Regulation of endothelial nitric oxide synthase by PGD$_2$ in the developing choroid

Isabelle Dumont, Pierre Hardy, Krishna G. Peri, Xin Hou, Stéphane Molotchnikoff, Daya R. Varma, and Sylvain Chemtob.

The choroid is a vascular tissue that provides the principal supply of O$_2$ and nutrients to the retina (4). Despite the lower tissue O$_2$ consumption of the perinate, its choroidal blood flow (ChBF) is relatively high compared with that in the adult, partly to compensate for the developing retinal vascular bed (19). Choroidal vasculature of the newborn, in contrast to that of the adult, also fails to constrict appropriately in response to augmented O$_2$ and perfusion pressure (18, 19, 24). This failure to adequately control O$_2$ delivery to the eye of the newborn could favor O$_2$ toxicity (28) and has also been suggested to contribute to predisposing to retinopathy of prematurity (19, 20, 28). The relatively increased basal ChBF and lack of the latter to exhibit O$_2$- and pressure-induced autoregulation in the newborn largely results from excess endothelial (e) nitric oxide synthase (NOS) activity, which generates higher levels of the vasorelaxant nitric oxide (NO; see Refs. 18 and 19). The mechanisms that regulate the ontogeny of NOS activity, particularly in the choroid, are not yet known.

In the developing subject, prostaglandins and NO seem to exhibit a comparable regulation of ChBF (10, 18). As seen with NO, prostaglandin formation is also increased in perinatal ocular tissues (1, 17). A role for prostaglandins in the regulation of inducible (i) NOS expression has been reported (3, 12, 27, 30). However, whether prostaglandins regulate the expression of the constitutive eNOS, specifically in the developing choroid, and the type of prostaglandin involved in this regulatory process are not known.

We therefore determined whether and which type of prostaglandin modulates eNOS expression, activity, and function in the choroid of newborn and juvenile pigs. Our data reveal that, specifically, PGD$_2$ regulates the expression of eNOS in the developing choroid, which in turn affects vasomotor tone and ChBF auto-regulation. These observations disclose a new regulatory mechanism of eNOS expression via a novel function for PGD$_2$ via its receptor (DP).

MATERIALS AND METHODS

Animals. Newborn (1-day-old) and juvenile (6- to 8-wk-old) Yorkshire pigs were used according to a protocol approved by the Animal Care Committee of Hôpital Sainte-Justine in accordance with the principles of the Guide to the Care and Use of Experimental Animals and guidelines of the Canadian
Council on Animal Care. For in vitro experiments, anesthetized (2% halothane) pigs were killed with pentobarbital sodium (120 mg/kg intracardiac), and the eyes were quickly removed and placed in ice-cold Krebs buffer (pH 7.4) of the following composition (mmol/l): 120 NaCl, 4.5 KCl, 2.5 CaCl₂, 1.0 MgSO₄, 27 NaHCO₃, 1.0 K-HPO₄, and 11 glucose, to which was added 1.5 U/ml heparin. On choroids from these eyes, eNOS mRNA, immunoreactivity, and activity, and nitrite production and prostaglandin levels were measured. Other choroids were first incubated with modulators of prostaglandin levels described below.

In vitro incubation of choroids. Isolated newborn choroids were incubated for 24 h in DMEM culture medium in the presence or absence of the prostaglandin synthase inhibitor ibuprofen (10 µM), a combination of ibuprofen (10 µM) and either PGD₂, 16,16-dimethyl-PGE₂, or carboxaprostacyclin (stable PGI₂ analog; all at 1 µM), or only with the selective DP antagonist BW A868C (1 µM; see Refs. 8 and 13). Choroids of juveniles were similarly treated with PGD₂ or a combination of PGD₂ with BW A868C. The 24-h treatment duration was based on pilot experiments which revealed that acute (<2 h) administration of those agents were ineffective in altering eNOS expression. At the end of the incubation, tissues were preincubated for 30 min; the presence or absence of the prostaglandin was determined as the L-NA (1 mM)-sensitive production of L-[³H]citrulline from L-[^³H]arginine as previously described (1); constitutive Ca²⁺-dependent NOS activity (largely eNOS in choroid; see Ref. 1) was determined after subtraction of Ca²⁺-independent iNOS activity (in presence of 0.5 mM EGTA) from total NOS activity.

