Carprofen, PGD₂, 16,16-dimethyl-PGE₃, 11-deoxy-PGE₂, 17-phenyl trion PGE₂, sulprostone, and U46619 were obtained from Cayman (Ann Arbor, MI). Polyclonal rabbit antibody specific to eNOS was obtained from Calbiochem-Novabiochem (San Diego, CA). α-[³²P]CTP (3000 Ci/mmol) and enhanced chemiluminescence kit were purchased from Amersham (Mississauga, Ontario, Canada). Pepstatin and leupeptin were purchased from Boehringer Mannheim (Montreal, Quebec, Canada). Ribonuclease A and T₄, sequencing kit were obtained from Pharmacia Biotech (Montreal, Quebec, Canada). pGEM4 plasmid vector and in vitro transcription kit were purchased from Promega (Madison, WI). Protein assay and electrophoretic reagents were purchased from BRL Life Technologies (Burlington, Ontario, Canada). Guanidinium isothiocyanate, T₄ DNA ligase, and restriction enzymes were purchased from Fisher Scientific (Montreal, Quebec, Canada) and Sigma-Aldrich (Oakville, Ontario, Canada).

Animals. Newborn (1-day-old) Yorkshire pigs were used according to a protocol of the Hôpital Sainte-Justine Animal Care Committee. Animals were anesthetized with 2% halothane and then sacrificed with 120 mg/kg intracardiac pentobarbital, and the brains were removed. Brains from adult (6- to 8-month-old) pigs were collected from an abattoir immediately after sacrifice and transported on ice to the laboratory.

Preparation of Microvessels. Cerebral microvessels were prepared as described previously (Li et al., 1994). To compare eNOS mRNA and immunoreactivity, as well as nitrite production in newborn and adult microvessels, brains were gently homogenized with a Wheaton pestle in 5 mM Tris-HCl buffer (pH 7.4) containing 1.1 mM acetylthioleucyl acid (ASA), 0.5 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μM soybean trypsin inhibitor; for all other experiments that involved the effects of modulation of prostaglandin levels on eNOS expression, ASA was omitted from the buffer for the microvessel preparations. ASA was added to avoid potential effects of prostaglandins on eNOS activity (Sasz, 1969; Golstein et al., 1975). The activity of γ-glutamyl transpeptidase was greater in the microvessels (5.6–5.9 mU/mg protein) than in brain parenchyma (0.30–0.35 mU/mg protein).

Incubation of Microvessels. Cerebral microvessels of newborn pigs were incubated for 24 h in Dulbecco’s modified Eagle’s medium in the absence or presence of 10 μM ibuprofen or 10 μM ibuprofen plus 1 μM concentration of one of the following agents: carbaprostacyclin (stable PGL₂ analog), PGD₂, 16,16-dimethyl-PGE₃ (stable PGE₂ analog), 11-deoxy-PGE₂, 17-phenyl trion PGE₂, sulprostone, or M&B 28,767. Microvessels of adult pigs were similarly treated with 16,16-dimethyl-PGE₂ and M&B 28,767. Concentrations of agents used have been previously reported to be effective (Kennedy and Doktorick, 1988; Ando et al., 1995; Li et al., 1995). After incubation, eNOS mRNA and nitrite production were determined.

In Vivo Experiments. Newborn pigs were anesthetized with 2% halothane, and polyethylene catheters were placed in the jugular vein for i.v. injection. Animals were randomly assigned to receive every 8 h for 24 h i.v., saline, ibuprofen (40 mg/kg), or ibuprofen plus EP₂ agonist sulprostone (10 μg/kg; Coleman et al., 1994). Animals were sacrificed at the end of the 24-h period, and the brain was immediately removed for NADPH-diaphorase staining and vasomotor experiments as described below; experiments were conducted on frontoparietal cortex because of its neuronal organization and segregated vasculature.

eNOS and Destrin Ribonuclease Protection Assays. The primer pair for porcine eNOS synthesized by RT-PCR was 5'-GGTTTTCCCTCAGAGGAGCAGC-3' and 5'-GCCAGTCTCAGCTCTTG-3' (Zhang et al., 1996). The primer pair for (loading control) destrin was 5'-ATGATGAACTTTGAAAC-3' and 5'-GGAGGCATGCTCTGTG-3'. The amplified products (0.4 kb) were digested with appropriate restriction enzyme (underlined sequences in the primers denote the restriction sites) and cloned into pGEM4 vector. The nucleotide sequences of eNOS and destrin partial cDNAs were determined using a T7 sequencing kit. The 32P-labeled cRNA probes for eNOS and destrin were prepared with the use of an in vitro transcription kit (Promega).

