PGE_2-Mediated eNOS Induction in Prolonged Hypercapnia

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Purpose. Because prostaglandins (PGs) are implicated in acute hypercapnia-induced hyperemia, this study was conducted to test the hypothesis that prolonged hypercapnia may cause a sustained increase in retinal blood flow (RBF) through a PG-dependent induction of endothelial nitric oxide synthase (eNOS).

Methods. Time-dependent RBF (microsphere technique), PGE_2, nitrite (NO_2^-), and NOS protein (reduced nicotinamide adenine dinucleotide phosphate [NADPH]-diaphorase staining) production were measured in hypercapnia (6% CO_2)-treated piglets. From the same species, PGE_2, eNOS mRNA, NOS protein, and vasomotor responses were measured in eyecup preparations, as were Ca^{2+} transients in neuroretinovascular endothelial cells.

Results. Hypercapnia caused biphasic (at 0.5 hours and 6–8 hours) increases in RBF that were abolished with normalization of the pH. The early phase (0.5 hour) was associated with an increase in PGE_2 levels and the latter phase (6–8 hours) with an increase in NO_2^- and NOS protein. Inhibition of cyclooxygenase by diclofenac prevented the early and late increase in RBF. NOS inhibitor L-nitro-arginine prevented only the latter. Hypercapnic acidosis increased retinal PGE_2 levels and eNOS-dependent vasorelaxation ex vivo. The ex vivo time course of eNOS mRNA expression corresponded with the late-phase increase in RBF and was blocked by the transcription inhibitor actinomycin D and the receptor-operated Ca^{2+} channel blocker SK&F96365. In neuroretinovascular cells, acidosis increased Ca^{2+} transients, which were inhibited by SK&F96365, but not diclofenac.

Conclusions. This study discloses a previously unexplored mechanism for late retinal hyperemia during sustained hypercapnia that appears secondary to the induced expression of eNOS mediated by PGE_2. (*Invest Ophthalmol Vis Sci.* 2002;43:1558–1566)

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Hyperoxia and ensuing oxidant stress are major factors involved in the pathogenesis of retinopathy of prematurity (ROP). Other factors regularly encountered in neonates with bronchopulmonary dysplasia, such as hypercapnia and associated acidosis, have also been shown to play a role in the development of ROP.[1–4] Hypercapnia causes retinal vasodilatation leading to increased retinal blood flow (RBF) and, in turn, oxygenation.[5–12] However, the ocular hemodynamic effects of hypercapnia have been reported only in acute conditions that are insufficient to account for the development of ROP. Evidence in brain tissue suggests that acute hypercapnia-induced hyperemia is transient.[13,14] Because sustained hypercapnic acidosis has been alleged to increase retinal oxygenation,[3,15] a delayed second phase of hyperemia is thought to explain the induced retinovascular injury.[3,15] Prolonged exposure to hypercapnia has recently been shown in the brain to be associated with a second increase in cerebral blood flow, and both the initial and second cerebral hyperemias are significantly accounted for by increased NO generation.[16] Although the retina is part of the central nervous system, its vasculature may not respond similarly to physiological adaptations because of differential expression of receptors and enzymes involved in these processes.[17–19] Thus, the response of the eye to prolonged hypercapnia and the relative role of NO in this process remains unknown.

Acute hypercapnia-induced ocular hyperemia appears to be largely mediated by relaxant prostaglandins (PGs),[8,20] whereas the involvement of NO is minimal.[20–22] Complex interactions have been described between PGs and NO.[23,24] Among these interactions, PGE_2 has been found to induce endothelial nitric oxide synthase (eNOS) in brain microvessels,[16,25] whereas a dominant role for PGD_2 has been documented in the choroidal vasculature of the eye.[26] We therefore hypothesized that sustained hypercapnia may cause PG-dependent early and late phases of increased RBF. Our findings disclose for the first time that sustained hypercapnia induces a late retinal hyperemia that is in large part due to augmented NO release secondary to increased eNOS expression mediated by PGE_2.

Methods

Animals

Yorkshire piglets (≤6 days old) were used according to a protocol approved by the Animal Care Committee of Hôpital Ste. Justine, in conformity with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RBF Measurements

RBF was measured by the microsphere technique, as previously described by us.[27–29] Briefly, catheterization of the left ventricle through the right subclavian artery for injection of fluorescent microspheres; the left subclavian artery for the 70-second withdrawal of reference blood samples, beginning 10 seconds before the injection of each type of fluorescent microsphere; and the femoral artery for blood pressure recording were performed in animals under halothane (2.5%) anesthe-

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TABLE 1. Arterial Blood Pressure and Gas Levels in Piglets before and after Hypercapnia

