Signaling Pathways Triggered by Oxidative Stress That Mediate Features of Severe Retinopathy of Prematurity

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Oxidative stress has been implicated in the pathogenesis of retinopathy of prematurity (ROP) for decades. It is becoming increasingly understood that reactive oxygen species can trigger signaling pathways that have beneficial or pathologic outcomes. Broad inhibition of reactive oxygen species in the preterm infant may lead to unwanted consequences, as has been experienced with vitamin E studies in the past. In this study, we provide a current understanding of the role of oxidative stress in activating signaling pathways that cause pathologic features in severe retinopathy of prematurity as it manifests in the era of oxygen regulation.


Oxidative stress has been implicated in the pathogenesis of retinopathy of prematurity (ROP) for several decades.1 Early thinking focused on tissue damage from excessively generated reactive oxygen species (ROS);2 potentially due to various oxygen stresses,3-6 changes in photoreceptor metabolism during darkness,7,8 and the high concentration of polyunsaturated fatty acids in photoreceptors.9 In fact, research was performed decades ago to test vitamin E as an antioxidant to reduce ROP, but these investigations were stopped because of complications of sepsis and necrotizing enterocolitis.10

It is becoming increasingly recognized that ROS activate signaling pathways that result in physiologic or pathologic effects. Of particular interest in ROP are those signaling pathways involved in apoptosis and angiogenesis. Apoptosis is natural to the developing neural and vascular retina through ganglion cell loss and vascular remodeling,11 as examples. Reactive oxygen species can cause apoptosis,12 which is associated with and in some studies is causative of delayed retinal vascular development in models of ROP.12,13 Angiogenesis is essential to retinal vascular development but, when aberrant or disordered, can lead to disoriented division of endothelial cells13 in the form of intravitreous neovascularization.

In addition, bursts of leukocyte-generated superoxide are important to fight off invading microorganisms, and this may be particularly important in the immune-suppressed preterm infant. However, when uncontrolled, ROS may lead to chronic pathologic conditions. Therefore, it is helpful to study the signaling cascades activated by ROS that mediate pathologic features in ROP to find potential safer therapies rather than broadly proposing antioxidants.

TWO PHASES OF OXYGEN-INDUCED RETINOPATHY AND THE STUDY OF HUMAN ROP

The description of 2 phases of ROP was based on a 1954 study by Ashton et al,15 who exposed newborn kittens to oxygen stresses similar to what preterm infants experienced at that time before technology to regulate oxygen delivery. Kinsey,16 Patz,1 and others later studied oxygen effects in human infants and found that time in oxygen therapy and low birth weight were strong predictors of severe ROP. Today, a common example based on oxygen lev-
els similar to those used by Ashton et al\textsuperscript{15} is the mouse model of oxygen-induced retinopathy (OIR) developed by Smith and colleagues.\textsuperscript{17} In this model, newly developed capillaries regress, leaving central areas of vaso-obliteration following exposure to constant, high oxygen (75% oxygen) from postnatal day 7 (p7) to p12. This oxygen level causes arterial oxygen greater than 300 mm Hg.\textsuperscript{18,19} Following return to room air, a relative hypoxia occurs in the vaso-obliterated retina, which stimulates the release of angiogenic factors to cause vasoproliferation of blood vessels into the vitreous. This model portrays ROP of the 1950s before the ability to regulate oxygen levels and may still potentially have a role in neonatal units today in which technology to regulate oxygen delivery is not implemented or in which high oxygen must be used for other reasons.\textsuperscript{1}
The mouse OIR model is important in the study of genetic mechanisms of high oxygen-induced vessel loss and in the recovery of lost vasculature,\textsuperscript{20} as well as in angiogenic processes.

Since Ashton et al,\textsuperscript{15} changes have been made in oxygen exposure to preterm infants and in recognition of earlier stages in the development of ROP. In ROP, rather than vaso-obliteration, delayed physiologic retinal vascular development (PRVD) occurs, which causes peripheral avascular retina. Animal models of OIR were developed that recreate features seen in human ROP. The rat model of fluctuating oxygen concentrations developed by Penn et al\textsuperscript{21} causes mainly a delay in PRVD in the peripheral retina, as well as some central capillary constriction, following 14 days of fluctuations between 50% and 10% inspired oxygen every 24 hours. The pups were brought into room air and then developed vasoproliferation at the junction of vascularized and avascular retina at p18. The 50/10 OIR model shares several similarities with human ROP. The model produces an appearance similar to stage 3 ROP that occurs in neonatal units in which oxygen delivery is regulated such as in the United States, United Kingdom, Canada, and Australia today.\textsuperscript{22} The rat pups were exposed to fluctuations in inspired oxygen,\textsuperscript{23} which cause arterial oxygen levels similar to transcutaneous oxygen measurements in human infants who developed severe ROP.\textsuperscript{23,24} The 50/10 OIR model is helpful to study delayed PRVD and vasoproliferation in severe ROP.