Total RNAs from brain microvessels was aliquoted and subjected to ribonuclease protection assays according to a published protocol (Bordonaro et al., 1994) with minor modifications. Briefly, 20 μg of total RNA was mixed with 105 cpm of eNOS and destrin probes in 20 μl of hybridization buffer [80% deionized formamide, 40 mM piperezine-N,N'-bis(2-ethanesulfonic acid), pH 6.8, 1 mM EDTA, and 0.4 M NaCl], denatured at 90°C for 5 min, and incubated overnight at 50°C. The RNA hybrids were digested with ribonuclease A (10 μg/ml) and ribonuclease T₁ (200 U/ml) in 200 μl of digestion buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 0.3 M NaCl) for 30 min at 25°C. Proteinase K treatment followed by precipitation of protected fragments was conducted exactly as described (Bordonaro et al., 1994). The protected RNA fragments were resolved on urea-6% polyacrylamide gels, and the hands were visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA) and quantified by densitometry.

Western Blotting. Western blotting for eNOS was performed using a method described previously (Abran et al., 1997). Cerebral microvessels were homogenized in a buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 137 mM NaCl, 1% Nonidet P-40, 10 μg/ml each of leupeptin, pepstatin, and soybean trypsin inhibitor, and 0.2 mM PMSF. After centrifugation at 13,000g for 10 min to remove fibrous material, the supernatants were denatured by boiling for 5 min and resolved by electrophoresis on 8% SDS-polyacrylamide gels. The transfer of tissue lysate proteins to membranes and immunoblotting using specific antibodies against eNOS (1:1000 dilution) and NADPH-diaphorase histochemistry were conducted exactly as described previously (Abran et al., 1997). Immunoreactive bands were visualized by chemiluminescence (Amersham) as recommended by the supplier.

Nitrite Production. NO formation was estimated by determination of its stable metabolite, nitrite (Verdon et al., 1995), as reported previously (Abran et al., 1997). NOS-dependent generation of NO was estimated as the difference in nitrite production in the absence or presence of 1 mM N⁵-nitro-L-arginine.

NADPH-Diaphorase Histochemistry. NADPH-diaphorase reactivity was performed using a method described previously (Kuchiiwa et al., 1994). Tissue from frontoparietal cortex was fixed by immersion in 4% paraformaldehyde in 0.1% PBS (pH 7.4) overnight at 4°C and then placed in 30% sucrose buffer for 2 days. Tissues were sectioned (40 μm) using a microtome. The free-floating sections were incubated in 0.1 M phosphate buffer (pH 7.4) containing 0.1% β-NADPH at 37°C for 60 min. After the reaction, the sections were rinsed in phosphate buffer and mounted on slides. The slides were air dried, treated in chloroform for 30 min to remove background staining, and counterstained with neutral red. Densitometry of tonality was analyzed after normalization adjustment of background tone; tonality was determined on similar number of
pixels delineating microvessels (which differed by ≤1% between photographs) using software program Photoshop 5 (Adobe).

Vasomotor Response of Brain Microvessels. Newborn pigs treated with ibuprofen in combination or not with sulprostone were prepared as described above; adult animals were not treated (tissues obtained from abattoir). Brains from these animals were sectioned (1 mm thick) to study vascular responses using videomaging techniques as reported previously (Li et al., 1997). Cumulative concentration-relaxant response curves to NO-dependent substance P (Jansen et al., 1991; Rosenblum et al., 1993) were determined on tissues precontracted with U46619 to approximately 75% of maximum contraction; relaxant response was calculated as percent decrease in the induced-tone.

Statistical Analysis. Data were analyzed by ANOVA, comparison among mean values test (Tukey-Kramer method), and Student’s t test. Statistical significance was set at \( p < .05 \). Data are presented as mean ± S.E.

Results

Prostaglandin Level, eNOS Expression, and Nitrite Generation in Newborn and Adult Brain Microvessels. PGE\(_2\) levels were approximately 6-fold higher in newborn...
than in adult cerebral microvessels (Fig. 1A). This was associated with 3- to 5-fold greater eNOS mRNA, immunoreactive protein, and nitrite production in newborn compared with adult (Fig. 1, B–F).

