*Am. J. Physiol. Heart Circ. Physiol.*
278: H60–H66, 2000.

# Regulation of endothelial nitric oxide synthase by $PGD_2$ in the developing choroid

ISABELLE DUMONT,[1,2] PIERRE HARDY,[1] KRISHNA G. PERI,[1] XIN HOU,[1] STÉPHANE MOLOTCHNIKOFF,[2] DAYA R. VARMA,[3] AND SYLVAIN CHEMTOB[1,3]
[1]*Departments of Pediatrics, Ophthalmology, and Pharmacology, Research Center of Hôpital Sainte-Justine, Montreal H3T 1C5;* [2]*Faculty of Biological Sciences, University of Montreal, Montreal H3C 3J7; and* [3]*Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada H3G 1Y6*

**Dumont, Isabelle, Pierre Hardy, Krishna G. Peri, Xin Hou, Stéphane Molotchnikoff, Daya R. Varma, and Sylvain Chemtob.** Regulation of endothelial nitric oxide synthase by $PGD_2$ in the developing choroid. *Am. J. Physiol. Heart Circ. Physiol.* 278: H60–H66, 2000.—We investigated if prostaglandins might regulate the increased choroidal endothelial (e) nitric oxide synthase (NOS) expression in the perinate. Prostaglandins, eNOS mRNA, immunoreactive protein and activity, and nitrite [stable metabolite of nitric oxide (NO)] production were markedly higher in newborn (1 day old) than juvenile (6–8 wk old) pig choroid. Treatment of isolated newborn choroids with the prostaglandin synthase inhibitor ibuprofen for 24 h reduced eNOS mRNA and nitrite production to values in juveniles. This effect was equally observed with the $PGD_2$ receptor (DP) blocker BW A868C and was prevented by cotreatment with $PGD_2$ but not other prostaglandins; similar observations were made on NOS activity in vivo. $PGD_2$ also increased eNOS expression on choroids of juveniles, and this effect was blocked by BW A868C. The manifestation of this upregulation of eNOS by $PGD_2$ on the control of choroidal vasomotor response was tested by using NO-dependent vasorelaxants, ACh, bradykinin (Bk), and substance P (SP). ACh-, Bk-, and SP-elicited choroidal vasorelaxation was greater in saline-treated newborn than juvenile pigs. Ibuprofen (24 h) decreased ACh-, Bk-, and SP-evoked vasorelaxation in newborns, whereas $PGD_2$ increased that in juveniles and prevented the ibuprofen-induced attenuated relaxation in newborns; infusion of $N^{\omega}$-monomethyl-L-arginine in choroids of those animals treated with $PGD_2$ reversed the augmented vasorelaxation to ACh, Bk, and SP. Finally, $PGD_2$-induced upregulation of NOS in the perinate was also reflected by curtailed choroidal blood flow autoregulatory response to increased perfusion pressure. In conclusion, $PGD_2$ exhibits a major role in upregulating eNOS expression and activity in the choroid, which in turn results in greater NO-mediated vasorelaxation; a new mechanism for eNOS regulation via DP is hereby disclosed. The relationship between $PGD_2$ and eNOS in the developing subject provides an explanation for the interactive role of these two factors in the absent choroidal blood flow autoregulation in the perinate.

endothelial nitric oxide synthase; newborn

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 autoregulation. 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

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

  

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 $CaCl_2$, 1.0 $MgSO_4$, 27 $NaHCO_3$, 1.0 $KH_2PO_4$, 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 $PGD_2$, 16,16-dimethyl-$PGE_2$, or carbaprostacyclin (stable $PGI_2$ 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 $PGD_2$ or a combination of $PGD_2$ 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 processed to measure eNOS mRNA and nitrite production; neuronal (n) NOS is not detectable in the isolated choroid (1).

*Animal preparation for in vivo experiments*. Pigs were anesthetized with 2% halothane. A catheter (Cathlon; Johnson & Johnson, Arlington, TX) was placed in a femoral vein and secured to the animal with tape. Newborn animals were randomly assigned to receive intravenously every 8 h for 24 h, saline, ibuprofen (40 mg/kg), a combination of ibuprofen (40 mg/kg) with either $PGD_2$, 16,16-dimethyl-$PGE_2$, or carbaprostacyclin (each at 10 μg/kg), or the $PGD_2$ receptor blocker BW A868C (10 μg/kg); a few animals treated with both ibuprofen and $PGD_2$ also received the NOS inhibitor $N^{\omega}$-monomethyl-L-arginine (L-NMMA; 1 mg/kg) 30 min before hemodynamic studies. The dose of ibuprofen was previously shown to decrease neonatal prostaglandin levels to those of the adult (1, 2) and those of prostaglandins and analogs to change prostaglandin levels and/or cause effects in vivo (5, 23, 25). Juvenile pigs were treated with saline or $PGD_2$. At the end of the 24-h period, animals were either kept alive to study ChBF autoregulation or were killed (120 mg/kg iv pentobarbital sodium) to obtain eyes to measure choroidal NOS activity or perform vasomotor studies.