Treatment	Baseline	0.5 h	3 h	6 h	8 h
Control					
MABP (mm Hg)	58.3 ± 5.4	63.1 ± 3.8	60.2 ± 4.7	63.5 ± 5.3	65.2 ± 3.5
Arterial pH	7.38 ± 0.04	7.19 ± 0.03*	7.23 ± 0.02*	7.26 ± 0.03*	7.29 ± 0.02*‡
$PaCO_2$ (mm Hg)	41.2 ± 1.5	68.7 ± 2.1*	65.1 ± 1.6*	66.8 ± 2.7*	64.6 ± 1.8*
PaO_2 (mm Hg)	90.0 ± 8.6	89.3 ± 4.3	88.4 ± 4.1	86.1 ± 8.5	91.1 ± 8.5
Diclofenac					
MABP (mm Hg)	63.2 ± 5.4	62.7 ± 3.4	68.2 ± 6.3	69.1 ± 2.5	64.0 ± 2.6
Arterial pH	7.40 ± 0.02	7.19 ± 0.02*	7.23 ± 0.03*	7.25 ± 0.06*	7.27 ± 0.03*‡
$PaCO_2$ (mm Hg)	41.8 ± 3.4	65.3 ± 3.1*	67.1 ± 3.4*	68.2 ± 2.5*	66.3 ± 2.6*
PaO_2 (mm Hg)	96.9 ± 7.9	91.8 ± 8.5	91.5 ± 4.5	86.1 ± 8.8	88.5 ± 8.0
L-NA					
MABP (mm Hg)	86.7 ± 3.1†	85.5 ± 2.9†	84.2 ± 3.9†	84.9 ± 4.1†	86.7 ± 2.7†
Arterial pH	7.39 ± 0.03	7.20 ± 0.01*	7.22 ± 0.01*	7.23 ± 0.02*	7.28 ± 0.03*‡
$PaCO_2$ (mm Hg)	42.4 ± 1.8	68.7 ± 3.3*	67.5 ± 3.3*	69.5 ± 2.6*	69.3 ± 2.8*
PaO_2 (mm Hg)	96.8 ± 9.5	97.8 ± 9.0	99.2 ± 8.4	86.7 ± 9.0	90.3 ± 8.4
TRIM					
MABP (mm Hg)	61.4 ± 2.3	65.3 ± 3.4	66.5 ± 2.2	61.4 ± 1.9	63.5 ± 3.5
Arterial pH	7.41 ± 0.03	7.21 ± 0.02*	7.21 ± 0.03*	7.19 ± 0.02*	7.25 ± 0.02*‡
$PaCO_2$ (mm Hg)	44.8 ± 3.1	71.0 ± 3.1*	72.4 ± 2.4*	72.7 ± 2.6*	70.5 ± 3.3*
PaO_2 (mm Hg)	91.4 ± 7.6	93.3 ± 8.4	91.3 ± 5.1	93.5 ± 5.2	92.4 ± 4.5
Bicarbonate					
MABP (mm Hg)	63.4 ± 2.9	66.1 ± 2.1	61.9 ± 4.0	65.5 ± 3.3	62.3 ± 2.5
Arterial pH	7.40 ± 0.03	7.41 ± 0.01	7.39 ± 0.03	7.38 ± 0.02	7.40 ± 0.02
$PaCO_2$ (mm Hg)	43.4 ± 2.0	69.9 ± 2.7*	71.9 ± 3.0*	70.5 ± 2.1*	72.1 ± 3.3*
PaO_2 (mm Hg)	88.3 ± 9.6	91.1 ± 6.4	89.3 ± 6.2	93.5 ± 5.6	91.3 ± 7.5

Data are the mean ± SEM of four to five eyes from different animals in each treatment group. Pigs were ventilated with 6% CO_2, 21% O_2, and 73% N_2 to adjust CO_2 to ≈65 mm Hg.

* $P < 0.05$ compared with corresponding baseline.

† $P < 0.05$ compared with corresponding data in other groups.

‡ $P < 0.05$ compared with corresponding data at 0.5 hours (trend analysis).

sia. In addition, a 27-gauge butterfly needle, attached to a catheter, was introduced into the anterior chamber of the eye, through the cornea, to measure intraocular pressure.

Upon cessation of halothane, sedation was continued with α-chloralose (50 mg/kg bolus followed by an infusion of 10 mg/kg · h). Animals were allowed to stabilize for 1.5 hours before experiments began. After baseline RBF measurements under 21% O_2 and 79% N_2, the gas mixture was changed to 6% CO_2, 73% N_2, and 21% O_2 to obtain stable $PaCO_2$ (≈65 mm Hg), which is commonly encountered in the clinical setting. RBF measurements were repeated 0.5, 3, 6, and 8 hours after hypercapnia was initiated.

Animals were randomly assigned to pretreatment (30 minutes before blood flow measurements) with one of the following: the PG synthase inhibitor diclofenac (5 mg/kg), the NOS inhibitor L-nitroarginine (L-NA; 3 mg/kg), the inducible and neuronal NOS (iNOS and nNOS, respectively) inhibitor 1-(2-trifluoromethylphenyl) imidazole (TRIM; 1 mg/kg followed by 50 μg/kg · min),[30] bicarbonate (8.4% solution) to normalize the pH, or saline.[16,31,32] After the experiment, the animals were killed with intravenous pentobarbital (120 mg/kg) and the eyes dissected to remove the retina. Tissues were digested, and flow cytometric analysis (Interactive Medical Technologies, Inc., Los Angeles, CA) was used to count the fluorescence intensities in the retinal and reference blood samples. Blood flow was calculated as the product of the microsphere count per minute per gram tissue and reference blood withdrawal rate, divided by the microsphere count in the reference blood.[27–29] Retinal PGE_2 levels, nitrite (NO_2^-) production, and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase reactivity were also measured in some animals.

Tissue Preparations

Isolated eyecups were placed in buffer of the following composition (in millimolar): 132 NaCl, 3.0 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 24.6 $NaHCO_3$, 1.2 KH_2PO_4, 20 glucose, 6.6 urea, and 0.5% fetal bovine serum. Tissues were incubated at 37°C for 6 hours under normocapnic, hypercapnic acidosis, hypercapnic nonacidosis, and normocapnic acidosis, as in in vivo conditions. Conditions were set by bubbling CO_2 (3%–10%) and adjusting pH with HCl and $NaHCO_3$. Preparations were treated with diclofenac (100 μM), with or without (1 μM) 16,16-dimethyl PGE_2, the selective PGD_2 agonist BW245C, $PGF_{2\alpha}$, or carbaprostacyclin or with the nonselective Ca^{2+} channel blocker SK&F96365 (10 μM) or the transcription inhibitor actinomycin D (2 μM). Tissues were used to measure eNOS mRNA and PGE_2 levels.