Modulation of eNOS mRNA and Nitrite Production on Isolated Microvessels by Prostaglandins. Incubation of cerebral microvessels from newborn pigs with ibuprofen for 24 h (but not acute, ≤2 h) caused a significant reduction in the expression of eNOS mRNA and nitrite production (Fig. 2, A–C). Effects of ibuprofen were prevented by concurrent treatment with 16,16-dimethyl-PGE₂ but not with other major prostaglandins, carbaprostacyclin, and PGD₂.

Fig. 3. Modulation of eNOS mRNA expression in pig cerebral microvessels by PGE₂ analogs. Isolated newborn cerebral microvessels were incubated 24 h with saline, ibuprofen (10 μM), or a combination of ibuprofen with 1 μM concentration of one of the following: 16,16-dimethyl-PGE₂, 11-deoxy-PGE₂, 17-phenyl trinor PGE₂, sulprostone (Sulp), or M&B 28,767. Isolated cerebral microvessels of adult pig were treated with saline, 16,16 PGE₂, or M&B 28,767. Values are mean ± S.E. of three or four experiments. *, Different (p < 0.01) from values without an asterisk.
Effects of PGE$_2$ Receptor Agonists on eNOS mRNA on Isolated Microvessels. Incubation of newborn pig cerebral microvessels with 11-deoxy-PGE$_1$ (EP$_2$/EP$_3$/EP$_4$ agonist), EP$_1$/EP$_3$ agonist sulprostone, or selective EP$_3$ receptor agonist M&B 28,767 prevented ibuprofen-induced decrease in eNOS mRNA (Fig. 3, A and B); the effects of M&B 28,767 on eNOS mRNA expression were concentration dependent (Fig. 3C). In contrast, 17-phenyl trinor PGE$_2$ (EP$_1$ agonist), even at relatively high concentrations (1 µM), did not modify the effect of ibuprofen on eNOS mRNA. Furthermore, treatment of adult cerebral microvessels with 16,16-dimethyl-PGE$_2$ and M&B 28,767 increased eNOS mRNA values to those in saline-treated newborns (Fig. 3, A and B).

In Vivo Modulation of Brain Tissue NADPH Diaphorase Staining by Prostaglandins. To determine whether findings ex vivo also applied in vivo, newborn pigs were treated with ibuprofen with or without sulprostone; ibuprofen reduced PGE$_2$ levels in brain cortex from $201 \pm 54$ to $87 \pm 14$ pg/mg protein, comparable to levels in adult brain. In ibuprofen-treated animals, there was a decrease in overall NADPH-diaphorase staining of brain microvessels (as well as neurons), which was prevented by sulprostone (Fig. 4); these changes can be further appreciated by tonality densitometry of microvessels (Fig. 4). Hematoxylin and eosin staining revealed no adverse effects of prostaglandin modulation on cell number (data not shown) as reported previously (Patel et al., 1993).

Effects of Prostaglandin Modulation on NO-Dependent Vasorelaxant Response of Brain Vasculature. To assess whether modulation of eNOS on vessels by prostaglandins was reflected functionally, newborn pigs were treated with ibuprofen with or without sulprostone, and the vasomotor effects of NO-dependent substance P (Jansen et al., 1991; Rosenblum et al., 1993) were tested. The treatment of newborn pigs with ibuprofen decreased vasorelaxant response to substance P (Fig. 5); this effect was prevented by concomitant treatment with sulprostone. Correspondingly, in adult animals, substance P caused less vasorelaxation than that seen in saline-treated newborns and approached dilatation of ibuprofen (alone)-treated newborns. Vasorelaxation to substance P was inhibited by the NOS blocker L-nitro-monomethyl arginine (l-NMMA; Fig. 5).

Discussion

NO has been attributed important roles in the regulation of cerebral hemodynamics (Prado et al., 1992; Lo et al., 1996), particularly in the newborn when the metabolic demand is high (Northington et al., 1996, 1997). Similar functions and ontogenic changes have been observed for prostaglandins (Leffler et al., 1993; Peri et al., 1995; Busija, 1997). Thus far, the factors that regulate cerebrovascular eNOS expression in the perinate were not known. Based on evidence that prostaglandins can regulate NOS activity in macrophages (mostly iNOS), we investigated whether these prostanoids contribute to the perinatal regulation of eNOS expression and activity in cerebral vasculature. Our findings reveal that through actions on EP$_3$ receptors, high levels of PGE$_2$ in cerebrovascular tissues of the perinate regulate eNOS expression and NO generation in brain microvessels, and this in turn affects vasomotor responses.

