Review

Oxidants, nitric oxide and prostanoids in the developing ocular vasculature: a basis for ischemic retinopathy

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Abstract

The choroid is the main source of oxygen to the retina. In contrast to the adult, the absence of autoregulation of choroidal blood flow in the newborn leads to hyperoxygenation of the retina. In the immature retina which contains relatively low levels of antioxidants this hyperoxygenation favors peroxidation including the generation of biologically active isoprostanes, and results in vasoconstriction and vascular cytotoxicity leading to ischemia, which predisposes to the development of a vasoproliferative retinopathy, commonly termed retinopathy of prematurity. During frequently encountered oxidative stress to the perinate, the combined absence of vascular autoregulation and excessive oxygen delivery to the eyes of the developing subject is largely the result of a complex epigenetic and genetic interplay between prostanoids and nitric oxide (NO) systems on vasomotor regulation. The effects of certain prostaglandins are NO-dependent; conversely, those of NO have also been found to be largely prostaglandin \textit{I\textsubscript{2}}-mediated in the eye; and NO synthase expression seems to be significantly regulated by other prostaglandins apparently through activation of functional perinuclear prostanoid receptors which affect gene transcription. The increased production of both prostaglandins and NO in the perinate augment ocular blood flow and as a result oxygen delivery to an immature retina partly devoid of antioxidant defenses. The ensuing peroxidation results in impaired circulation (partly thromboxane \textit{A\textsubscript{2}}-dependent) and vascular integrity, leading to ischemia which predisposes to abnormal preretinal neovascularization, a major feature of ischemic retinopathy. Because tissue oxygenation is largely dependent upon circulation and critical in the generation of reactive oxygen species, and since the latter exert a major contribution in the pathogenesis of retinopathy of prematurity, it is important to understand the mechanisms that govern ocular blood flow. In this review we focus on the important and complex interaction between prostanoid, NO and peroxidation products on circulatory control of the immature retina. © 2000 Elsevier Science B.V. All rights reserved.

\textit{Keywords}: Blood flow; Free radicals; Nitric oxide; Prostaglandins

1. Introduction

Vascular pathologies induced by ischemia/reperfusion result in part from the production of reactive oxygen species (ROS). Several evidences indirectly suggest that ROS play a major role in the genesis of vasoproliferative retinopathy [1–7]. Increased retinal oxygenation which results in augmented ROS generation is a major factor in the pathogenesis of retinopathy of the immature subject, commonly termed retinopathy of prematurity (ROP) [4,8–10]. In the initial phase of development of ROP the retinal vasculature constricts markedly and is subsequently associated with degeneration of endothelium resulting in vas-
oobliteration which leads to cessation of the vascular front to progress towards the periphery [11,12]; this results in retinal ischemia which predisposes to abnormal preretinal (intravitreal) neovascularization in the later phase [11,13–17].

In the newborn, in contrast to the adult [18–20], there is an inability of retinal and choroidal circulation to limit excess delivery of O$_2$ [21–23], partly due to insufficient constrictors but mostly secondary to increased formation of vasorelaxants in neonates [23–25]. As a result retinal oxygenation increases, and combined with a restricted ability of the newborn to readily dispose off free radicals [26,27], it facilitates the generation and propagation of peroxides [23]. The free radicals in turn are involved in a cascade of events in the retina which culminates in vasocostriction and ultimately in endothelial cell degeneration (vasoobliteration) [28–33], a major feature which precedes neovascularization in ROP [11,15,16,34]. Hence, retinal hyperoxegenation leads to free radical generation which is a major factor in the genesis of ROP [1–11,35,36]. Since oxygen delivery is largely dependent upon circulation, it is important to understand the mechanisms that govern ocular blood flow.

Oxidative stress causes the release of prostaglandins (PGs) and nitric oxide (NO) from the ocular vascular endothelium, both of which participate significantly in the regulation of ocular vascular smooth muscle tone. PG levels and NO synthase (NOS) activity are relatively high during the perinatal period [23,37,38]. A complex epigenetic and genetic interaction between these two systems has been uncovered. The effects of some PGs are NO-dependent [39,40], those of NO are largely mediated via PGIs, in the eye [41], and specific PGs regulate endothelial NOS (eNOS) expression and activity in ocular blood vessels [42]; moreover, the transcriptional regulation of eNOS by PGs seems to be mediated via recently identified functional perinuclear PG receptors [43–45]. These factors augment ocular blood flow and in turn oxygen delivery to the retina, such that because of its immature antioxidative systems free radical generation is facilitated in the newborn. The ensuing peroxidation causes opposite vascular effects, namely an impairment in ocular circulation (partly thromboxane A$_2$ (TXA$_2$)-dependent [30]) and in vascular integrity, resulting in ischemia. The latter predisposes to abnormal preretinal neovascularization seen in ischemic retinopathies including ROP [15,16].

This review discusses mechanisms of ocular blood flow regulation during conditions of oxidant stress by focusing on the roles of and interaction between free radicals, PGs and NO in the ocular vascular system, which contribute in retinal hyperoxegenation and vasculopathy of the developing subject, namely ROP [21,23].

2. The choroid and oxygen supply to the retina

Two separate vascular systems are primarily involved in supplying the eye with nutrition and oxygen: the retinal vessels and the uvea, which contains the choroid in its posterior segment. The retinal vessels supply the inner retina, whereas the outer layers that include the photoreceptors, the portion that consumes most of the oxygen, are nourished by the choroid [46]. Because vascularization of the retina is incomplete in the preterm subject whereas choroidal vascularization is completed early in gestation, this tissue plays a more important role in supplying the neural retina in the immature newborn [18]. Indeed choroidal blood flow (ChBF) has clearly been shown to be extremely high with a low oxygen extraction making the choroid a tissue of great importance for the supply of oxygen and nutrients to the retina [21,46–48]. In contrast, retinal blood flow (RBF) is markedly lower [21,46]. This high ChBF appears to be needed during the development of the retinal vascular bed.