Vasomotor Response of Retinal Vessels

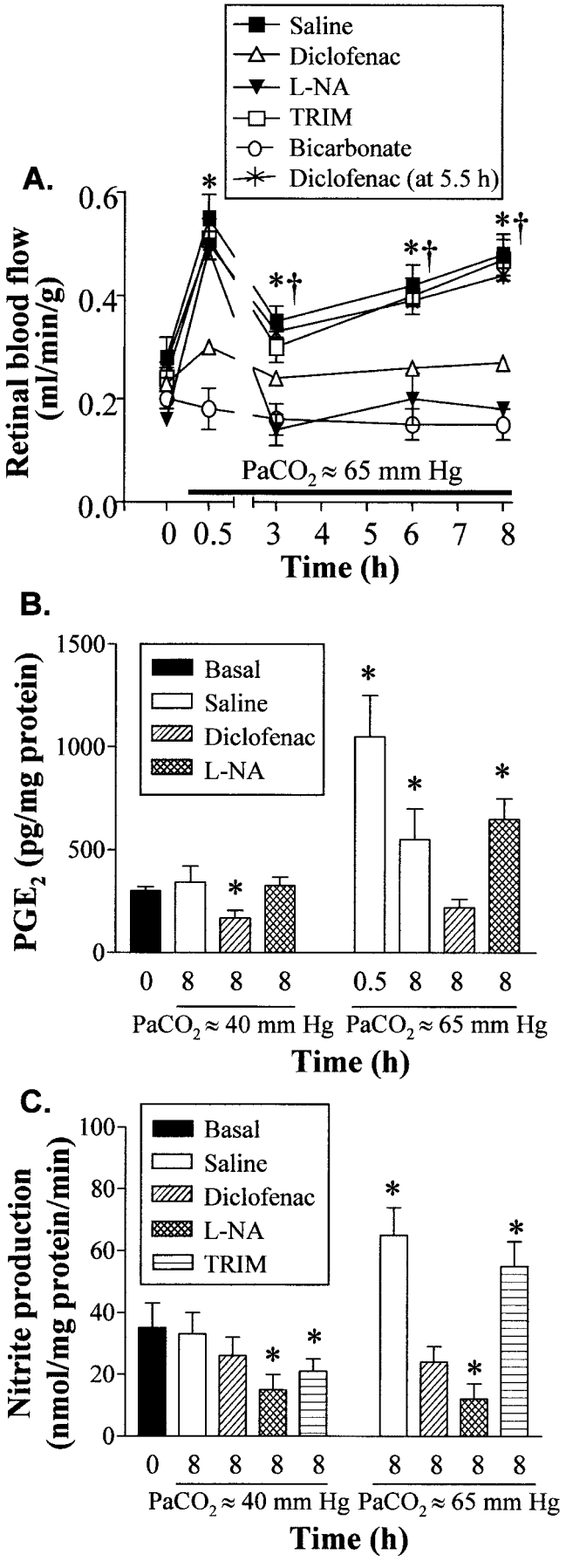
Eye cups were prepared, to study vasomotor responses of the relatively undisturbed retinal vasculature, as previously described,[28,29,33] and were incubated for 4 hours in normocapnic or hypercapnic conditions, with or without diclofenac (100 μM), as described earlier. Thereafter, they were placed in physiological buffer (as described in Tissue Preparations) with normal CO_2, to determine vasorelaxant responses to eNOS-dependent substance P,[34] using video imaging techniques.[28,29] Effects of substance P were also tested in some eyecups treated with L-NA (1 mM) 20 minutes before determining the vasomotor response to substance P.

NADPH-Diaphorase Histochemistry

In situ NOS expression was assessed using NADPH-diaphorase staining, as described.[35] The intensity of blood vessel staining was analyzed digitally by computer (ImagePro Plus, ver. 4.1; Media Cybernetics, Silver Spring, MD). After normalizing for background tone, densitometry of tonality was determined in an equal number of pixels from each of the treatment groups, as reported by us.[16]

eNOS mRNA Detection by RNase Protection Assay

eNOS and destrin (loading control) RNase protection assays were conducted as described.[25,26] The primer pair for porcine eNOS was 5′-GCTTTTCC*CTGCAG*GAGCGAC-3′ and 5′-GCCAGTCT*CTGCAG*ACT-



CTGG-3′; the primer pair for porcine destrin was 5′-ATGATG-*CAAGCTT*TGAAACC-3′ and 5′-GG*AAGCTT*TCGATCTGTGG-3′.[25] The amplified products (0.4 kb) were digested with appropriate restriction enzymes (italic sequences in the primers denote the restriction sites) and cloned into the pGEM4 vector. The ^{32}P-labeled cRNA probes for eNOS and destrin were prepared with an in vitro transcription kit (Promega, Madison, WI).

Briefly, 20 μg total retinal RNA was mixed with 10^5 cpm eNOS and destrin probes in 20 μL hybridization buffer (80% deionized formamide, 40 mM piperazine-*N,N*′-bis(2-ethanesulfonic acid) [pH 6.8], 1 mM EDTA, and 0.4 M NaCl), denatured for 5 minutes at 90°C, and incubated overnight at 50°C. The RNA hybrids were digested with RNase A (10 μg/mL) and RNase T_1 (200 U/mL) in 200 μL digestion buffer (10 mM Tris-HCl [pH 7.5], 5 mM EDTA, and 0.3 M NaCl) for 30 minutes at 25°C, followed by precipitation of protected fragments. The protected RNA fragments were resolved on urea-6% polyacrylamide gels, and the bands were visualized by phosphorimaging (Molecular Dynamics, Sunnyvale, CA) and quantified by densitometry.

PGE_2 and Nitrite Measurements

PGE_2 levels were determined with a radioimmunoassay (RIA) kit (Advanced Magnetics, Boston, MA) as previously described.[27,32] Formation of NO by the retinas of piglets ventilated and treated as detailed was estimated by measuring the stable metabolite NO_2^-.[36]

Calcium Transients in Cultured Endothelial Cells

Porcine neuroretinovascular endothelial cells were prepared as previously described.[32,37] $[Ca^{2+}]_i$ was measured by the fura-2-acetoxymethyl ester technique[32] in cells pretreated for 15 minutes with diclofenac (100 μM), SK&F96365 (10 μM), or EGTA (5 mM). The $[Ca^{2+}]_i$ was calculated according to a published method.[32,38]