The ability of ibuprofen (which reduced prostaglandin levels) to decrease eNOS expression and NO formation in the newborn cerebral microvessels suggests that prostaglandins regulate eNOS; because this effect of ibuprofen could be prevented by PGE$_2$ and not by other major prostanoids (Fig. 4).

Fig. 4. In vivo modulation of NADPH-diaphorase staining by prostaglandins. Newborn animals were treated every 8 h for 24 h with saline, ibuprofen (40 mg/kg), or a combination of ibuprofen plus sulprostone (Ibu and Sulp, respectively; 10 µg/kg). At the end of treatment period, animals were sacrificed, and the brain was stained for NADPH-diaphorase. Tonality densitometry was analyzed as described in the text ($n = 3$ for each treatment); note that higher arbitrary tonality units correspond to reduced densitometry, and vice versa for lower units. Large arrows point to individual NADPH-diaphorase-positive vessels, and small arrows point to neurons. 1 cm represents 200 µm. * Different ($p < .05$) from values without an asterisk.
EP3 PGE2 may be involved in the concerted regulation of NADPH-diaphorase positivity, could suggest that via prostaglandins. The spatial relationship between cerebral vessels and newborn, respectively. A, NB-Saline; B, NB-Ibu; C, NB-L-NMMA; D, Adult-L-NMMA. Values are mean ± S.E. of four experiments each, different (p < .01) from adult and ibuprofen (alone)-treated newborns and from L-NMMA-treated preparations (two-way ANOVA factoring for substance P concentration and group).

2), it seems that PGE2 is the major prostaglandin playing a critical role in eNOS regulation (Fig. 2). This regulation of eNOS expression in the newborn cerebral microvessels by PGE2 seems to be mediated via EP3 receptor because EP3 receptor agonists sulprostone and M&B 28,767 prevented the effect of ibuprofen as did PGE2 (Figs. 2 and 3). As a corollary to observations made in the newborn, PGE2 and M&B 28,767 increased eNOS expression in adult microvessels to values in the newborn (Fig. 3). Moreover, the regulation of eNOS expression by EP3 stimulation both ex vivo and in vivo was also manifested functionally by studying the vasorelaxant response to NO-dependent substance P (present study; Jansen et al., 1998). A vasorelaxation was diminished by treatment (24 h) of newborn pigs with ibuprofen to values seen in the adult (Fig. 5) and could be prevented by concomitant treatment with ibuprofen plus sulprostone. It is of interest to point out in this regard that EP3 receptors were among the two PGE2 receptors detected in porcine cerebral microvessels, with the other one being EP1 (Li et al., 1994); EP2 and EP4 are not detectable in porcine brain microvessels (Li et al., 1994). Altogether, findings suggest that via EP3 PGE2 positively regulates NOS expression as well as NO-dependent functions in cerebral vessels.

NADPH-diaphorase staining of cerebral vessels in the cerebral cortex was modulated by prostaglandins (Fig. 4). Interestingly, a majority of the NADPH-diaphorase positive cerebral vessels were found near neurons, which also exhibited strong NADPH staining and were also affected by prostaglandins. The spatial relationship between cerebral vessels and neurons, together with the fact that prostaglandins modulate NADPH-diaphorase positivity, could suggest that via EP3 PGE2 may be involved in the concerted regulation of cerebral blood flow and neuronal development; this inference has been proposed for nNOS (Dumont et al., 1998).

The mechanism by which PGE2 induces eNOS expression is not clear; however, certain inferences can be drawn. EP3 stimulation may lead to the activation of protein kinases (Burkey and Regan, 1995); eNOS promoter contains a site for activator protein-1 that could be stimulated by phosphorylation (Barchowsky et al., 1995). Alternatively, we recently reported the activation directly of functional perinuclear PGE2 receptors that can induce gene transcription (Bhattacharya et al., 1998). In support of this suggestion, inhibition of prostaglandin transporter using bromocresol green (Kanai et al., 1995) prevented PGE2-induced up-regulation of eNOS expression (data not shown).

In conclusion, our results reveal an important mechanism for the developmental regulation of eNOS by PGE2 through its action on EP3 receptors in cerebral microvessels. Because parturition is associated with significant increases in PGE2 levels in brain (Jones et al., 1993), we speculate that PGE2 is developmentally required in the perinate to maintain via increased NO formation adequate brain circulation, particularly at the end of labor, when fetal blood oxygen tension decreases (Dildy et al., 1994).

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References


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