The immature retina is sensitive to oxygen toxicity [49]. Increased retinal oxygenation (hyperoxia) is a critical factor in the development of vasoproliferative ROP [50,51]. Both the absolute oxygen tension of gas used and the duration of its administration are believed to be important components in the genesis of ROP. Control of retinal oxygenation is mostly dependent upon choroidal circulation [52]; thus, retinal hyperoxia can occur provided the choroid, a vascular tissue and the major supply of oxygen to the retina, is not able to limit oxygen delivery. In fact, the newborn choroid in contrast to the adult is unable to autoregulate during hyperoxia, which leads to an increase in oxygen delivery to the retina which in turn favors lipid peroxidation [23]. Hence, the choroidal circulation seems to play a key role in the early phase of the pathogenesis of ROP.

3. Autoregulation of retinal and choroidal blood flow

RBF is maintained constant over a wide range of perfusion pressure (45–145 mmHg) in the adult [18,25,46,53–56]. In contrast, RBF is autoregulated over a very narrow range of perfusion pressure (45–85 mmHg) in the newborn [21] (Fig. 1A). This limited range of autoregulation in the newborn suggests that there is insufficient vasoconstriction when perfusion pressure is raised.

ChBF is also autoregulated in the adult over a relatively wide range of perfusion pressure [52,57,58]. However, there is almost complete absence of autoregulation of ChBF in the newborn [21–23,25]. Consequently, when blood pressure is modestly increased (above the upper limit of autoregulation) as it often happens in the sick newborn due to iatrogenic manipulations such as endotracheal intubation and aspiration [59], ChBF and oxygen delivery increases. The excess oxygen supplied to the retina is not consumed and results in the generation of reactive oxygen species that may be deleterious to the eye [22,28,60].

Ocular blood flow autoregulation also operates in re-
response to changes in blood oxygen tension. Both in the adult and the newborn [14,20,23,61–64], the retinal vasculature constricts comparably in response to hyperoxia. However, ChBF decreases during hyperoxia in the adult [23,65], but in the newborn it increases (Fig. 1B). As a result of the retinal hyperoxia peroxidation is triggered and propagates in the newborn devoid of completely developed antioxidant systems (Fig. 1C) [23]. In the clinical setting, premature infants are subject to rises in blood oxygen tension which frequently lead to hyperoxygenation. Hence, the absence of a fully developed autoregulatory control of ocular blood flow in the newborn favors retinal hyperoxygenation and peroxidation [21–23].

The mechanisms that govern ocular blood flow autoregulation are not yet fully understood. We will focus on the interactive role of free radicals, prostaglandins and NO on this major vascular physiological event, namely autoregulation of blood flow.

4. Prostanoids and ocular circulation

Prostanoids are among the most important autacoids that exert a remarkably large variety of physiological and pathophysiological actions in nearly all mammalian tissues [66]. Major prostanoids are derived from arachidonic acid mainly by enzymatic catalysis [67,68]. Prostanoid-like substances termed isoprostanes can also be produced by free radical-mediated peroxidation [69–71]; these will be discussed in Section 6.4. The initial step in the synthesis of major prostanoids is mediated by cyclooxygenase (COX) [72]. COX converts arachidonic acid to PGH₂, which is then acted upon by distinct prostaglandin synthases to yield different prostanoids (PGD₂, PGE₂, PGF₂α, PGI₂, and TXA₂) [73]. COX enzymes are membrane-bound hemoproteins and include two isozymes. The constitutive form (COX-1) is almost ubiquitously expressed and is responsible for the low prostaglandin synthesis required for cell homeostasis. The inducible form (COX-2) is synthesized de novo in response to a wide range of extracellular and intracellular stimuli (cytokines, growth factors and tumor promoters) in the course of inflammation or other cellular stresses [73,74]; in addition, COX-2 is developmentally regulated [37]. Oxidant stress may induce COX-2 since it can be activated by intracellular peroxides whereas much higher peroxide levels are needed to activate COX-1 [75,76]. Both COX-1 and COX-2 are located on the luminal surface of the endoplasmic reticulum and in inner and outer nuclear membranes [77,78].

Prostanoids are charged anions at physiological pH and
diffuse poorly across biological membranes; their flux across the plasma membrane is largely controlled by a prostaglandin transporter (PGT), which is discussed in Section 5.6. Prostanoids are presumed to act on G-protein-coupled receptor [66] classified as FP for PGF_{2α}, DP for PGD_{2}, IP for PGI_{2}, TP for TXA_{2} and EP for PGE_{2} [79–81]. EP receptors have been further subdivided into EP_{1}, EP_{2}, EP_{3} and EP_{4} subtypes [80–82]; in addition, eight isoforms of EP_{3} have been cloned in humans [83]. Activation of FP and EP receptors increases inositol 1,4,5-triphosphate (IP_{3}) production and that of EP_{2} and EP_{4} increases adenosine 3′,5′-cyclic monophosphate (cAMP) formation [84–91]. Stimulation of EP_{3} receptors may decrease cAMP formation or increase IP_{3} production [92]. EP receptors, mainly of the EP_{3} subtype, are the most diverse of the prostaglandin receptors and are found in nearly every tissue [81].

4.1. Retinal and choroidal prostanoids in control of ocular circulation

Prostanoids are produced by the retina and choroid [93–96]; their production is significantly higher in perinatal than in adult ocular tissues [38,39]. Prostanoids play important roles during hypotension, hypertension and in response to changes in blood gases but do not seem to control basal circulation [21,22,30,97]. Prostanoids exert major effects on the autoregulatory range of RBF and ChBF in the newborn animal [21,22]. During a rise in perfusion pressure PGE_{2} and PGF_{2α} are abundantly released by the ocular vasculature causing vasoconstriction in the adult [22]; PGI_{2} and PGD_{2} are also released during acute hypertension albeit to a lesser extent, whereas TXA_{2} is not. In the newborn PGF_{2α} exerts negligible effects and PGE_{2} produces primarily a choroidal vasorelaxation [39]. In addition, PGI_{2} and PGD_{2} cause greater relaxation in the newborn than in the adult [40]. However, the age-dependent difference in response to constrictor prostanoids (PGE_{2} and PGF_{2α}) is greater than that to dilators (PGI_{2} and PGD_{2}) [39,98], such that overall the immature subject responds more to relaxants and less to constrictors than the adult. Hence, developmental divergence in the vasomotor actions of PGs seem to contribute to the ontogenic differences in ocular blood flow autoregulation [38,39].