*eNOS and destrin RNase protection assays*. Partial eNOS and destrin cDNAs were synthesized by RT-PCR from porcine cerebellar total RNA. The primer pair for porcine eNOS was 5′-GCT TTT CCC TGC AGG AGC GAC-3′ and 5′-GCC AGT CTC TGC AGA CTC TGG-3′ (35). The primer pair for porcine destrin was 5′-ATG ATG CAA GCT TTG AAA CC-3′ and 5′-GGA AGC TTT CGA TCT GTG G-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 pig eNOS and destrin partial cDNAs were determined by sequencing multiple clones using the T7 sequencing kit (BRL Life Technologies, Burlington, ON, Canada). [$^{32}$P]cRNA probes for eNOS and destrin were prepared using an in vitro transcription kit (Promega).

Total RNAs from choroid were separated into aliquots and subjected to RNase protection assays according to a published protocol with minor modifications (9). Briefly, 20 μg of total RNA were mixed with $10^5$ counts/min of eNOS and destrin probes in 20 μl of hybridization buffer (80% deionized formamide, 40 mM PIPES, 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 $T_1$ (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, followed by precipitation of protected fragments (9). The protected RNA fragments were resolved on urea-6% polyacrylamide gels, and the bands were visualized by phosphorimaging (Molecular Dymamics) and quantified densitometrically.

*eNOS Western blotting*. Western blotting for choroidal eNOS was performed exactly as previously described (1).

*Nitrite production*. NO production was estimated by determination of its stable metabolite, nitrite (33). Measurement of 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 $N^{\omega}$-nitro-L-arginine (L-NA; 1 mM).

*NOS activity*. Total NOS activity in choroid was determined as the L-NA (1 mM)-sensitive production of L-[$^3$H]citrulline from L-[$^3$H]arginine as previously described (19); constitutive $Ca^{2+}$-dependent NOS activity (largely eNOS in choroid; see Ref. 1) was determined after subtraction of $Ca^{2+}$-independent iNOS activity (in presence of 0.5 mM EGTA) from total NOS activity.

*Prostaglandin measurements*. Choroidal levels of $PGE_2$, $PGD_2$, and 6-keto-$PGF_{1\alpha}$ (stable metabolite of $PGI_2$) were measured by RIA (1, 15).

*Choroidal vasomotor responses*. Choroidal vasomotor response to agents that elicit their effects mostly via NO was studied as previously described (1, 15). Hence, effects of NO-dependent vasorelaxants ACh (14), bradykinin (Bk; see Ref. 37), and substance P (SP; see Ref. 31) were determined on vascular tone of choroids from newborn animals treated for 24 h with saline, ibuprofen, or a combination of ibuprofen and $PGD_2$; juvenile pigs were treated with saline or $PGD_2$. The choroid was perfused using a pulsatile minipump (Gilson) 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 vasorelaxation and an increase reflects vasoconstriction. 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-NMMA (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 (nonreceptor mediated), which exerts 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 reported (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

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), ~$10^6$ 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. 1*A*). 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 $Ca^{2+}$ 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 $PGD_2$ but were unaltered by stable analogs of other major prostaglandins, 16,16-dimethyl-$PGE_2$ 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, $PGD_2$, but not other prostaglandins, increased nitrite production and eNOS mRNA, and this effect was prevented by cotreatment of $PGD_2$ 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 $PGD_2$ on eNOS mRNA and nitrite production are reflected more specifically on $Ca^{2+}$-dependent NOS activity in vivo in the newborn. Treatment of neonatal pigs for 24 h with ibuprofen reduced $PGE_2$, 6-keto-$PGF_{1\alpha}$, and $PGD_2$ levels in choroid, respectively, to 1,408 ± 351,