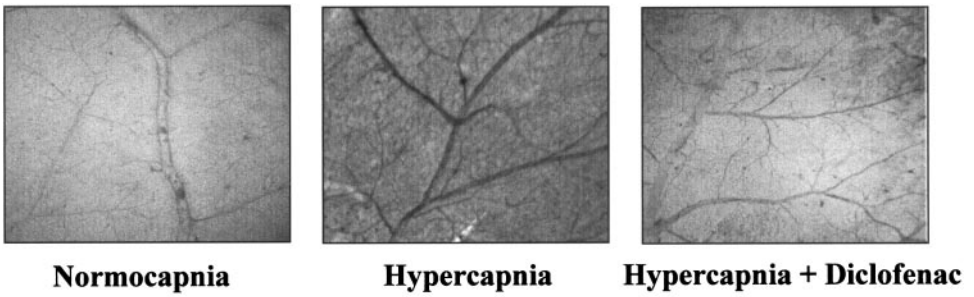
Chemicals

The following chemicals were purchased: α-chloralose, actinomycin D, diclofenac, EGTA, L-NA, and substance P (Sigma-Aldrich, Oakville, Ontario, Canada); BW245C, carbaprostacyclin, 16,16-dimethyl-PGE_2, $PGF_{2\alpha}$, and U46619 (Cayman, Ann Arbor, MI); TRIM (Tocris, Ballwin, MO); SK&F96365 (Biomol, Plymouth Meeting, PA); fura-2-acetoxymethyl (Calbiochem, La Jolla, CA); PGE_2 RIA kits (Cedarlane, Hornby, Ontario, Canada); fluorescent microspheres (Interactive Medical Technologies); pGEM4 plasmid vector (Promega); [α-^{32}P]CTP (3000 Ci/mmol; Amersham, Mississauga, Ontario, Canada); and RNase A (Pharmacia Biotech, Montreal, Québec Canada). All other high-purity chemicals were from Fisher Scientific (Montreal, Québec, Canada).

Statistical Analysis

RBF was analyzed by 2-way ANOVA, factoring for time and treatment followed by Dunnett's multiple comparison test. All other data were analyzed by one-way ANOVA with comparison among means being performed by Dunnett or Tukey multiple comparison test, as appro-

FIGURE 1. (**A**) Time course of RBF in piglets during an 8-hour exposure to hypercapnia by ventilation with 6% CO_2 (indicated by *horizontal bar*). One group of animals was treated with intravenous diclofenac (5 mg/kg) 5.5 hours after exposure to hypercapnia, and another group was given sodium bicarbonate (8.4%) to normalize pH. All other animals were pretreated with diclofenac (5 mg/kg), L-NA (3 mg/kg), TRIM (1 mg/kg followed by 50 μg/kg · min), or saline. At time 0, RBF was basal (normocapnia). Data are the mean ± SEM of four or five eyes of different animals. *$P < 0.05$ compared with basal levels; †$P < 0.05$ compared with corresponding levels in animals treated with diclofenac or L-NA. Retinal PGE_2 levels (**B**) and NO_2^- production (**C**) are from animals exposed in vivo to normocapnic and hypercapnic conditions. Data are the mean ± SEM of three or four eyes from different piglets. *$P < 0.05$ compared with 8-hour values at P_{ACO_2} of approximately 40 mm Hg.



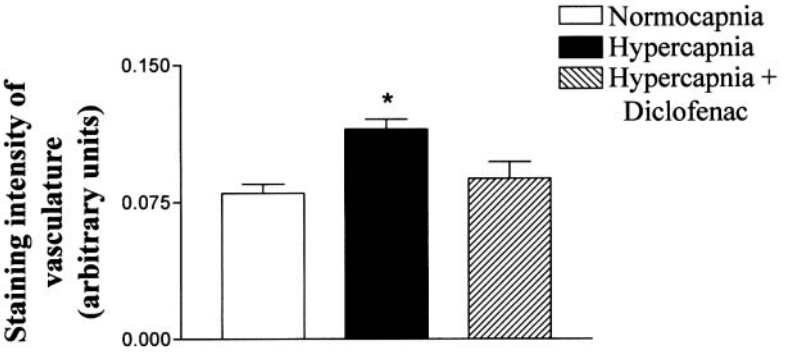


FIGURE 2. In vivo modulation of NADPH-diaphorase staining of retinas by exposure to hypercapnia. Piglets were ventilated and treated as in Figure 1, and retinal wholemounts were fixed for NADPH-diaphorase reactivity (*top*). Individual pixel tonality of densitometry of vasculature was analyzed by correcting for background tone (*bottom*). Data are the mean ± SEM of four retinal preparations from eye cups of different animals. $^*P < 0.05$ compared with data without *asterisks*.

priate. Statistical significance was set at $P < 0.05$. Data are expressed as the mean ± SEM.

RESULTS

Time Course of Retinal Response to Hypercapnia

During the 8-hour ventilation of animals with 6% CO_2, $Paco_2$ remained high, whereas pH decreased initially and then tended to increase over time (Table 1). Mean arterial blood pressure (MABP) and Pao_2 remained unchanged (Table 1), and intraocular pressure remained constant (14.5 ± 1.53 mm Hg). There was no difference in these parameters between treatment groups, with the exception of the expected increase in blood pressure with L-NA (Table 1). Hypercapnia produced a marked increase in RBF at 0.5 hour in control animals (Fig. 1A), which decreased significantly by 3 hours and gradually increased again between 6 and 8 hours (Fig. 1A). Normalization of the pH with bicarbonate abolished the retinal hemodynamic changes to hypercapnia at all time points (Fig. 1A). Both the early and late increases in RBF were blunted by diclofenac. L-NA prevented only the late-phase increase in RBF (Fig. 1A); whereas, the selective iNOS and nNOS inhibitor TRIM did not affect retinal hemodynamics. Injection of diclofenac at 5.5 hours of hypercapnia also did not alter the hemodynamic response (Fig. 1A).

The early increase in RBF coincided with an increase in retinal PGE_2 levels under hypercapnic conditions, which diminished by 8 hours, but remained higher than basal values (Fig. 1B). Under normocapnia, PGE_2 levels and NO_2^- production remained stable up to 8 hours. NO_2^- production was also increased by hypercapnia (Fig. 1C). Diclofenac reduced PGE_2 levels and prevented the increase in NO_2^- production observed under hypercapnia (Figs. 1B, 1C); L-NA reduced NO_2^- production but not PGE_2 levels (Figs. 1B, 1C); and TRIM reduced (slightly) NO_2^- production during normocapnia but negligibly during hypercapnia (Fig. 1C).