4.2. Prostaglandin receptors in the ocular vasculature

The retinal microvasculature contains all prostaglandin receptors with the exception of EP_{1} [24]; the choroidal vasculature includes EP_{4} [39]. A decrease in retinal and choroidal vasoconstriction to PGE_{2} and PGF_{2α} in the newborn was found to be mostly associated with a reduction in corresponding receptors when compared to the adult [24]; however, relaxation to DP, EP_{2} and EP_{4} was also augmented in the newborn tissues. The neonatal deficiency in choroidal FP, EP_{1} and EP_{3} receptors, which are coupled to vasoconstriction, was found to be secondary to their homologous down-regulation by high PG levels in the perinatal period [39,40]. Accordingly, inhibition of COX for 24 h in newborn pigs results in an upregulation of PGE_{2} and PGF_{2α} receptors, receptor-coupled transduction mechanisms and vasomotor response to values comparable to the adult [24,39,40].

In contrast to constrictor PGs, increased relaxation to IP, DP, EP_{2} and EP_{4} is secondary to augmented coupling to adenylyl cyclase (for IP) and more importantly to nitric oxide synthase (NOS); interestingly, the regulation of this increased coupling action in response to stimulation of IP, DP, EP_{2} and EP_{4} is itself governed by prostanoids [42,45,99] (discussed in Section 5), without affecting receptor expression itself [40].

In summary, high perinatal levels of PGs exhibit an important role in regulating the expression of their receptors and coupling, such that on the one hand it leads to down regulation of receptors coupled to vasoconstriction, and on the other hand to increased coupling action of receptors involved in vasodilatation. In the immature subject, when released during autoregulatory adjustments PGs outdo the effects of constrictors [21]. Altogether these observations are consistent with an inability of the newborn RBF and ChBF to autoregulate, which results in excess delivery of oxygen to retina [21,23].

4.3. Eicosanoids in the ocular vasculature

Eye tissue responds to physiological and pathological stimuli by the activation of phospholipases and the consequent release from membrane phospholipids of biologically active metabolites. Activation of phospholipase A_{2} is the first step in the synthesis of two important classes of lipid second messengers, the eicosanoids and platelet-activating factor (PAF). In addition to the metabolism of arachidonic acid (AA) into major prostanoids by the COX pathway, AA can also be metabolized by lipoxygenases and monoxygenases, and separately non-enzymatically (into isoprostanes; see Section 6.4.). Lipoxygenases (LOX) convert AA into leukotrienes, hydroperoxyeicosatetraenoic acids (HPETEs) which are reduced into hydroxyeicosatetraenoic acids (12-HETE, 15-HETE), and other monohydroxy isomers [93,100,101]. Activation of another metabolic pathway, cytochrome P450 enzymes, catalyze monoxygenation of AA into (a) epoxidation giving rise to four regioisomers, 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acid (EETs), (b) allylic oxidation to produce six regioisomers, 5-, 8-, 9-, 11-, 12- and 15-hydroxyeicosatrienoic acid (HETEs), and (c) ω/ω1 hydroxylation to produce 19- and 20-HETEs [102]; in ocular tissue 12-HETE seems to be a major product including in vascular endothelium [103,104]. However, the physiologic role of these autacoids in ocular vasculature remains for the most part to be determined; albeit proposed roles have been suggested for some.
LOXs are highly regulated lipid-peroxidizing enzyme whose expression and metabolites are implicated in several important inflammatory conditions. Peptido-leukotrienes (C4/E4/D4) enhance microvascular permeability [105]. 12-HETE is a potent vasoconstrictor, chemotactic and angiogenic factor whose synthesis is induced in inflamed tissues [106]. The 15-HETEs produced mainly by the vascular endothelial cells are known to evoke contractions in a variety of isolated blood vessels in a dose-dependent manner [107]. The 15-HETE-induced vasoconstriction is likely to occur by direct activation of thromboxane A2 receptors on smooth muscle and the vasorelaxation probably via the release of an endothelium-derived relaxing factor including COX metabolites [108]. In addition, 15-HPETEs seem to decrease cell proliferation whereas 15-HETEs produce opposite effects; concentrations of the latter have been reported to be significantly increased compared to 15-HPETEs in patients with proliferative vitreoretinal diseases [109].

PAF also accumulates in the eyes in response to inflammatory reactions and ischemia [96,110]. PAF is a potent vasoconstrictor and a modulator of vasomotor tone and blood pressure [111]. Some of the effects of PAF could be mediated by AA metabolites generated after PAF-receptor interaction with the COX and LOX pathways [112,113]. As for the LOX metabolites the role of PAF in the ocular vasculature needs to be further studied.

Since LOX products have potent chemotactic, hypertrophic, and mitogenic effects in vascular cells, and that PAF can enhance LOX and COX activities and expression and contribute to the angiogenic effect of vascular endothelial growth factor (VEGF), the combine interaction of LOX- and COX-derived metabolites and PAF during oxidative stress may be one of the mechanism, at least in part, involved in angiogenesis [114].

5. Nitric oxide

NO plays an important role in the control of ocular vascular tone and blood flow of the newborn and adult [23,25,115–117]. NO is a radical gas albeit not very reactive. NO formation is catalyzed by NOS enzymes in the process of conversion of arginine to citrulline. NO is a potent signaling molecule in blood vessels, where a continuous formation from endothelial cells acts on the underlying smooth muscle to maintain vasodilatation and blood flow. NO stimulates the production of cGMP via guanylyl cyclase activation in smooth muscle cells; more recently a major PGI2-mediated action of NO has been uncovered in ocular vasculature [41]. NO can also affect the vascular system through its ability to inhibit platelet aggregation and adhesion [118]. Under physiological conditions, NO can be found among three redox forms (i) nitrosonium (NOO−), (ii) nitric oxide (NO), and (iii) nitroxy anion (NO2−) favoring different interactions [119]. Rapid removal of NO by oxygen radicals and metalloprotein limits its spread to a few hundred microns and shortens its half-life to seconds. Three isoforms of NOS have been identified to date. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutive isoforms that produce rapidly small amounts of NO (picomolar concentrations within few seconds or minutes) in response to an increase in intracellular calcium concentration. Inducible NOS (iNOS), independent of calcium, catalyzes generation of large amounts of NO (nanomolar concentrations) over extended periods (hours or days) in response to inflammatory stimuli such as cytokines and lipopolysaccharides [120].