**ROLE OF OXYGEN IN ROS GENERATION**

Reactive oxygen species are oxygen-containing atoms, ions, or molecules and include hydroxyl radical, superoxide radical, and hydrogen peroxide, as examples. Therefore, it is helpful to review the role of oxygen concentration in the generation of ROS. Hypoxia is generally accepted as a mechanism to enhance ROS generation by increasing superoxide. Besides hypoxia, hypoxia can (in theory) increase ROS generation by slowing upstream events in the electron transport chain and raise the concentration of oxygen donors that promote electron transfer to oxygen.\textsuperscript{25} Hypoxia can also lead to the activation of nitric oxide synthase (NOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase,\textsuperscript{26} which are enzymes that generate ROS and are involved in oxygen-induced retinopathy.\textsuperscript{13,27}

**Figure 1.** Postnatal oxygen stress induces oxidative stress and nitro-oxidative stress via activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and endothelial nitric oxide synthase (eNOS), as well as via hypoxia-stabilized or reactive oxygen species–stabilized hypoxia-inducible factor 1α (HIF1α). ↑ NO indicates increased nitric oxide.

**ROS GENERATORS IN THE PRETERM INFANT**

Within the cell, the mitochondrion is recognized as a key source of superoxide radical, but some enzymes are also involved in ROS generation, including NADPH oxidase and nitric oxide synthetase (Figure 1). Antioxidant enzymes, such as superoxide dismutases, glutathione peroxidase, and catalase, quench oxidative products, rendering them less reactive. A balance between generation and quenching of ROS is important for physiologic metabolism, while minimizing pathologic processes.

In the events surrounding the early life of the preterm infant, increased oxygen content is postulated to generate ROS directly or perhaps from tissue hypoxia that develops when hyperoxia injures newly developed capillaries in vulnerable immature tissue beds. Besides increased ROS generation, the preterm infant has reduced ability to produce antioxidant enzymes\textsuperscript{3} and to quench ROS.\textsuperscript{28}

**EVIDENCE FROM ANIMAL MODELS THAT ROS-ACTIVATED SIGNALING CAUSES AVASCULAR RETINA OR INTRAVITREOUS NEOVASCULARIZATION**

Using several OIR models, studies\textsuperscript{29,30} showed that retinal ROS were generated in association with or had a causal role in avascular retina or intravitreous neovascularization. In the 50/10 OIR model, a trend was observed toward increased retinal end products of ROS (lipid hydroperoxides) in temporal association with delayed PRVD and intravitreous neovascularization.\textsuperscript{12} However, neither intravitreous neovascularization nor delayed PRVD was affected by systemic administration of the broad antioxidant N-acetylcysteine at a dose that significantly inhibited retinal lipid hydroperoxides.\textsuperscript{12} Yet in the mouse model of OIR, daily N-acetylcysteine by intraperitoneal injection given to pups exposed to hyperoxia inhibited central vaso-obliteration by 42% at p12; when pups that were exposed to hyperoxia (75% oxygen) and then relative hypoxia (21% oxygen) received daily N-acetylcysteine from p7 to p17, a 62.1% reduction in intravitreous neovascularization was observed at p17.\textsuperscript{11}

Treatment with vitamins C or E\textsuperscript{30} or liposomal superoxide dismutase\textsuperscript{31} improved PRVD and reduced vascu-
lar leakage compared with sham-injected controls. However, the therapy did not reduce pathologic intravitreous neovascularization in the 50/10 OIR model.