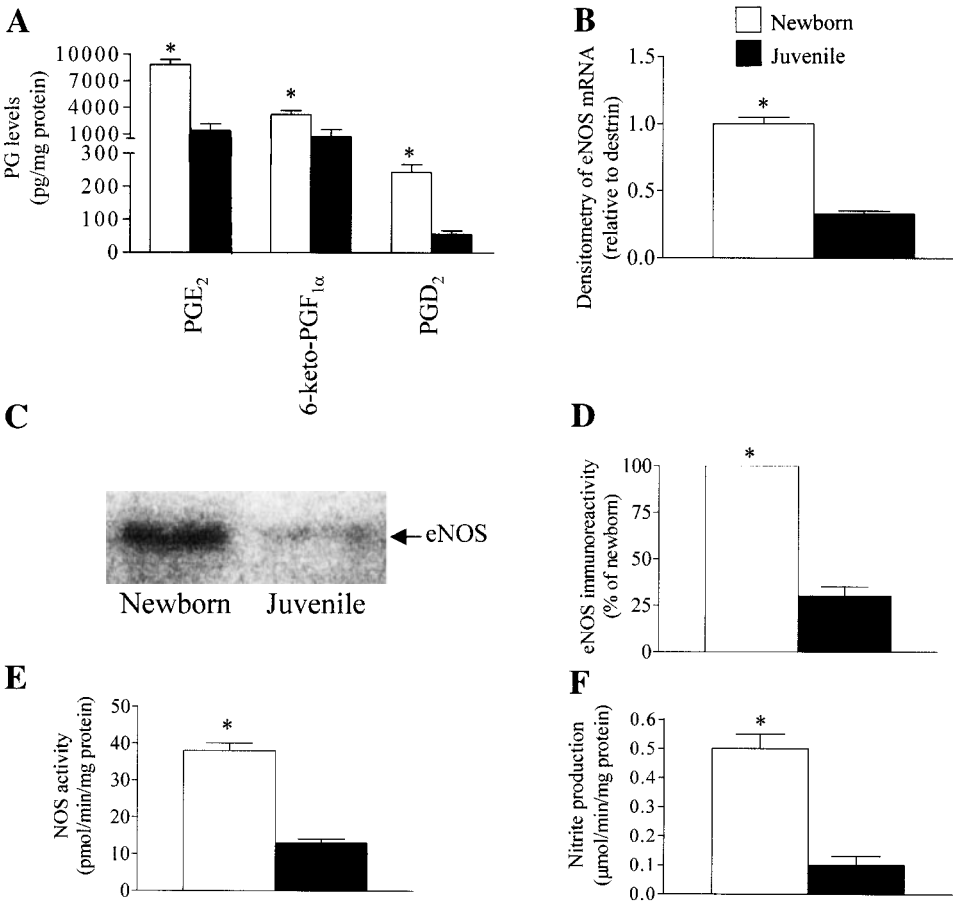


Fig. 1. Prostaglandin (PG) levels (*A*) and endothelial (e) nitric oxide synthase (NOS) mRNA (*B*), eNOS protein (*C* and *D*), NOS activity (*E*), and nitrite production (*F*) in newborn (1 day old) and juvenile (6–8 wk old) pig choroid. *B*: 20 μg total RNA were subjected to RNase protection assay. *C* and *D*: Western blot lanes were each loaded with 100 μg protein; arrow points to the eNOS 140-kDa protein, the only band detected in the range of interest, 120–180 kDa. *E*: NOS activity was measured as $Ca^{2+}$-dependent $N^{\omega}$-nitro-L-arginine (L-NA)-sensitive production of L-[$^3$H]citrulline from L-[$^3$H]arginine. Likewise, in *F*, nitrite production was measured as that sensitive to L-NA. Values are means ± SE of 3–4 experiments for values each performed in duplicate. *$P < 0.01$ compared with value for the juvenile.