5.1. Localization of nitric oxide synthase in the eye

NADPH diaphorase staining (a histochemical reaction which reflects NOS activity) is present throughout the visual system [121]. NOS has been detected in retinal neurons and pigment epithelium [122,123], in amacrine cells and ganglion cells [115,124,125], in nerve fibers in the outer and inner plexiform layers, in photoreceptor ellipsoids and in perivascular nerve fibers of the choroid [115,116,126]. Neuronal NOS immunoreactivity is present in amacrine cells, horizontal cells and in photoreceptor cells in different species [115,127,128]. Endothelial NOS immunoreactivity is present in the vascular endothelium of the retina and the choroid [129]. Inducible NOS has been found to be constitutively expressed in retinal pigment epithelium, ciliary epithelial cells, Müller cells, retinal parenchyma, choroid vasculature and pericytes [23,115,129–132].

5.2. Nitric oxide in the control of ocular circulation

As indicated above, both eNOS and nNOS are present in retina and choroid. NO from eNOS is largely involved in resting circulation [23,25,116,117,133–136]. NO from nNOS arises mostly from the non-adrenergic and non-cholinergic parasympathetic nerve fibers innervating the choroid [126,137], and seems to affect blood flow response to adaptive circulatory changes such as acute alterations in perfusion pressure [116], consistent with recent observations suggesting a role for nNOS in cerebral blood flow response to blood pressure changes in the newborn [138]. Nonetheless, one cannot exclude in this process a role for microvascular eNOS which is activated by shear stress [139].

Both eNOS and nNOS expression and activity are increased in choroidal and neuroretinal tissue of the perinatal subject [23,25,42,99,138]. Increased NO formation in the newborn exerts an important effect on vasomotor tone which masks those of constrictors implicated in autoregulatory responses [23,25,140]. Accordingly, inhibition of NOS enhances ChBF response to hyperoxia as well as RBF and ChBF response to acute hypertension in the
newborn animal, to levels approaching those of the adult [23,25]; interestingly, the enhancement in ocular blood flow autoregulation by NOS inhibition is similar to that produced by COX inhibition [21,38]. This improved autoregulation exerted by NOS inhibition stabilizes oxygen delivery (Fig. 2) and prevents retinal peroxidation during hyperoxia [23,25]. In contrast, NO does not seem to contribute to ocular blood flow adaptation to hypotension or hypoxia [136,141]. Therefore, excess relaxation secondary to increased NO synthesis seems to curtail a relatively adequate RBF and ChBF autoregulatory response to increased perfusion pressure and oxygen tension in the newborn. Thus a reduction in NO synthesis in the newborn could improve the control of $O_2$ delivery to the retina without apparently compromising the lower limit of autoregulation.

5.3. Interaction between prostaglandins and nitric oxide

Data reported to date reveal that both PGs and NO play a significant and comparable role in the control of RBF and ChBF autoregulation. Hence, possible interactions between these systems are conceivable and have in fact been uncovered. The oculovasorelaxant effects in response to stimulation of the DP, EP$_2$ and EP$_3$ receptors have all been partly attributed to NO generation [39,40]. NO has also been suggested to stimulate COX pathway which in turn affects the autoregulation of ocular blood flow [23,142,143]. But this effect is not mediated through direct interaction of NO with COX [144]. Rather, NO has been shown to stimulate prostaglandin (mainly PGI$_2$) formation via successive stimulation of $K_{Ca}^+$ and non-voltage-gated Ca$^{2+}$ channels localized on the endothelium [41]. In contrast to its primary action of guanylate cyclase, this mode of action via PGI$_1$ turns out to mediate $>75\%$ of the action of NO on the ocular vasculature (Fig. 3). Thus, acute vasomotor effects of specific prostaglandins (PGD$_2$ and PGE$_2$) are NO-dependent, and vice versa those of NO are mediated by distinct prostaglandins, PGI$_2$. These findings highlight significant bidirectional interactions.

![Fig. 2](https://academic.oup.com/cardiovascres/article-abstract/47/3/489/344428)
Fig. 3. Postulated mechanisms of NO-induced ocular vasorelaxation during an oxidative stress involving an interaction between cGMP, cyclooxygenase-derived free radicals (R⁺), prostacyclin (PGI₂), calcium-dependent potassium channels (KCa), non-voltage operated calcium channels (NVOC) and voltage-dependent potassium channels (Kv). The abbreviation MBP, AC, GC, AA, R and FR, ROP, refers, respectively, to mean blood pressure, adenylate cyclase, guanylate cyclase, arachidonic acid, free radical, and retinopathy of prematurity.

between these two systems; genetic interactions between PGs and NOS regulation are presented below (Section 5.5).

5.4. Regulation of nitric oxide synthase

The analysis of the loci of the three distinct genes encoding the family of human NOS proteins reveals that mechanisms implicated in controlling mRNA expression and structure are unique for the different NOS isoforms. iNOS is an immediate early gene product and is transcribed readily in response to various inflammatory cytokines, endotoxins and oxidants [145]. Although the nNOS and eNOS isoforms were initially described as constitutive, both can also be induced. Expression of nNOS can be regulated by various physiological and pathophysiological conditions, including sympathetic activity, acute heat stress and estrogen [146–148]. The eNOS gene is also subject to expression regulation in response to various physiological or pathological stimuli with important consequences on vascular homeostasis [149,150].