Retinal NADPH-Diaphorase Reactivity and eNOS mRNA

In vivo and ex vivo exposure to 6 hours of hypercapnia evoked increased NADPH-diaphorase reactivity that was largely localized to the vasculature, compared with the normocapnic control (Figs. 2, 3A). These changes in NOS expression were inhibited by diclofenac (Figs. 2, top right, 3A, top right). eNOS mRNA also increased in the retinas of eye cups exposed to hypercapnia. This was blocked by diclofenac, but not by L-NA (Fig. 3B, bottom). Diclofenac did not affect NOS expression during normocapnia (data not shown).

Effects of High CO_2 on NO-Dependent Retinal Vasorelaxation

Experiments were conducted to assess whether changes in hypercapnia-induced eNOS expression were reflected functionally. The retinal vessels of eye cups exposed to high CO_2 for 4 hours exhibited a marked increase in vasorelaxation to eNOS-dependent substance P when compared with the response of retinal vasculature maintained in normal CO_2 tension (Fig. 4). Cotreatment (4 hours) with diclofenac or actinomycin D prevented this augmented vasorelaxation induced by high CO_2. Agents did not affect vasomotor response during normal CO_2. Acute (15–30 minutes) exposure to high CO_2 did not modify the response to substance P.

Concentration- and Time-Dependent Effects of CO_2 on eNOS mRNA Expression

Exposure of isolated eye cups to increasing CO_2 tension for 6 hours produced a concentration-dependent increase in retinal eNOS mRNA (Fig. 5A). Acidosis in the presence of normal CO_2 induced a comparable time-dependent increase in eNOS expression (Figs. 5A, 5B), which was blocked by the transcription inhibitor actinomycin D. Normalization of the pH prevented changes in eNOS mRNA (Fig. 5A). The acidosis-induced increase in eNOS expression was also associated with an increase in PGE_2 levels, and both were prevented by the PG synthase inhibitor diclofenac, as well as the Ca^{2+} channel blocker SK&F96365 (Figs. 5C, 5D) consistent with calcium requirements for formation of PG.

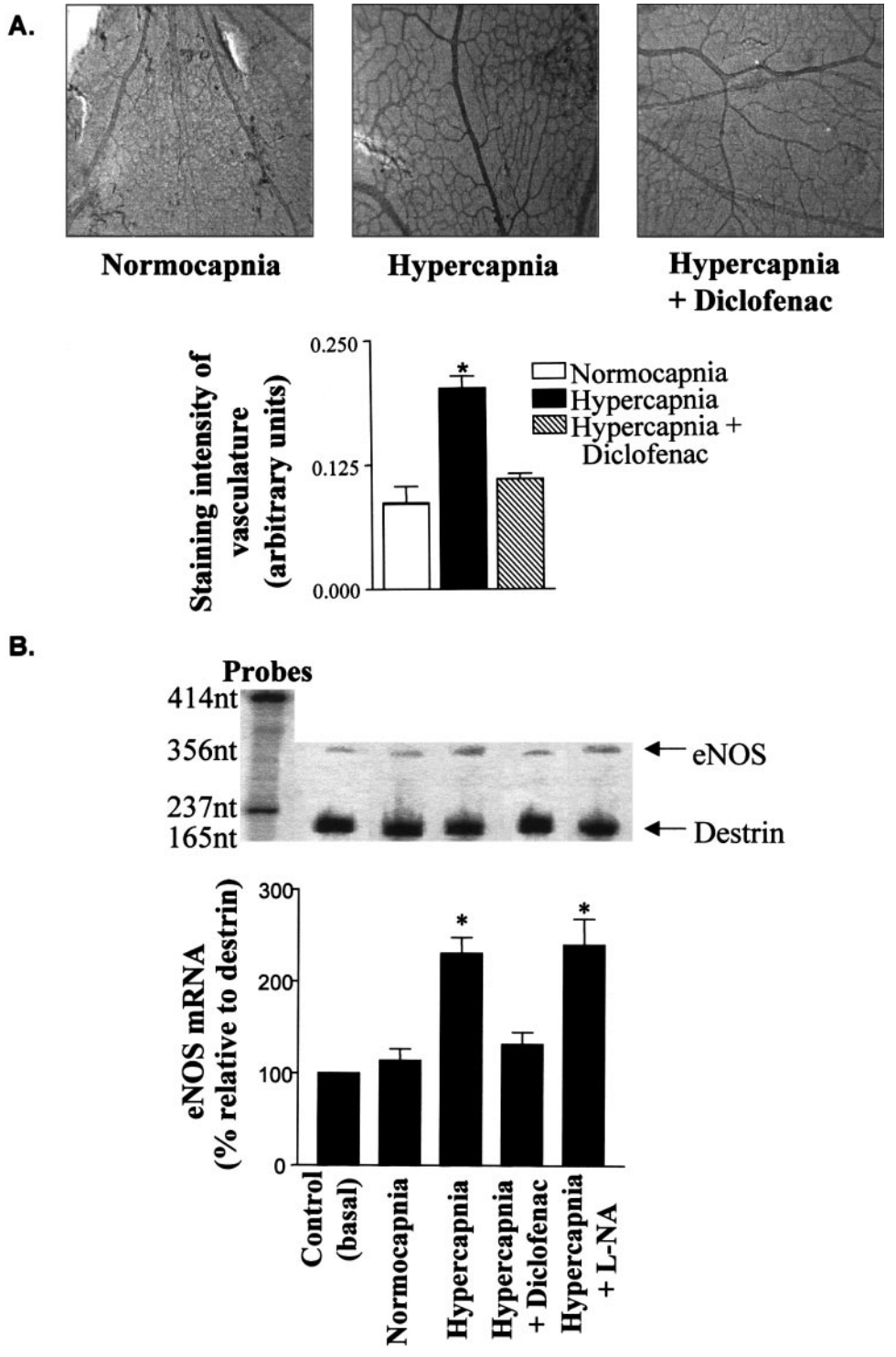


FIGURE 3. Ex vivo modulation of NADPH-diaphorase staining and eNOS mRNA expression after exposure to hypercapnia. Isolated eyecups were exposed for 6 hours to 5% CO_2 ($PaCO_2 \approx 40$ mm Hg, pH 7.4) or 9% to 10% CO_2 ($PaCO_2 \approx 65$ mm Hg, pH 7.15–7.2) in absence or presence of diclofenac (100 μM) or L-NA (1 mM). (**A**) Retinal wholemounts were fixed for NADPH-diaphorase reactivity (*top*) and individual pixel tonality of densitometry of vasculature was analyzed by correcting for background tone (*bottom*). (**B**) eNOS mRNA blot from RNase protection assay (*top*) and semiquantitative analysis with densitometry (*bottom*) relative to destrin (control). Unprotected and protected fragments for eNOS are 414 and 356 nucleotides (nt), and for destrin, 237 and 165 nt. Data in both histograms are the mean ± SEM from three retinas of different animals. $^*P < 0.05$ compared with data without *asterisks*.