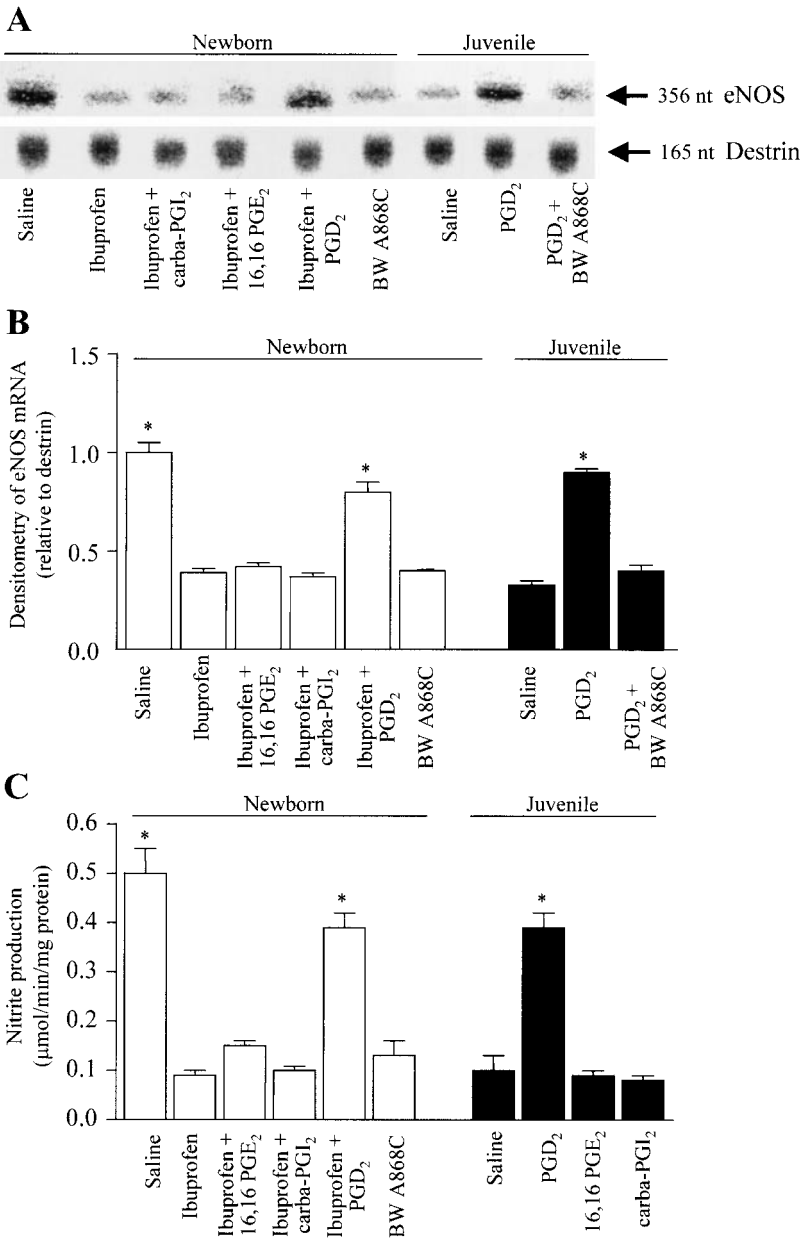


Fig. 2. Modulation of eNOS mRNA expression (*A* and *B*) and nitrite production (*C*) by prostaglandins in isolated choroids. Choroids of newborn pigs were incubated for 24 h with saline, ibuprofen (10 μM), a combination of ibuprofen and $PGD_2$, 16,16-dimethyl-$PGE_2$ (16,16-$PGE_2$), or carbaprostacyclin (carba-$PGI_2$; all at 1 μM), or with BW A868C (1 μM); choroids of juveniles were treated with saline, $PGD_2$, or a combination of $PGD_2$ with BW A868C. RNase protection assays were performed as in Fig. 1. Protected RNA fragment of eNOS contained 356 nucleotides (nt), and that of destrin contained 165 nt. Autoradiographic exposure was overnight and was visualized by phosphorimaging. Values are means ± SE of 3–4 experiments each performed in duplicate. $*P < 0.01$ compared with all other values.

704 ± 106, and 56 ± 11 pg/mg protein from those in saline-treated newborns (see Fig. 1*A*). 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 $PGD_2$ but not with 16,16-dimethyl-$PGE_2$ 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 $PGD_2$ 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 $PGD_2$, consistent with increased $PGD_2$-dependent NOS activity (Figs. 2 and 3). Correspondingly, juvenile animals treated (24 h) with $PGD_2$ exhibited increased vasorelaxation to ACh, Bk, and SP, as seen in saline-treated newborns. Infusion of L-NMMA in choroids of animals treated with $PGD_2$ 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-

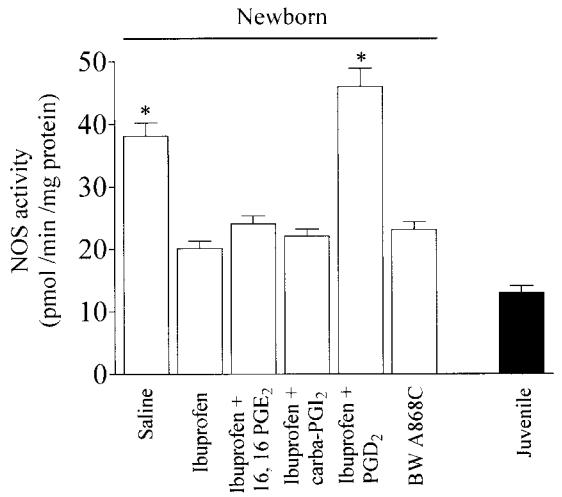


Fig. 3. In vivo modulation of NOS activity in newborn pig choroid by prostaglandins. Newborn pigs were treated every 8 h for 24 h with saline, ibuprofen (40 mg/kg), a combination of ibuprofen with $PGD_2$, 16,16-dimethyl-$PGE_2$, or carbaprostacyclin (all at 10 μg/kg), or with BW A868C ($PGD_2$ receptor antagonist, 10 μg/kg). Juvenile animals were treated with saline. $Ca^{2+}$-dependent constitutive NOS activity was determined as the L-NA (1 mM)-sensitive production of L-[$^3$H]citrulline from L-[$^3$H]arginine after subtracting $Ca^{2+}$-independent activity (see MATERIALS AND METHODS). Values are means ± SE of 3–4 experiments. *$P < 0.01$ compared with all other values.