Myristylation, palmitoylation and tyrosine phosphorylation target eNOS to the Golgi membrane and plasmalemmal caveolae that are critical for endothelial NO production [151]. The promoter region of the eNOS gene contains consensus sequences for the binding of transcriptional factors such as AP-1, AP-2, NF-1, NF-κB, shear stress- and cAMP response elements as well as half sites of estrogen-responsive elements which can modulate the expression of this gene during different conditions [152]. In addition to its effects on NOS activation [139], shear stress increases eNOS mRNA and protein [153], whereas TNF-α decreases NOS mRNA posttranscriptionally [154]. The expression of the eNOS gene has been shown to be reduced during hypoxia by transcriptional and posttranscriptional mechanisms resulting in suppression of NO release [155,156]. Endothelial NOS expression and activity are also upregulated by estrogen which contributes to the high eNOS expression in the fetal pulmonary endothelium during the perinatal period [140,150].

5.5. Prostaglandin-induced regulation of NOS expression

More recently a major role for PGD₂ acting via DP
receptors has been shown to govern developmental increases in eNOS expression in choroid [42]. This regulation of eNOS by PGD₂ was manifested in the control of vasomotor tone as well as on autoregulation of ChBF, such that sustained inhibition of DP led to a decrease in eNOS expression, reduced vasorelaxation and relatively enhanced vasoconstriction in response to increases in perfusion pressure [5,42]. On the other hand, in neurons and neurovascular endothelium eNOS and nNOS are, respectively, developmentally regulated mainly by PGE₂ acting via EP₂ receptors and not by DP despite its presence in these tissues [45,99]; this EP₂-dependent regulation of e- and nNOS also affects neurovascular tone. Reasons for distinct receptor involvement on such similar functions on separate tissues are not clear at this point; expression of different EP₂ subtypes coupled to dissimilar second messengers may explain in part the role of different prostaglandin receptors in NOS regulation.

The mechanism by which PGs act to induce NOS expression has recently begun to be addressed. So far the biological actions of PGs have been attributed to result from their interaction with cell surface receptors [81]. However, several lines of evidence suggest that prostanooids may also act intracellularly. (1) The enzymes implicated in the synthesis of prostanooids, namely COX-1, COX-2, and phospholipase A₂ (PLA₂), have been found to be localized at the nuclear envelope [77,78,157]. (2) Intracellular binding of prostanooids has been detected [158]. (3) A PGT which plays a key role in prostaglandin transport has recently been identified [159–161]. In addition to transporting prostaglandins for intracellular metabolism, the PGT may also facilitate intracellular actions of circulating prostanooids. The PGT preferentially transports PGE₂, PGF₂α, PGD₂ with high affinity and to a lesser extent, TXA₂ and PGI₂ [160].

Extensive studies have clearly demonstrated the existence of prostaglandin receptors at the perinuclear envelope in a variety of cells and tissues [43,44]. Moreover, stimulation of isolated intact nuclei from vascular endothelial cells with PGE₂ increased transcription of iNOS [43]. Furthermore, PGE₂-evoked eNOS transcription in neurovascular endothelial cells was inhibited by PGT blockers, suggesting a major role for the intracellular receptors in mediating eNOS regulation in this tissue in response to released PGs [45]. This novel concept describing functional perinuclear G protein-coupled prostanooid receptors sets forth new perspectives for the biological actions of PGs. A model depicting the mechanism of action of PGs in modulating eNOS expression is presented in Fig. 4.

5.6. Interaction of nitric oxide with oxidant species: peroxynitrite

During oxidant stress superoxide is readily generated. Under basal condition, nitric oxide undergoes a rapid biradical reaction with superoxide anions to form peroxynitrite [162]. This reaction, and hence the formation of peroxynitrite is augmented in inflammatory conditions such as ischemia–reperfusion injury when both substrates are present in favorable concentrations. NO is the only currently known biological molecule produced in high enough concentrations to react fast enough with superoxide to outcompete endogenous superoxide dismutase [163,164]. The formation of peroxynitrite is complex and the reader is referred to major recent reviews [165–168].

Peroxynitrite interacts with a number of biotargets, such as heme containing proteins where the iron is in its ferrous state, peroxidases, seleno-proteins such as glutathione peroxidase, proteins with zinc-thiolate centers such as DNA-binding transcription factors [144]. In contrast to mostly beneficial and cytoprotective effects of NO, the generation of peroxynitrite has mainly been attributed with cytotoxic effects [169–172]; however, in vivo when thiol-containing agents (glutathione, albumin, cysteine) are available to convert the peroxynitrite anion to nitrosothiols and related products it may exhibit protective properties [173]. Peroxynitrite formation, which is regulated by tetrahydrobiopterin and arginine supply, is largely dependent upon superoxide availability [174–178]. However, the ratio of superoxide to NO cannot be excessive otherwise peroxynitrite is limited [168]; such is observed during abundant neutrophil generation of superoxide. Potential pathological consequences for the action of peroxynitrite on ocular blood vessels and tissues are numerous. The ischemia–reperfusion that predisposes to vasoproliferative retinopathy elicits the formation of superoxide, that causes loss of the vasodilatory action of NO and at the same time yields peroxynitrite. Subsequently, peroxynitrite nitrates and inactivates PGI₂ synthase but not thromboxane synthase causing vasospasm, platelet aggregation, and thrombus formation via thromboxane A₂ [179]. The generation of peroxynitrite can also initiate lipid peroxidation which results in impaired circulation and vascular integrity [180]. Peroxynitrite can affect mitochondrial respiration causing cellular energy failure, contractile dysfunction and cell death [181–184]. During an oxidative stress, the interaction of peroxynitrite and thromboxane can cause a loss of intercellular communication which impair enhancement of vascularization of ischemic tissues [185,186]. In retinal tissue peroxynitrite also contributes to increase the permeability of microvascular endothelium during oxidant stress [187]. Furthermore, peroxynitrite-mediated protein nitration may participate in photoreceptor degeneration [188], a dysfunction recently identified in ROP and in oxygen-induced retinopathy (OIR) model of ROP [189–193]. Hence, one could speculate that peroxynitrite, as is the case with other oxidants, partakes in the genesis of ischemic retinopathies such as ROP.