Measurement of nitrite production. Nitrite production in isolated choroids was performed as previously reported (1). NOS-dependent formation of NO was estimated as the difference in nitrite production in the absence or presence of N-nitro-L-arginine (L-NA; 1 mM).

Chorioallantoic vascular responses. Choroidal vasomotor responses were assessed in separate experiments in vivo, in which choroidal blood flow was measured. Isolated newborn choroids were first incubated with modulators of prostaglandin levels described above. The choroid was perfused using a pulsatile minipump (Gilmont, Graduate School of Medicine, University of California, San Francisco) with Krebs buffer at physiological (19) constant flow rates of ~0.20 ml/min in the newborn and at ~0.57 ml/min in juveniles to produce a perfusion pressure of 60 and 67 mmHg (10, 19), respectively. Perfusion pressure was continuously recorded using a pressure transducer (Perceptor DT) connected immediately afferent to the choroid; accordingly, a decrease in perfusion pressure reflects vasoconstriction and an increase reflects vasodilation. After stabilization of the preparation (~30 min), U-46619 (0.1 µM) was added to the perfusate to evoke constriction; thereafter, cumulative concentrations (10⁻¹² to 10⁻⁵ M) of ACh, Bk, or SP were added to the perfusate in some tissues, the perfusate contained L-NAME (1 mM). Relaxation was calculated as the percent reversal of U-46619-induced constriction, which was ~75–85% of maximal U-46619-evoked constriction in both newborn and juvenile preparations; constriction to U-46619 is unaffected by ibuprofen (2). To ascertain that NO-dependent vasorelaxants produced a similar comparative profile of action on newborn and juvenile preparations, effects of ACh were tested on tissues preconstricted with 8 µM phorbol 12-myristate 13-acetate (non-receptor mediated), which exhibits similar (80% of maximum) constriction in choroids of newborns and juveniles (2); results were comparable to those with U-46619.

Measurement of ChBF. Animals were prepared to measure ChBF using the radiolabeled microsphere technique exactly as described in detail elsewhere (10, 16, 18, 19). ChBF as a function of changes in perfusion pressure was studied as previously described (17, 18). Increased ocular perfusion pressure [OPP; mean blood pressure (MBP)–intraocular pressure (IOP)] was produced by inflating a balloon-tipped catheter placed in the distal thoracic descending aorta through a femoral artery. Each animal was subjected to stepwise acute increases in mean blood pressure (MBP)
OPP preset at 90, 105, and 125 mmHg. These values varied by <5 mmHg on different animals; baseline MBP was 68 ± 5 mmHg for all animals and was unaffected by treatments. Once MBP remained steady (within 30 s after balloon inflation), ~10⁶ microspheres (15 µm diameter) labeled with ^{141}Ce, ^{113}Sn, and ^{85}Sr (NEN, Boston, MA) were injected in a random sequence into the catheterized left ventricle. Reference samples were appropriately collected over the following 70 s. After the experiment, pigs were killed (120 mg/kg pentobarbital sodium). Radioactivity in the choroid and the reference blood samples was counted in a gamma scintillation counter (Cobra II; Canberra Packard, Meridien, CT), and blood flow was calculated using an on-line computer program (PCG-ERDA).

Statistical analysis. Data were analyzed by ANOVA, comparison among means test (Tukey-Kramer method), and Student's t-test. ChBF was analyzed by regression analysis as previously described (17, 18). The Pearson's product moment coefficient (r) was calculated. Linear regressions were compared by the regression equality test using the method of least squares. Data are presented as means ± SE. Statistical significance was set at P < 0.05.

RESULTS

eNOS expression and activity in choroid of newborn and juvenile pig. Prostaglandin levels were four- to sixfold higher in newborn than juvenile choroid (Fig. 1A). This was associated with three- to fivefold greater eNOS mRNA, immunoreactive protein and activity, and nitrite production in newborn compared with juvenile tissue (Fig. 1, B-F); >90% of NOS activity was Ca²⁺ dependent (constitutive), and, as we reported, nNOS was not detectable using selective nNOS blockers and by immunoreactivity, confirming dominance of eNOS in this vascular tissue (1, 19).