Nicotinamide adenine dinucleotide phosphate oxidase-dependent ROS generation was found to result in apoptosis and delayed PRVd at p14 in the 50/10 OIR model.12 Treatment with apocynin, an inhibitor of NADPH oxidase, significantly improved PRVd by reducing activated caspase 3 in the retina. Repeated oxygen fluctuations led to increased retinal vascular endothelial growth factor (VEGF) expression, which activated Janus kinase and signaling transducer and activator of transcription 3 (STAT3).33,34 Translocation of STAT3 into the nucleus downstreamregulated Müller cell–derived erythropoietin, which contributed to delayed PRVd at p14 in the 50/10 OIR model (Figure 2). Exogenous erythropoietin given at p2, p4, and p6 significantly increased retinal vascularization approximately 40%,34 suggesting that increased retinal VEGF alone requires other angiogenic factors, such as erythropoietin, to support PRVd. However, exogenous erythropoietin given late at p12 was unable to improve PRVd in the 50/10 OIR model (M.E.H., unpublished data, 2009), indicating that timing of erythropoietin administration is critical, similar to findings using the mouse model of oxygen-induced retinopathy.34

Several studies have measured the effect of the NADPH oxidase inhibitor apocynin on intravitreous neovascularization. In a study of the mouse model of OIR,20 daily apocynin given to mice during relative hypoxia from p12 to p17 significantly reduced intravitreous neovascularization; however, treatment also increased the central vasoproliferated area more than 2-fold compared with controls. Apocynin also abolished the increase in retinal VEGF expression measured at p14. In another study,35 acti-

Figure 2. Signaling events regulated by stabilized hypoxia-inducible factor 1α (HIF1α) contribute to the pathogenesis of retinopathy of prematurity by increasing avascular retina in phase 1 and/or vasoproliferation in phase 2. EPO indicates erythropoietin; PRVd, physiologic retinal vascular development; STAT3, signaling transducer and activator of transcription 3; VEGF, vascular endothelial growth factor; †, increased; and ‡, decreased.

Figure 3. Signaling events regulated by oxidative stress contribute to the pathogenesis of retinopathy of prematurity by increasing avascular retina in phase 1 and/or vasoproliferation in phase 2. COX indicates cyclooxygenase; PC, prostacyclin; PGS, prostaglandins; PLA2, phospholipase A2; PPARγ, peroxisome proliferator–activated receptor γ; STAT3, signaling transducer and activator of transcription 3; TA, thromboxanes; and †, increased.
is that it generates hydrogen peroxide instead of superoxide; therefore, it does not produce damaging peroxynitrite in the presence of nitric oxide. Nox 4 seems to have a role in hypoxia-inducible factor 1α (HIF1α) stabilization and in VEGF upregulation. In addition, Nox 4 was implicated in STAT3-mediated VEGF expression in endothelial cells and is required for endothelial cell proliferation and migration. These results indicate a potential role of NADPH oxidase 4 in hypoxia-elicited proangiogenic responses of endothelial cells. The evidence of its role in apoptosis is conflicting. Nevertheless, how Nox is implicated in the pathogenesis of avascular retina and intravitreal neovascularization in ROP is of great interest and should be investigated in the future.

Other enzymes that catalyze ROS generation in endothelial cells and have been implicated in the phases of ROP include endothelial NOS and cyclooxygenase. Endothelial NOS catalyzes nitric oxide generation. Nitric oxide is an important vasodilator and has protective and proangiogenic properties in the eye that preserve endothelial cell barrier integrity by reducing apoptosis. However, nitric oxide can react with ROS and generate damaging compounds, including nitrites, nitrates, and peroxynitrite, by processing nitro-oxidative stress. Retinal peroxynitrite was significantly elevated in the mouse model of OIR and caused vaso-obliteration and subsequent vasoproliferation by enhancing VEGF signaling. In the 50/10 OIR model, phosphorylated endothelial NOS was found to be associated with increased arteriolar tortuosity, and evidence also suggests that excessive VEGFR2 signaling contributes to the formation of peripheral avascular retina by causing blood vessels to become disordered rather than oriented as necessary in fully vascularizing peripheral retina. Reactive nitrogen species, such as nitrogen dioxide, can isomerize arachidonic acid to trans-arachidonic acid, which contributed to vaso-obliteration by increasing the expression of the antiangiogenic agent thrombospondin 1 in the mouse OIR model. Retinal polyunsaturated fatty acids are susceptible to forming trans-arachidonic acids when arachidonic acid reacts with reactive nitrogen species (Figure 4). Therefore, evidence suggests that oxidative stress–regulated signaling can cause pathologic features independent of and in association with VEGF signaling. However, technologic limitations force the need to use whole retinas to measure signal activation; therefore, effects in individual cells can be missed.