The greatest reactivity of peroxynitrite in the physiological state seems to be with CO₂, which is present in high concentrations in intra- and extracellular compartments
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Fig. 4. Proposed mechanisms of developmental regulation of endothelial nitric oxide synthase by prostaglandins in the ocular vasculature. The abbreviation MBP, AA, PG, R*, EP, PGT refers, respectively, to mean blood pressure, arachidonic acid, prostaglandins, free radicals, PGE₂ receptor, and prostaglandin transporter.

[165]. Peroxynitrite anion reacts rapidly with CO₂, forming an adduct, nitrosoperoxocarboxylate (ONOOCO₂⁻), whose decomposition has been proposed to produce reactive intermediates such as the nitrogen dioxide NO₂ and carbonate radical CO₃⁻ [194]. Biologically important reactions of these free radicals are, for example, the nitration of tyrosine residues. These nitrations can be pathological by compromising the function of enzymes; however they may also play a role in signal transduction, since nitration of tyrosine can modulate phosphorylation and thus control enzymatic activity [195]. Nonetheless, because it is difficult to directly scavenge peroxynitrite in view of its rapid reaction with CO₂, other means of diminishing its toxic effects could perhaps be achieved by scavenging intermediates of the latter reaction, reducing the formation of NO, and lowering the concentrations of superoxide (such as with superoxide dismutase).

6. Free radicals

Excess delivery of oxygen to the retina of the newborn fuels the generation of ROS [23], which in turn have been found in humans and in animal models to exert a major role in the genesis of ROP [1–11,35,36]. Although oxygen is a necessity for the survival of aerobic organisms, it is also required for the generation of ROS which may cause cell damage. A single electron can be added to molecular oxygen to form superoxide anion, O₂⁻, which is often the first free radical produced. By itself superoxide is not very reactive, but in the presence of transition metals such as iron, superoxide can react directly with H₂O₂ to produce the highly reactive hydroxyl radical, which is capable of reacting in turn with almost every type of cell molecule [196]; under these conditions superoxide can also react with lipid peroxides to yield alkoxyl radicals. Radicals attack other biomolecules such as DNA, protein, and most commonly lipids and in doing so generate new radicals. In the presence of metal ions the interaction between lipid peroxides and hydrogen peroxide can lead to a metal-catalyzed Fenton reaction and this could form strong oxidizing agents capable of propagating lipid peroxidation. This leads to the production of toxic metabolites like aldehydes (malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE)), alcanes and alkoxyl radicals [6,197,198]. These products of peroxidation can themselves increase vascular permeability, produce edema, inflammation, promote cell death and can alter the func-
tions of membrane proteins like receptors, ion channels and enzymes [199–203].

6.1. Susceptibility of the developing retina to free radicals

The immature retina that originates from the same embryonic tissues as the brain is rich in polyunsaturated fatty acids and molecular photosensitizers and maintains a very high rate of oxidative metabolism [49,204]. These characteristics, in addition to an incompletely developed antioxidant system [26,27,205], render ocular tissues of the newborn very susceptible to free radical attack. Essentially all major components of the antioxidant system in neonate, including heme oxygenase-1, metallothionein, Cu–Zn superoxide dismutase, catalase, vitamins C and E and glutathione peroxidase, have been shown to be reduced in retinal tissues of the newborn [26,27,205,206]. Furthermore, in newborn, in contrast to the adult there is more free iron in neural tissue which becomes readily available to catalyze oxidizing reactions [207,208]. Altogether, these predispositions make the ocular tissues including the vasculature of the newborn more vulnerable to oxidative damage.

Free radical generation is not simply non-enzymatically catalyzed. In fact a variety of enzymes and transporters contribute to the generation of free radicals. These include the mitochondrial electron transport chain, endothelial cell xanthine oxidase activity, COX, NOS and to a lesser extent the lipoxygenase pathways. During inflammatory conditions as well as during ischemia–reperfusion injury NADPH oxidase is an important pathway for the formation of free radicals [209–212]. In ocular tissues during the neonatal period COX and NOS activities are high and shown to contribute significantly to peroxidation [22,23,37–39,204,213]; along these lines, inhibition of COX significantly attenuates the neovascularization in the OIR model of ROP [214].

6.2. Effects of free radical products on ocular blood vessels

The vascular system, and in particular the endothelium [215], is a prime target for ROS. ROS are involved in the regulation of vascular tone and in the local control of blood flow in a variety of tissues studied [216–223]. Free radicals and their metabolites affect vasomotor tone and blood flow regulation [22,28,30,218,224–228]. These vasomotor changes depend on the type of blood vessels, the type and concentration of free radical, the developmental stage, and the animal species. On retinal vasculature peroxides cause at lower concentrations a small vasodilatation [22]. Of interest, such peroxide-induced vasodilatation has been invoked during RBF and ChBF autoregulatory adjustments, mediated via a prostaglandin-dependent mechanism [22]. But as peroxide concentrations rise this vasodilatation readily switches to a marked TXA₂-dependent constriction [28–30]. In addition, in the immature subject peroxide-induced constriction is more pronounced and sustained compared to that in the older subject due to increased and unopposed (by PGI₂) TXA₂ generation [28,29]. This shift in ratio of PGI₂ towards TXA₂ has been previously explained by an endoperoxide steal whereby endotheliually generated PGH₂ is used by platelet TXA₂ synthase to generate TXA₂ in the presence of lipid peroxides [229,230]. Thus peroxidation significantly affects blood flow to the developing eye [30].

6.3. Interaction between free radicals and prostanoids

Several mechanisms have been suggested to explain the vasomotor effects of free radicals and peroxides. Peroxides and free radicals stimulate prostaglandin formation by increasing release of AA and by activating the COX pathway [231–234]. In endothelial cells prostaglandin synthesis is high compared to other vascular and perivascular cells [235]; both TXA₂ and PGI₂ are produced [28,41,202,222,227,236,237]. The regulation of COX-2 expression by ROS could also contribute to the formation of prostanoids [238,239]. As peroxidation propagates PGI₂ synthase is inactivated and TXA₂ preferentially increases [28–30,240].