In vitro modulation of eNOS mRNA and nitrite production by prostaglandins in the choroid. Incubation of isolated newborn choroid with ibuprofen (10 µM) for 24 h (but not ≤2 h) caused a significant reduction in the expression of eNOS mRNA and in nitrite production to levels observed in the juvenile (Fig. 2, A-C). Effects of ibuprofen were prevented by cotreatment with PDG, but were unaltered by stable analogs of other major prostaglandins, 16,16-dimethyl-PGE₂ and carbaprostacyclin, at similarly increased doses. Furthermore, the selective DP antagonist BW A868C decreased eNOS mRNA and nitrite production to levels found in saline-treated juvenile and ibuprofen-treated newborn choroids (Fig. 2, A-C). Moreover, in choroids of juveniles, PDG2, but not other prostaglandins, increased nitrite production and eNOS mRNA, and this effect was prevented by cotreatment of PDG2 with BW A868C to levels found in ibuprofen-treated newborn choroids.

In vivo modulation of NOS activity in newborn choroid. We examined whether in vitro effects of PGD₂ on eNOS mRNA and nitrite production are reflected more specifically on Ca²⁺-dependent NOS activity in vivo in the newborn. Treatment of neonatal pigs for 24 h with ibuprofen reduced PGE₂, 6-keto-PGF₁α, and PGD₂ levels in choroid, respectively, to 1,408 ± 351, 202 ± 51, and 62 ± 10 μg/g protein (Fig. 1, A-B). This was associated with three- to fivefold greater eNOS mRNA, and nitrite production was prevented by cotreatment of PGD₂ with BW A868C to levels found in ibuprofen-treated newborn choroids (Fig. 2, A-C).
704 ± 106, and 56 ± 11 pg/mg protein from those in saline-treated newborns (see Fig. 1A). This decrease in prostaglandin levels was associated with a decrement in NOS activity to levels found in the juvenile (Fig. 3). This reduction in NOS activity was prevented by cotreatment with PGD₂ but not with 16,16-dimethyl-PGE₂ or carbaprostacyclin. Once again, the selective DP blocker BW A868C reduced NOS activity to values in the juvenile and the ibuprofen-treated newborn.

Choroidal vasomotor responses. To determine if this upregulation of eNOS expression and activity by PGD₂ is manifested physiologically in the control of the choroidal vasomotor response, we tested if NO-dependent vasorelaxation was affected by modulation of eNOS expression. ACh, Bk, and SP caused NO-dependent vasorelaxation as it was inhibited by L-NMMA (Fig. 4). Treatment of newborns with ibuprofen decreased vasorelaxation to ACh, Bk, and SP to values in juveniles (Fig. 4); this effect was prevented by (24 h but not ≤2 h) cotreatment with PGD₂, consistent with increased PGD₂-dependent NOS activity (Figs. 2 and 3). Correspondingly, juvenile animals treated (24 h) with PGD₂ exhibited increased vasorelaxation to ACh, Bk, and SP, as seen in saline-treated newborns. Infusion of L-NMMA in choroids of animals treated with PGD₂ reversed the augmented vasorelaxation to ACh, Bk, and SP (Fig. 4).

ChBF autoregulation. Because failure of the newborn to autoregulate ChBF is largely due to increased NO formation (18, 19), we tested if modulation of NOS by prostaglandins affected, in turn, ChBF autoregulation; experiments were not conducted in juveniles because other factors such as increased efficacy of vasoconstrictors participate in the complex autoregula-
tery control of the older subjects (25). Basal ChBF was 32 ± 6 and 29 ± 6 ml·min⁻¹·g⁻¹, respectively in newborn and juvenile saline-treated pigs; blood gases, heart rate, and IOP remained stable throughout experiments. In saline-treated newborn pigs, in contrast to juveniles (r = 0.13–0.22, P > 0.3; Fig. 5F), ChBF increased linearly as a function of OPP over the entire range of OPP studied (r = 0.82–0.96, P < 0.01; Fig. 5A), whereas treatment of newborns with ibuprofen or BW A868C (24 h) led ChBF to be maintained constant as a function of OPP (r = 0.07–0.31, P > 0.4; Fig. 5, B and D). Coadministration of PGD₂ with ibuprofen caused ChBF to increase linearly with OPP as seen in saline-treated newborns (r = 0.71–0.99, P < 0.05; Fig. 5C); addition of l-NMMA reduced basal ChBF to 16 ± 3 ml·min⁻¹·g⁻¹, increased MBP from 64 ± 4 to 83 ± 5 mmHg as expected (19), and caused ChBF to remain stable as a function of OPP (r = 0.10–0.30, P > 0.3; Fig. 5E). Regression coefficients for newborn pigs treated with saline or ibuprofen plus PGD₂ differed significantly from juveniles and from newborns treated with ibuprofen, BW A868C, or ibuprofen plus PGD₂ plus l-NMMA (P < 0.05, by regression equality test).