Effect of Acidosis on Ca^{2+} Transients in Endothelial Cells

Acidosis-induced Ca^{2+} influx, an essential cofactor for phospholipase A_2-dependent yield of PGs, was studied in endothelial cells, which are an important source of PGs under these conditions.[39] Acidification of the medium with HCl or NaH_2PO_4 to an approximate pH of 7.10 to 7.15 caused a rapid and marked increase in Ca^{2+} transients measured directly on neuroretinovascular endothelial cells (Figs. 6A, 6B). This was prevented by bicarbonate (data not shown), SK&F96365, and EGTA, but not by diclofenac (Figs. 6A, 6B), further suggesting that the Ca^{2+} transients precede the increase in PGs (Fig. 5C).

Effects of PG Analogues on Acidosis-Induced Changes in eNOS mRNA Levels

To establish which PG is involved in the acidosis-induced eNOS expression, eyecup preparations were exposed to acidified buffer (pH ~7.15) and pretreated, or not, with diclofenac in the absence or presence of PGs. Inhibitors of selective PGE_2, $PGF_{2\alpha}$, PGD_2, and PGI_2 synthases are not yet available. The

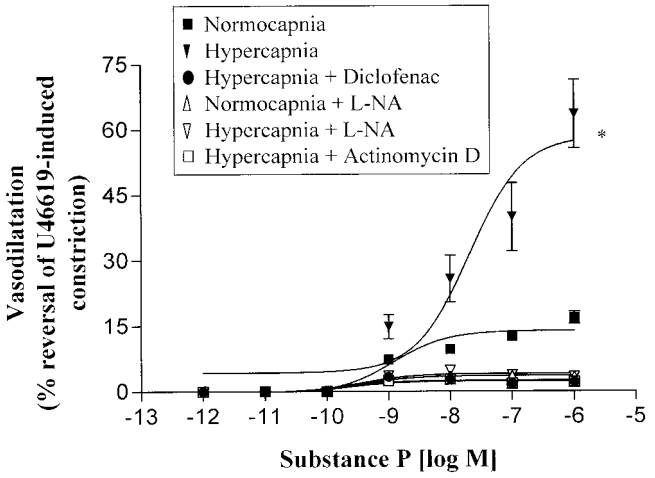


FIGURE 4. Effects of exposure to high CO_2 on the retinal vasorelaxant response to substance P. Eye cups were incubated for 4 hours in buffer bubbled with 5% or 9% to 10% CO_2 in absence or presence of diclofenac (100 μM) or actinomycin D (2 μM), as described in Figure 3. Some preparations were treated with L-NA (1 mM) 20 minutes before administration of substance P. Data are the mean ± SEM of three or four eyes of different animals. $^*P < 0.01$ compared with other curves.

inhibitory effect of diclofenac on the acidosis-induced retinal eNOS mRNA increase was prevented by concurrent treatment with 16,16-dimethyl-PGE_2, but not with $PGF_{2\alpha}$ or the stable analogues of PGD_2 and PGI_2, BW245C and carbaprostacyclin, respectively (Fig. 7).

DISCUSSION

Although hypercapnia has been found to affect ocular blood flow,[5–12] the hemodynamic changes that occur during prolonged hypercapnia and the underlying mechanisms of these changes have been unknown thus far. Piglets were used in this study to measure hemodynamic parameters more accurately, because the larger size of their ocular tissues allows for the minimum number of microspheres (~400) to be present without impairing tissue blood flow.[40] Although piglets, in contrast to rats, have a nearly mature retina at birth, oxygen-induced retinopathy has been produced in these animals.[41,42] Moreover, the early changes we observed in RBF in piglets and the role of PGs in this process are consistent with those previously reported in the same and other (monkeys) species.[8,20] During prolonged hypercapnia, the present study showed a second increase in RBF and expected O_2 delivery that was NO-mediated and associated with increased eNOS expression induced by an earlier augmentation in PGE_2 levels.

The effects of high CO_2 on vascular tone have largely been ascribed to acidosis.[43–45] The same appears to be true of prolonged exposure to hypercapnia, because normalization of the pH abolished all changes in RBF (Fig. 1A). During the 8-hour exposure to high CO_2, pH increased slightly, and MABP remained stable (Table 1), yet RBF continued to increase (Fig. 1A). This suggests that changes in RBF can be attributed to