tory control of the older subjects (25). Basal ChBF was 32 ± 3 and 29 ± 4 $ml \cdot min^{-1} \cdot g^{-1}$, 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. 5*F*), ChBF increased linearly as a function of OPP over the entire range of OPP studied ($r$ = 0.82–0.96, $P < 0.01$; Fig. 5*A*), 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 $PGD_2$ with ibuprofen caused ChBF to increase linearly with OPP as seen in saline-treated newborns ($r$ = 0.71–0.99, $P < 0.05$; Fig. 5*C*); addition of L-NMMA reduced basal ChBF to 16 ± 3 $ml \cdot min^{-1} \cdot g^{-1}$, 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. 5*E*). Regression coefficients for newborn pigs treated with saline or ibuprofen plus $PGD_2$ differed significantly from juveniles and from newborns treated with ibuprofen, BW A868C, or ibuprofen plus $PGD_2$ 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 $O_2$ 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 $PGE_2$, 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 $PGD_2$ 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 $PGD_2$, 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

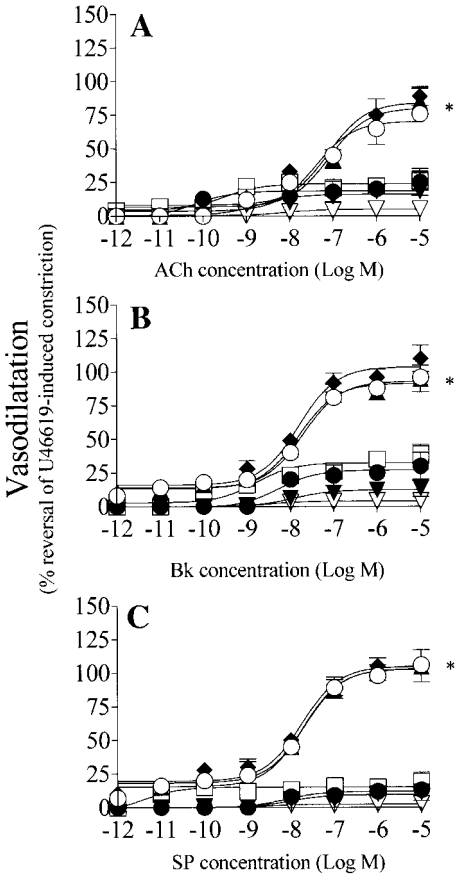


Fig. 4. Vasorelaxation of choroids to ACh (*A*), bradykinin (Bk; *B*), and substance P (SP; *C*). Newborn animals were treated every 8 h for 24 h with saline (○), ibuprofen (40 mg/kg; ●), or a combination of ibuprofen and $PGD_2$ (10 μg/kg; ▲); juvenile pigs were treated similarly with saline (□) or $PGD_2$ (◆). Some preparations of newborn and juvenile animals were infused with $N^{\omega}$-monomethyl-L-arginine (L-NMMA; 1 mM). To avoid overcrowding, data are shown only for $PGD_2$-treated newborn (▼), although results were similar for $PGD_2$-treated juvenile and saline-treated newborn; L-NMMA-infused preparations of saline-treated juveniles are presented (▽). Values are means ± SE of 4 experiments expressed as percent reversal of constriction induced by U-46619 (0.1 μM). *$P < 0.01$ compared with juveniles and with L-NMMA- and ibuprofen-treated newborn pigs (by ANOVA).