**DISCUSSION**

Increased NOS activity in the newborn choroid exerts important functions by maintaining adequate ocular circulation during the development of the retinal vascular bed (18, 19, 21). However, as a result of this increased NO formation, the ChBF autoregulatory response to increased O₂ and perfusion pressure is absent in the perinate (18, 19). The mechanisms that regulate NOS expression and activity in choroid during development are not known. Prostaglandin levels in choroid are also increased in the neonate, and these have equally been found to curtail ChBF autoregulation (1, 2, 10, 21). Prostaglandins, primarily PGE₂, have been reported to regulate iNOS expression (3, 12, 27, 30). We therefore investigated if and which type of prostaglandins might govern the expression, specifically of eNOS in the developing choroid. Our findings reveal that high levels of PGD₂ through its actions on DP regulate eNOS expression and activity in the choroid of the neonate, and this in turn affects choroidal vasomotor regulation.

Evidence that high levels of prostaglandins, specifically PGD₂, modulate eNOS expression in the newborn choroid is based on the following observations. 1) Reduction in prostaglandin levels of the newborn by ibuprofen [sustained (24 h), but not acute] to levels in the juvenile caused a decrease in eNOS mRNA, protein, and NOS activity (Figs. 2 and 3) to values in the older...
This modulation of eNOS by PGD₂ was observed in vitro; prostaglandins (even at high concentrations, 1 µM) inhibit eNOS expression by PGD₂ in the choroid is more pronounced in juveniles (which have low prostaglandin and NO formation); our observations supported this inference (Fig. 2). One may suggest that the reported role of DP stimulation is mostly coupled to cAMP formation (11), a cAMP response element is not present on the eNOS promoter (32, 36), albeit the latter does contain a site for activator protein-1, which may be activated by cAMP-dependent protein kinase A-induced phosphorylation (6). Alternate possibilities include the activation directly of functional perinuclear prostanoid receptors, which have been shown to induce gene transcription (7). In support of this suggestion, inhibition of the prostaglandin transporter using bromcresol green (22) prevented PGD₂-induced upregulation of eNOS expression in the choroid (unpublished observation).

In conclusion, our results reveal an important mechanism for the developmental regulation of eNOS by PGD₂ in the choroid, which in turn confers a major role on vasomotor tone. The findings disclose a new mechanism for the regulation of eNOS expression, namely by PGD₂ via DP. This relationship between PGD₂ and eNOS provides an explanation for the interactive role of these two factors in curtailed ChBF autoregulation in the newborn (10, 18, 19). The findings may also have implications for understanding of retinal hyperoxygenation (10, 19), a predisposition to retinopathy of prematurity.

We are grateful to Hendrika Fernandez for technical assistance.

This work was supported by grants from the Medical Research Council of Canada, the Heart and Stroke Foundation of Québec, the Hospital for Sick Children Foundation, the March of Dimes Birth Defects Foundation, the United Cerebral Palsy Foundation, the Fonds pour la Formation de Chercheurs et l’Aide à la Recherche, and the Fonds de la Recherche en Santé du Québec. I. Dumont is a recipient of a studentship from the Ministry of Indian and Northern Affairs, Canada, and P. Hardy of a fellowship award from the Medical Research Council of Canada.

Address for reprint requests and other correspondence: S. Chemtob, Research Center, Hôpital Sainte-Justine, Depts. of Pediatrics, Ophthalmology, and Pharmacology, 3175, Côte Sainte-Catherine, Montreal, Quebec, Canada, H3T 1C5 (E-mail: chemtobs@ere.umontreal.ca).

Received 19 April 1999; accepted in final form 11 August 1999.

REFERENCES