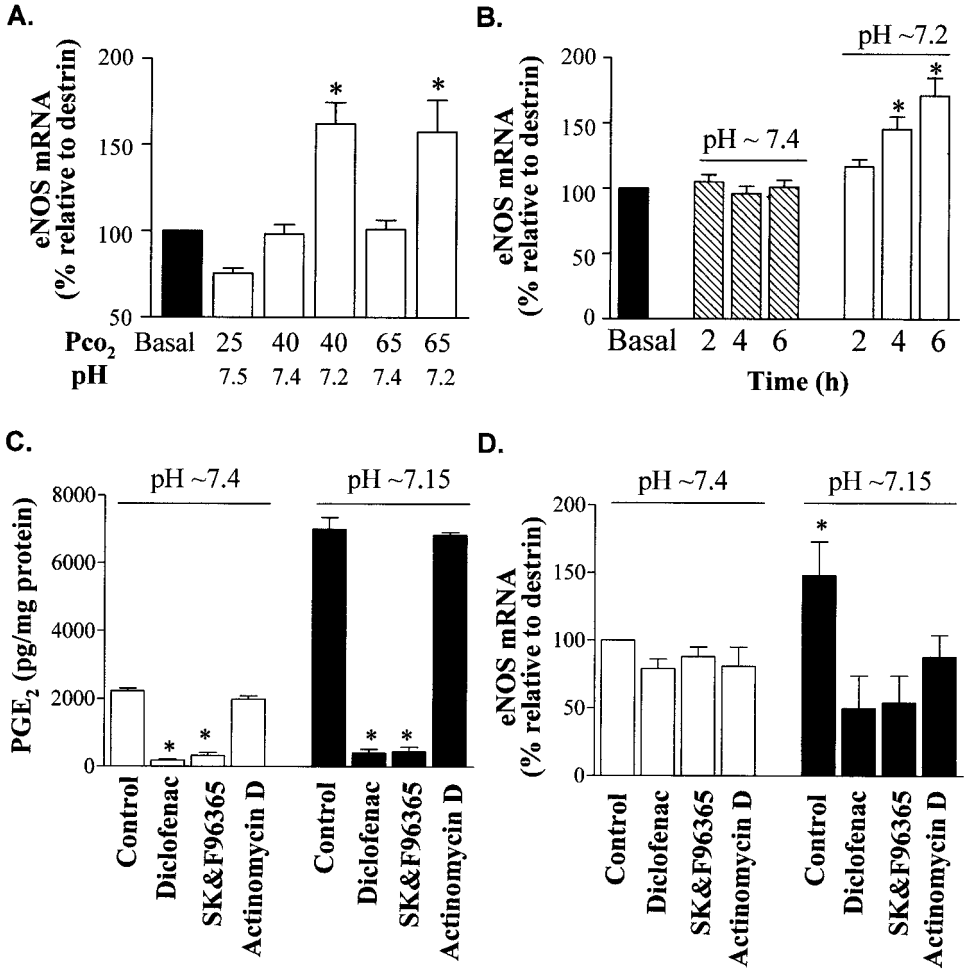


FIGURE 5. Concentration- and time-dependent effects of hypercapnic acidosis on eNOS mRNA expression in piglet retinas. (**A**) Eye cups were incubated in physiological buffer for 6 hours with 3% CO_2 ($Paco_2 \approx 25$ mm Hg, pH ~7.5), 10% CO_2 ($Paco_2 \approx 65$ mm Hg, pH ~7.2), 10% CO_2 with normalized pH ($Paco_2 \approx 65$ mm Hg, pH ~7.4) and 5% CO_2 with acidosis ($Paco_2 \approx 40$ mm Hg, pH ~7.2) or not incubated (basal). Acidosis with normal CO_2 was adjusted by addition of NaH_2PO_4 or HCl. (**B**) Time-dependent changes in eNOS mRNA after 2, 4, and 6 hours of exposure to normocapnic acidosis. PGE_2 levels (**C**) and expression of eNOS (**D**) in retinas isolated from eyecups incubated for 6 hours in normocapnic acidosis conditions in the presence or absence of diclofenac (100 μM), SK&F96365 (10 μM), or actinomycin D (2 μM). mRNA was subjected to RNase protection assay for eNOS. Data are the mean ± SEM of five experiments on eyes from different piglets. $^*P < 0.05$ compared with all other data.

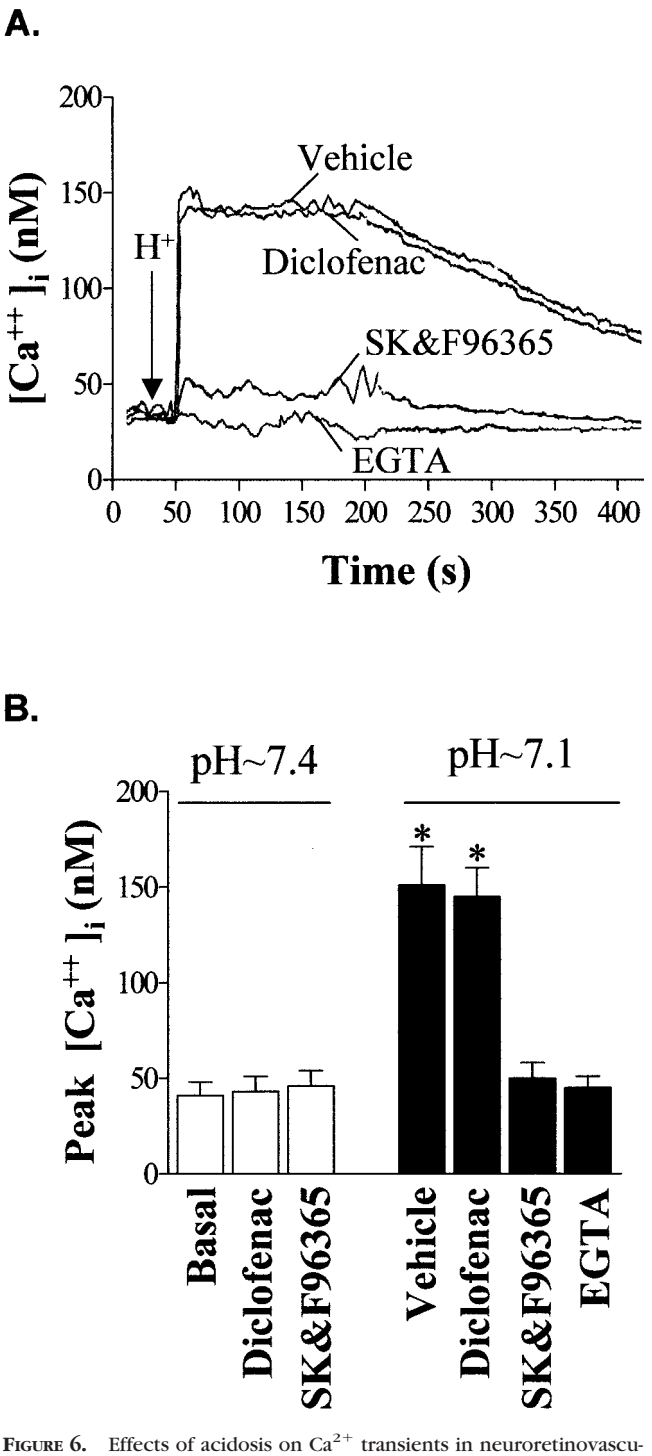


FIGURE 6. Effects of acidosis on Ca^{2+} transients in neuroretinovascular endothelial cells. Ca^{2+} transients were measured by the fura-2-acetoxymethyl ester technique after acidification of the medium by the addition of HCl or NaH_2PO_4 (pH ~7.10–7.15). (**A**) Typical tracing. (**B**) Histogram presenting peak $[Ca^{2+}]_i$. Media were pretreated with vehicle, diclofenac (100 μM), SK&F96365 (10 μM), or EGTA (5 mM). *Arrow*: time of administration of acidifying agents (H^+). Data are the mean ± SEM of three or four experiments. $^*P < 0.01$ compared with all other data without *asterisks*.