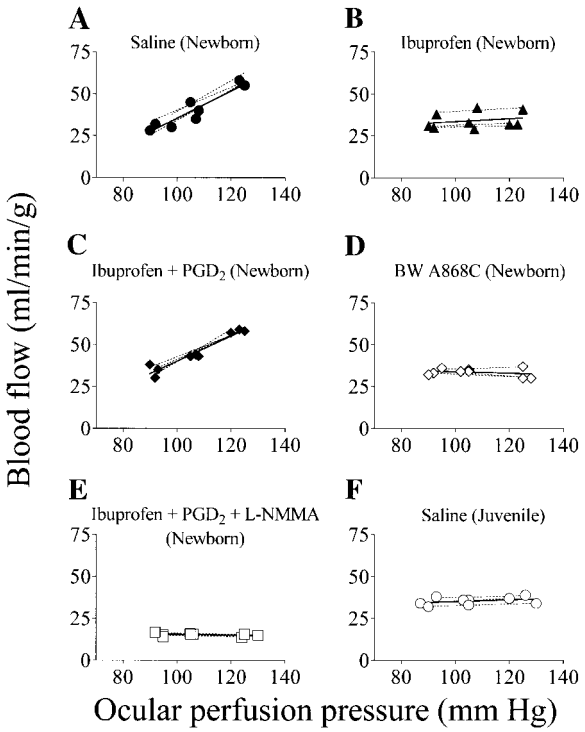


Fig. 5. Choroidal blood flow (ChBF) as a function of ocular perfusion pressure (OPP). Animals ($n$ = 3 in each group) were treated as described in Figs. 3 and 4; an additional 3 animals treated with ibuprofen and $PGD_2$ also received L-NMMA (1 mg/kg) 30 min before ChBF measurements. Broken lines correspond to the regressions for individual animals, and solid lines are mean regressions for all animals in the group. In newborn pigs treated with saline (*A*) or a combination of ibuprofen and $PGD_2$ (*C*), ChBF increased linearly with OPP ($r$ = 0.71–0.99, $P < 0.01$). In saline-treated juvenile pigs (*F*) and in newborns treated with ibuprofen, BW A868C, or a combination of ibuprofen + $PGD_2$ + L-NMMA (*B*, *D*, and *E*), ChBF did not change as a function of OPP ($r$ = 0.07–0.31, $P > 0.4$).

subject. *2*) Effects of ibuprofen were reproduced by the selective $PGD_2$ receptor blocker BW A868C. *3*) Ibuprofen-induced inhibition of eNOS expression in newborns was prevented specifically by $PGD_2$ but not by other prostaglandins (even at high concentrations, 1 μM); this modulation of eNOS by $PGD_2$ was observed in vitro and in vivo. *4*) Because our data suggested that high $PGD_2$ levels in the newborn upregulate eNOS expression, we tested if $PGD_2$ can increase eNOS activity in the juveniles (which have low prostaglandin and NO formation); our observations supported this inference (Fig. 2). One may suggest that the reported role of estrogens in regulating eNOS activity in lung tissue and cells may in part be attributed to prostaglandins (26, 29); alternatively, prostaglandins and estrogens may facilitate each other in coordinating the control of eNOS expression.

An important finding in this study is that the regulation of eNOS expression by $PGD_2$ in the choroid is reflected in the developmental control of vasomotor tone. It has previously been shown that increased NO formation in the newborn exerts a greater contribution on basal choroidal vascular tone than in that of the juvenile adult and also curtails the autoregulatory response (18, 19). Accordingly, a reduction in NOS activity after ibuprofen or BW A868C decreased effects of NO-dependent vasorelaxants and enhanced ChBF autoregulation (Figs. 4 and 5) as seen after treatment with NOS inhibitors (Refs. 18 and 19 and the present study). Conversely, addition of $PGD_2$ (24 h) evoked a choroidal vasomotor control as seen in the saline-treated newborns. Interestingly, sustained inhibition of prostaglandin synthesis has been reported to improve the regulation of choroidal vasomotor tone in vivo in the newborn (10). The present study provides a mechanism by which prostaglandins, specifically $PGD_2$, interact with NOS in the control of choroidal vascular tone, such that prostaglandins regulate NO formation and the latter exerts a major role in governing ChBF.

The mechanism responsible for $PGD_2$ in inducing eNOS expression is not clear; however, certain inferences can be made. Although 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 $PGD_2$-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 $PGD_2$ 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 $PGD_2$ via DP. This relationship between $PGD_2$ 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

1. **Abran, D., I. Dumont, P. Hardy, K. G. Peri, D. Y. Li, S. Molotchnikoff, D. R. Varma, and S. Chemtob.** Characterization and regulation of prostaglandin $E_2$ receptor and receptor coupled functions in the choroidal vasculature of the pig during development. *Circ. Res.* 80: 463–472, 1997.