ROS activate the formation of prostanoids by increasing COX activity [233,234,241], COX-2 expression [238,239], and by stimulating the activity of the rate-limiting enzyme, PLA₂ [236,237,242]. It is believed that ROS activate COX in the following manner. COX exhibits two activities: a cyclooxygenase which yields PGG₂, and a peroxidase activity which forms PGH₂. Catalysis of PGH₂ formation by COX involves a radical at the active site on tyrosine at position 385 which needs to be regenerated by peroxides during the peroxidase step for continued activity of the enzyme [243,244]. In the usual setting the endoperoxide, PGG₂, can serve as the electron donor resulting in PGH₂ formation to generate the tyrosyl radical and in turn initiate COX activation [243].

Induction of COX-2 expression by ROS has been demonstrated [245], and is consistent with a redox-sensitive consensus sequence for NF-κB in its promoter [246]. As far as PLA₂ activation is involved, several mechanisms have been proposed. ROS have been found: (1) to increase cellular calcium by stimulating intracellular release and by activating surface channels [247,248]; (2) increase the conversion of inactive form of PLA₂ into its active form through direct stimulation [249,250]; (3) augment calmodulin activity [247]; (4) activate protein kinases C and tyrosine kinases which in turn affect PLA₂ activity [242]; and (5) by increasing the proportion of oxidized fatty acids in phospholipids as preferential substrates for PLA₂ [251]. A small proportion of AA is also released by an activation of PLC by hydroperoxides [247,252].
6.4. Free radical-mediated generation of isoprostanes

A major product of peroxidation is a group of compounds commonly termed isoprostanes [253–255]. Isoprostanes are mainly derived by free radical-mediated oxidation of ubiquitous fatty acid arachidonic acid [256]. In contrast to PGs synthesized by COX, the isoprostanes are formed in situ on esterified phospholipids and are released in free form, presumably by phospholipases. In oxidant stress formation of isoprostanes exceeds that of COX-derived PGs. At present, four series of arachidonic acid-derived isoprostanes have been discovered: F₂, E₂, D₂, and thromboxane-isoprostanes; in view of its commercial availability, the one most studied has been 8-iso-PGF₂α. The isoprostanes seem to exert significant biological activity. They produce potent vascular smooth muscle contraction including of retinal vasculature and have been found to stimulate smooth muscle cell proliferation [254,255,257,258]. Although it was shown that effects of 8-iso-PGF₂α can be abrogated by the TXA₂ receptor antagonist, SQ29548, binding studies suggest they interact with a receptor (or binding site) distinct from the TXA₂ receptor [259–262]. A novel mechanism of action of 8-iso-PGF₂α was described on neural vasculature including that of retina, whereby 8-iso-PGF₂α elicits vasoconstriction by causing the release of TXA₂ from the endothelium and the parenchyma via a mechanism dependent upon activation of cell surface calcium channels [257,263]; interestingly, the effect of this product of peroxidation (8-iso-PGF₂α) mimicked that of various peroxides and oxidant stresses tested on the retina [28–30]. In addition, vasoconstrictor efficacy of 8-iso-PGF₂α was inversely related to developmental age, such that it was most augmented in fetal tissue [263].

![Fig. 5. Effects of 8-iso-PGF₂α on retinal microvascular cell death. (A) Isolated small retinal microvessels (±25 μm) were incubated with 8-iso-PGF₂α (50 nM), and 48 h later stained with membrane permeable Hoechst 33342 and normally membrane impermeable propidium iodide (PI), respectively; PI incorporates in cells with disrupted membranes. One notes a larger proportion of cells treated with 8-iso-PGF₂α that incorporate PI, reflecting disruption of cell membrane indicative of cell death. (B) Effects of 8-iso-PGF₂α on vascular degeneration in retina. 8-iso-PGF₂α (0.5 μl containing 0.5 μmol [total eye volume ~50 μl]) was injected daily into the preretinal vitreous (in proximity of the optic nerve) of one eye of rats on postnatal days 9–11 and the other eye received saline. The degree of vascularity was assessed on postnatal day 13 by adenosine diphosphatase staining. Saline-treated eyes did not differ from untreated ones. One notes markedly reduced vascular density in 8-iso-PGF₂α-treated eyes. Magnification: 35×.](https://academic.oup.com/cardiovascres/article-abstract/47/3/489/344428)
Based on substantial evidence that free radicals are involved in retinal microvascular degeneration [4,9,10], a potential role for 8-iso-PGF$_{2\alpha}$ in retinal microvascular cell death was recently proposed [264]. 8-Iso-PGF$_{2\alpha}$ was found to cause cell death of isolated retinal microvascular cells (≈25 μm) (Fig. 5A). A similar vascular degeneration was also detected by injecting in vivo into the preretinal vitreous 8-iso-PGF$_{2\alpha}$ in young rat pups (Fig. 5B); injection of saline in the contralateral eye had no effect. Because 8-iso-PGF$_{2\alpha}$ causes proliferation of smooth muscle cells it is presumed that its cytotoxicity is exerted on the microvascular retinal endothelial cells [259]. The mechanisms of this cell death remain unknown and are being elucidated. Altogether, isoprostanes not only compromise circulation by diminishing blood flow, but also seem to contribute to the oxidant stress-induced vascular injury as seen in ischemic retinopathies.

7. Vascular endothelial growth factor in neovascularization of ROP

The ischemia induced by peroxidation leads to an abnormal preretal neovascularization [11,13–17]. Several growth factors have been shown to possess some angiogenic properties [265]. One of these factors, namely VEGF or vascular permeability factor is an endothelial cell-specific mitogen with a complex molecular heterogeneity as determined by molecular cloning [266–268]. There exists four subtypes of VEGF isoforms generated by alternate splicing, which contain 121, 165, 189 and 206 amino acids, of which the first two are signaling peptides [268]; other homologous proteins (placental growth factor, VEGF-B, VEGF-C and VEGF-D) have recently been identified [269] but their roles are not yet well characterized. The biological response of cells to VEGF is mediated through high affinity cell surface receptors which belong to the superfamily of tyrosine kinase receptors and are classified as Flk-1 and Flt-1 [270]. VEGF is up-regulated in glioblastoma cells and its receptors, are expressed in the tumor endothelial cells in vivo suggesting that VEGF functions as a paracrine angiogenic factor [271]. Likewise, in the retina VEGF is predominantly produced by the neuroglia (first astrocytes and later Müller cells) adjacent to the retinal vasculature which contains the Flk-1 receptor [17,272].