local vasomotor alterations. Once the hyperemic process is triggered, it cannot be prevented by a late normalization of the pH, and the effects of hypercapnic acidosis on RBF are delayed, perhaps because of a relatively slower process, such as gene transcription.

A number of observations imply a major role for eNOS induction in the second hypercapnia-induced hyperemia. A direct effect of acidosis on NOS activity is unlikely, because acidosis would tend to reduce it.[46] However, retinal NO_2^- production increased late into the hypercapnic exposure (Fig. 1C). Also, the nonselective NOS inhibitor L-NA prevented the late increases in RBF (Fig. 1A), whereas TRIM, a selective inhibitor of iNOS and nNOS,[30] did not affect the retinal hemodynamics (Fig. 1A). The second increase in RBF parallels the time course profile of eNOS mRNA expression (Fig. 5B), which was further manifested functionally by augmented NADPH-diaphorase activity in retinal vasculature (Figs. 2, 3) and eNOS-dependent retinal vasorelaxation to substance P (Fig. 4). Along these lines, the mechanisms underlying the effects of prolonged hypercapnia on RBF cannot be explained by the mere activation of PG and NO synthases. The inefficacy of late (at 5.5 hours) diclofenac administration on RBF, compared with its efficacy when administered at the onset of hypercapnia (Fig. 1A), supports this inference. Furthermore, the transcription inhibitor actinomycin D prevented both the delayed hypercapnia-induced increase in eNOS mRNA and vasorelaxation to substance P (Figs. 4, 5). Thus, collectively, the data are indicative of the triggering of de novo eNOS expression in retinal vasculature by hypercapnic acidosis.

The role of PGs in regulating eNOS expression in the retina during prolonged hypercapnic acidosis is a salient feature of this study. Evidence supporting this major role for PGE_2 includes an early increase in PGE_2 levels during hypercapnia and prevention by diclofenac of the hypercapnia-induced eNOS expression (mRNA and in situ protein activity), as well as retinal hyperemia and increased vasorelaxation in response to substance P (Figs. 1 to 5). In addition, PGE_2 was found to be the major PG that modulates eNOS expression in retina (Fig. 7), as reported in brain vasculature.[25] The mechanisms that

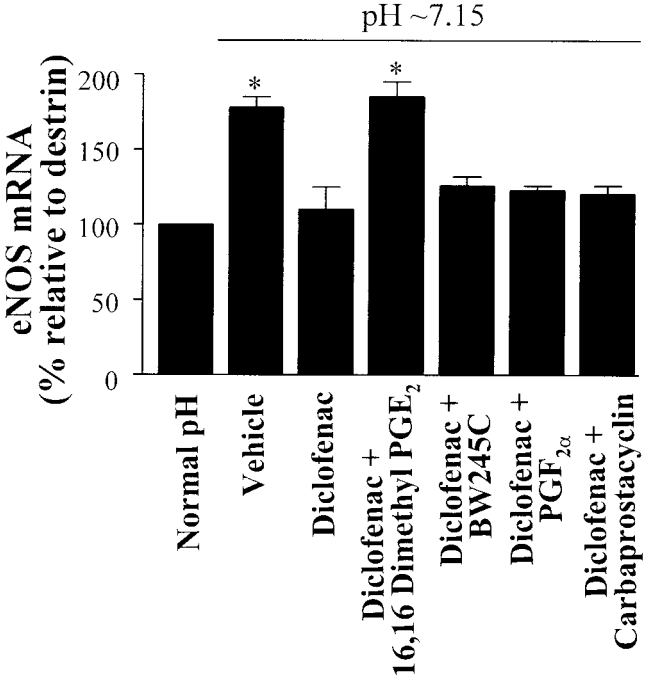


FIGURE 7. Effects of PG analogues on retinal eNOS mRNA expression from eyecups exposed to HCl-induced acidosis. Tissues were exposed for 6 hours to acidosis, as described in Figure 5 and treated with diclofenac (100 μM), with or without 16,16-dimethyl-PGE_2, BW245C, $PGF_{2\alpha}$, or carbaprostacyclin (1 μM each). Data are the mean ± SEM of three experiments conducted on eyes from different piglets. $^*P < 0.05$ compared with all other data without *asterisks*.

lead to stimulation of PG formation are not clear from this study. Phospholipase A_2 and cyclooxygenase are unlikely targets, because their activities are optimal at basic pH.[47] However, hypercapnia-induced Ca^{2+} influx as we observed (Fig. 5, 6), possibly mediated directly through stimulation of Ca^{2+} channels[48,49] or indirectly by K^+ channels,[50] would provide a necessary cofactor for formation of PG. Although endothelium is a likely important source of PGs during acidosis,[39] participation by specialized neurons[48,49] and vascular musculature[51] cannot be fully excluded; however, smooth muscle, astroglia, and neurons including those in the retina, either do not form PGs or exhibit a calcium influx.[52–54]

In conclusion, the present study disclosed a previously unexplored mechanism for late retinal hyperemia during sustained hypercapnia that appears secondary to the induction of eNOS expression and activity mediated by PGE_2, as recently described in brain tissue.[16] Therefore, PGs seem to be involved in both the acute and prolonged hypercapnia-induced increased retinal blood flow through direct and indirect vascular effects, respectively. The findings provide a mechanism for extended hypercapnia in predisposing to ROP[1–4] and are consistent with a role for PGs[55] and eNOS[56] in the development of this oculovascular disorder.

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