2. **Abran, D., D. R. Varma, and S. Chemtob.** Regulation of prostanoid vasomotor effects and receptors in choroidal vessels of newborn pigs. *Am. J. Physiol. Regulatory Integrative Comp. Physiol*. 272: R995–R1001, 1997.
3. **Aeberhard, E. E., S. A. Henderson, N. S. Arabolos, J. M. Griscavage, F. E. Castro, C. T. Barret, and L. J. Ignarro.** Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem. Biophys. Res. Commun*. 208: 1053–1059, 1995.
4. **Alm, A., and A. Bill.** The oxygen supply to the retina. I. Effects of changes in intraocular and arterial blood pressures, and in arterial $PO_2$ and $PCO_2$ on the oxygen tension in the vitreous body of the cat. *Acta Physiol. Scand*. 84: 261–274, 1972.
5. **Ando, T., T. Ichijo, T. Katafuchi, and T. Hori.** Intracerebroventricular injection of prostaglandin $E_2$ increases splenic sympathetic nerve activity in rats. *Am. J. Physiol. Regulatory Integrative Comp. Physiol*. 269: R662–R668, 1995.
6. **Barchowsky, A., S. R. Munro, S. J. Morana, M. P. Vincenti, and M. Treadwell.** Oxidant-sensitive and phosphorylation-dependent activation of NF-κB and AP-1 in endothelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol*. 269: L829–L836, 1995.
7. **Bhattacharya, M., K. G. Peri, G. Almazan, A. Ribeiro-da-Silva, H. Shichi, Y. Durocher, M. Abramovitz, X. Hou, D. R. Varma, and S. Chemtob.** Nuclear localization of prostaglandin $E_2$ receptors. *Proc. Natl. Acad. Sci. USA* 95: 15792–15797, 1998.
8. **Bhattacherjee, P., L. Rhodes, and C. A. Paterson.** Prostaglandin receptors coupled to adenylyl cyclase in the iris-ciliary body of rabbits, cats and cows. *Exp. Eye Res*. 56: 327–333, 1993.
9. **Bordonaro, M., C. F. Saccomanno, and J. L. Nordstrom.** An improved T1/A ribonuclease protection assay. *Biotechniques* 16: 428–430, 1994.
10. **Chemtob, S., K. Beharry, J. Rex, T. Chatteejee, D. R. Varma, and J. V. Aranda.** Ibuprofen enhances retinal and choroidal blood flow autoregulation in newborn piglets. *Invest. Ophthalmol. Vis. Sci*. 32: 1799–1807, 1991.
11. **Coleman, R. A., W. Smith, and S. Narumiya VIII.** International Union of Pharmacology Classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev*. 46: 205–229, 1994.
12. **Gaillard, T., A. Mulsch, H. Klein, and K. Decker.** Regulation by prostaglandin $E_2$ of cytokine-elicited nitric oxide synthesis in rat liver macrophages. *Biol. Chem*. 373: 897–902, 1992.
13. **Giles, H., P. Leff, M. L. Bolofo, M. G. Kelly, and A. D. Robertson.** The classification of prostaglandin DP-receptors in platelets and vasculature using BW A868C, a novel, selective and potent competitive antagonist. *Br. J. Pharmacol*. 96: 291–300, 1989.
14. **Granstam, E., S. O. Granstam, B. Fellstrom, and L. Lind.** Endothelium-dependent vasodilation in the uvea of hypertensive and normotensive rats. *Curr. Eye Res*. 17: 189–196, 1998.
15. **Hardy, P., D. Abran, X. Hou, I. Lahaie, K. G. Peri, P. Asselin, D. R. Varma, and S. Chemtob.** A major role for prostacyclin in nitric oxide-induced ocular vasorelaxation in the piglet. *Circ. Res*. 83: 721–729, 1998.
16. **Hardy, P., D. Abran, D. Y. Li, H. Fernandez, D. R. Varma, and S. Chemtob.** Free radicals in retinal and choroidal blood flow autoregulation in the piglets interaction with prostaglandins. *Invest. Ophthalmol. Vis. Sci*. 35: 580–591, 1994.
17. **Hardy, P., M. Bhattacharya, D. Abran, K. G. Peri, P. Asselin, D. R. Varma, and S. Chemtob.** Increases in retinovascular prostaglandin receptor functions by cyclooxygenase-1 and -2 inhibition. *Invest. Ophthalmol. Vis. Sci*. 39: 1888–1898, 1998.
18. **Hardy, P., A. M. Nuyt, D. Abran, J. St.-Louis, D. R. Varma, and S. Chemtob.** Nitric oxide in retinal and choroidal blood flow autoregulation in newborn pigs: interactions with prostaglandins. *Pediatr. Res*. 39: 487–493, 1996.