Overlap exists in molecular signaling between oxidative and inflammatory compounds, in that complex networks of signaling pathways link oxidative agents and proinflammatory cytokines. Release and activation of phospholipid metabolites from cell membranes can potentiate or exacerbate inflammation and trigger signaling of angiogenic or apoptotic pathways. One family of metabolites includes the phospholipase A2 enzymes, which catalyze the hydrolysis of fatty acids from membrane phospholipids. Phospholipase A2 is activated by oxidative stresses and hypoxia and can lead to the release of arachidonic acid, platelet-activating factor, and lysophospholipids. From arachidonic acid, cyclooxygenase 1 and cyclooxygenase 2 can oxidize and catalyze arachidonic acid into the proangiogenic eicosanoids, which include the prostaglandins, prostacyclins, and thromboxanes. These effectors and their downstream signaling have been associated with both phases in models of ROP by inducing activation of VEGF signaling in vascular endothelial cells (Figure 3). Inhibition of phospholipase A2 significantly reduced proangiogenic prostaglandins and intravitreal neovascularization in the 50/10 OIR model.

The HIFs are important in retinal vascular development in part by enhancing transcription of important angiogenic factors, such as VEGF, during physiologic hypoxia and by increasing apoptotic effects caused by RTP801. Reactive oxygen species can also regulate HIFs by increasing transcriptional activity even in normoxia (Figure 1), whereas HIF1α is usually stabilized and transcriptionally activated in hypoxia. The von Hippel–Lindau protein regulates HIF1α stability via the formation of a ubiquitin ligase complex and prolyl hydroxylases are the enzymes that enable the interaction of HIF1α and von Hippel–Lindau protein. Using the mouse OIR model, inhibiting prolyl hydroxylase chemically during the hyperoxic phase reduced the breakdown of HIF1α and permitted intraretinal vascularization and angiogenesis. By promoting this physiologic retinal vascularization even in the presence of high oxygen, intravitreal endothelial budding during room air and relative hypoxia in phase 2 were also reduced. RTP801 is an HIF1α-responsive gene that is strongly upregulated in ischemic cells of neuronal origin or in conditions associated with increased oxidative compounds such as cigarette smoke, leading to neuronal apoptosis and alveolar septal cell apoptosis (Figure 2). RTP801 also can lead to inflammation and oxidative stress by promoting nuclear factor κB activation in cultured cells and in mouse lung. Retinal RTP801 expression was increased during relative hypoxia at p17 in wild-type mice exposed to the OIR model, whereas RTP801-deficient mice had reduced intravitreal neovascularization in association with reduced apoptotic cells in the inner nuclear layer of the retina.

Figure 4. Signaling events regulated by nitro-oxidative stress contribute to the pathogenesis of retinopathy of prematurity by increasing avascular retina in phase 1 and/or vasoproliferation in phase 2. VEGF indicates vascular endothelial growth factor; †, increased.
Evidence also shows that some cytokines and growth factors can lead to increased generation of ROS. For example, VEGF can induce NADPH oxidase activation via activation of its subunit Rac1 in endothelial cells to increase ROS generation.53,64

In summary, it is useful to understand the signaling pathways that can be activated by ROS and to relate these to the pathologic features seen in the phases of ROP. The first phase of delayed PRVD and avascular retina occurs in part because of increased apoptosis, which is supported by data from animal models implicating NADPH oxidase, endothelial NOS, and HIF1α-induced RTP801. In addition, signaling through Janus kinase–STAT can cause downregulation of the angiogenic factor erythropoietin in Müller cells, which contributes to delayed PRVD in phase 1. In phase 2, intravitreous neovascularization can be affected by increased generation of ROS caused by supplemental oxygen and mediated through the activation of STAT3. Reactive oxygen species also trigger the activation of inflammatory pathways, including those involving phospholipase A2, arachidonic acid, and angiogenic growth factors. Hypoxia or ROS can stabilize HIF1α, which increases transcription of angiogenic factors, including VEGF.

Broad inhibition of ROS generation as a therapy for ROP may not have been effective or safe because ROS can have beneficial effects as transcription factors in physiologic processes and are a first-line offense to invading microorganisms in the immune-suppressed preterm infant. Future therapies may target pathologic downstream effectors activated by ROS.

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