The expression of VEGF has been shown to be promoted primarily by hypoxic conditions and inhibited in the retina by hyperoxia [17,273]. In OIR in pups of various species including mouse, rat and cat, VEGF expression has been shown to be closely related to the neovascular front and its expression precedes new vessel formation [17,272,274]. In addition, Flk-1 and Flt-1 have also been found to be upregulated in OIR [275]. The biological importance of the VEGF–Flk-1 receptor system in retinal angiogenesis has been demonstrated using antibodies against VEGF and by injection of soluble Flk-1 receptors linked to IgG [276]. Furthermore, levels of VEGF have been reported to be elevated in the vitreous of patients with retinal neovascularization secondary to various ischemic retinopathies [277,278]. Altogether, there is strong evidence to suggest VEGF as a principal angiogenic factor in neovascularization of ROP.

The biochemical mechanisms implicated in VEGF induction are not yet well understood. A number of factors implicated in angiogenesis, such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), stimulate VEGF secretion [279]. It is of interest also in the context of this review to mention that PGE$_2$ can induce VEGF expression in osteoblasts [280]; moreover, this prostaglandin can produce retinal venule dilation [98], and vasodilation is an early event which precedes the emergence of the first capillary sprout [281]. In addition, a complex interaction between NO and VEGF has been reported [282–290]. VEGF-evoked increased vascular permeability and endothelial proliferation seems largely mediated by NO through activation and de novo expression of NOS following stimulation of its Flk-1/KDR receptor [282,284–288,290–295]; since VEGF is also capable of generating ROS a role for peroxynitrite can be proposed [290]. In a reciprocal manner NO can also affect the expression and release of VEGF, mostly by suppressing hypoxia-induced VEGF expression via cGMP-dependent and independent mechanisms [283,296]; in the latter case, it should be noted that the VEGF promoter contains NO-responsive cis-elements which are the hypoxia-inducible factor-1 (HIF-1) binding site and an adjacent sequence that is located immediately downstream within the hypoxia-response element (HRE) which is the primary target of NO [289].

Under other conditions NO can stimulate VEGF expression [297]. ROS are also important stimulants of VEGF [298,299]. All in all prostanooids, NO, ROS, along with other growth factors, namely PDGF and bFGF, all of which are modulated by hypoxia [300–303], act in a complex concerted fashion to regulate VEGF expression, a principal angiogenic factor in the neovascularization of ROP [17,272,276].

8. Summary

Although oxygen is required for the survival of all aerobic organisms, hyperoxia may be toxic particularly to tissues of immature subjects that have not yet fully developed their antioxidant defenses. The free radicals generated attack many biomolecules, in particular lipids. The retina is rich in polyunsaturated fatty acids, which are more prone to peroxidation. In the ocular tissues of the immature neonate the COX pathway is an important source of free radicals during oxidative stress.

In the perinate, COX activity is high, and as a result
 produces increased levels of prostaglandins; these products play a significant role in the regulation of ocular blood flow in the newborn. NOS activity is also high in the newborn choroid and generally exerts similar effects on ocular circulation of the developing ocular vascular bed. Prostaglandins and NO interact at various epigenetic and genetic levels in the developing ocular vasculature. As a result of increased prostaglandin and NO formation, the ChBF autoregulatory response to increased O₂ and perfusion pressure is absent in the perinate leading to retinal hypoxia and in turn to peroxidation which predisposes to impaired circulation and vascular integrity, and thus to ischemic retinopathy of the developing subject, namely ROP.

The normally observed high levels of PGs and NO in the ocular vasculature of the perinate cannot simply be regarded as a physiological anomaly which predisposes to vasoproliferative retinopathy of the perinate. Uterine contractions during advanced labor are associated with marked decreases in fetal blood oxygen tension [304,305]. Increased perinatal concentrations of PGs which are minimally contractant and mostly relaxants, as well as elevated levels of the potent vasorelaxant NO are capable of enhancing choroidal, retinal as well as brain circulation and consequently improve oxygen delivery to the central nervous system, of which the retina is an integral part. Hence, in the fully developed and stable term healthy neonate these factors would be beneficial. The problem arises when the subject is born prematurely and its retinal vasculature is still underdeveloped. The fetus is normally exposed to pO₂ values of 25–30 mmHg (corresponding to ~10% O₂); but, when born prematurely it is unprepared to encounter O₂ tensions at or above atmospheric values, and when further faced with stresses secondary to circulatory and respiratory instability, this child may be at serious risk of developing major complications such as ROP.

The cascade of events that leads to ROP illustrates the complexity in the action of free radicals. This is highlighted by the divergence of their effects on vasomotor tone as well as on vascular integrity and is depicted by the diverse actions of peroxides, isoprostanes and NO. In addition, the chronicity in the genesis of ROP further adds to the intricate dynamics of mechanisms involved. From a therapeutic standpoint, antioxidants can be considered an appropriate option. Administration of free radical scavengers and antioxidants such as vitamin E, C, and superoxide dismutase, or of COX inhibitors which reduce free radical generation [22,30,204], have all been shown to improve OIR [3,4,10,34,36,214]; however, the cellular distribution, kinetics and efficacy of these compounds complicate their choice. Likewise, because NO exerts beneficial as well as adverse effects depending on its levels and other conditions it is a difficult target to modulate at this point particularly in the newborn where respiratory problems can be aggravated by eNOS inhibition [306–308]. The same argument applies to prostanoids; although inhibition of COX reduces neovascularization, vasoobliteration is unaffected [214], probably because of the opposing actions of distinct prostanoids in cytoprotection [309,310]. Because retinal ischemia such as that seen in ROP leads to persistent retinal functional deficits prevention simply of the resultant abnormal preretal neovascularization cannot be of sufficient benefit [14,189–191,311,312]. One could speculate that perhaps the development of selective prostaglandin receptor blockers and NOS inhibitors administered early in the genesis of ROP may provide potential amelioration in the outcome of this disorder, awaiting effective tocolytics.

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