19. **Hardy, P., K. G. Peri, I. Lahaie, D. R. Varma, and S. Chemtob.** Increased nitric oxide synthesis and action preclude choroidal vasoconstriction to hyperoxia in newborn pigs. *Circ. Res*. 79: 504–511, 1996.
20. **Hardy, P., D. R. Varma, and S. Chemtob.** Control of cerebral and ocular blood flow autoregulation in neonates. *Pediatr. Clin. North Am*. 44: 137–152, 1997.
21. **Jacot, J. L., J. T. O'Neill, D. M. Scandling, S. D. West, and J. E. McKenzie.** Nitric oxide modulation of retinal, choroidal, and anterior uveal blood flow in newborn piglets. *J. Ocul. Pharmacol. Ther*. 14: 473–489, 1998.
22. **Kanai, N., R. Lu, J. A. Satriano, Y. Bao, A. W. Wolkoff, and V. L. Schuster.** Identification and characterization of a prostaglandin transporter. *Science* 268: 866–869, 1995.
23. **Kennedy, T. G., and P. E. Doktorcik.** Effects of analogues of prostaglandin $E_2$ and $F_{2a}$ on the decidual cell reaction in the rat. *Prostaglandins* 35: 207–219, 1998.
24. **Kiel, J. W., and W. A. J. van Heuven.** Ocular perfusion pressure and choroidal blood flow in the rabbit. *Invest. Ophthalmol. Vis. Sci*. 36: 579–585, 1995.
25. **Li, D., P. Hardy, D. Abran, A. K. Martinez-Bermudez, A. M. Guerguerian, M. Bhattacharya, G. Almazan, K. Menezes, K. G. Peri, D. R. Varma, and S. Chemtob.** Key role for cyclooxygenase-2 in $PGE_2$ and $PGF_{2\alpha}$ receptor regulation and cerebral blood flow of the newborn. *Am. J. Physiol. Regulatory Integrative Comp. Physiol*. 273: R1283–R1290, 1997.
26. **MacRitchie, A. N., S. S. Jun, Z. Chen, Z. German, I. S. Yuhanna, T. S. Sherman, and P. W. Shaul.** Estrogen upregulates endothelial nitric oxide synthase gene expression in fetal pulmonary artery endothelium. *Circ. Res*. 81: 355–362, 1997.
27. **Marotta, P., L. Sautebin, and M. Di. Rosa.** Modulation of the induction of nitric oxide synthase by eicosanoids in the murine macrophage cell line. *Br. J. Pharmacol*. 107: 640–641, 1992.
28. **McLeod, D. S., R. Brownstein, and G. A Lutty.** Vasoobliteration in the canine model of oxygen-induced retinopathy. *Invest. Ophthalmol. Vis. Sci*. 37: 300–311, 1996.
29. **Myers, S. I., R. H. Turnage, L. Bartula, B. Kalley, and Y. Meng.** Estrogen increases male rat aortic endothelial (RAEC) $PGI_2$ release. *Prostaglandins Leukot. Essent. Fatty Acids* 54: 403–409, 1996.
30. **Raddassi, K., J. F. Petit, and S. Lemaire.** LPS-induced activation of primed murine peritoneal macrophages is modulated by prostaglandins and cyclic nucleotides. *Cell. Immunol*. 149: 50–64, 1993.
31. **Rosenblum, W. I., T. Shimizu, and G. H. Nelson.** Endothelium-dependent effects of substance and calcitonin gene-related peptide on mouse pial arterioles. *Stroke* 7: 1043–1047, 1993.
32. **Venema, R. C., K. Nishida, R. W. Alexander, D. G. Harrison, and T. J. Murphy.** Organization of the bovine gene encoding the endothelial nitric oxide synthase. *Biochim. Biophys. Acta* 1218: 413–420, 1994.
33. **Verdon, C. P., B. A. Burton, and R. L. Prior.** Sample pretreatment with nitrate reductase and glucose-6-phosphate dehydrogenase quantitatively reduces nitrate while avoiding interference by $NADP^+$ when the Greiss reaction is used to assay for nitrite. *Anal. Biochem*. 224: 502–508, 1995.
34. **Zhang, J. L., J. M. Patel, and E. R. Block.** Molecular cloning, characterization and expression of a nitric oxide synthase from porcine pulmonary artery endothelial cells. *Comp. Biochem. Physiol. A Physiol*. 116: 485–491, 1996.
35. **Zhang, R., W. Min, and W. C. Sessa.** Functional analysis of the human endothelial nitric oxide synthase promoter. Sp1 and GATA factors are necessary for basal transcription in endothelial cells. *J. Biol. Chem*. 270: 15320–15326, 1995.
36. **Zhu, P., J. L. Beny, J. Flammer, T. F. Lüscher, and I. O. Haefliger.** Relaxation by bradykinin in porcine ciliary artery. Role of nitric oxide and $K^+$ channels. *Invest. Ophthalmol. Vis. Sci*. 38: 1761–1767, 1997.