Activated NAD(P)H Oxidase from Supplemental Oxygen Induces Neovascularization Independent of VEGF in Retinopathy of Prematurity Model

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Purpose. To study NAD(P)H oxidase-dependent outcomes after oxygen stresses that are similar to those experienced by preterm infants today using a rat model of retinopathy of prematurity.

Methods. Within 4 hours of birth, pups and their mothers were cycled between 50% and 10% oxygen daily for 14 days and were returned to room air (21% O_2, 50/10 oxygen-induced retinopathy [OIR]) or supplemental oxygen (28% O_2, 50/10 OIR+SO) for 4 days. Pups received intraperitoneal injections of the specific NAD(P)H oxidase inhibitor apocynin (10 mg/kg/d) or of PBS from postnatal day (P)12 to P17, and some received intraperitoneal injections of hypoxyprobe before kill. Intravitreous neovascularization (IVNV), avascular/total retinal areas, vascular endothelial growth factor (VEGF), NAD(P)H oxidase activity, or hypoxic retina (conjugated hypoxyprobe) were determined in neurosensory retinas. Human retinal microvascular endothelial cells (RMVECs) treated with apocynin or control were exposed to 1% or 21% O_2 and assayed for phosphorylated (p-)Janus kinase (JNK) and NAD(P)H oxidase activity.

Results. Retinas from 50/10 OIR+SO had increased NAD(P)H oxidase activity and lower VEGF than did retinas from 50/10 OIR. Apocynin treatment reduced the IVNV area and hypoxic retina in 50/10 OIR+SO. RMVECs treated with 1% O_2 had increased p-JNK compared with RMVECs exposed to room air.

Conclusions. Different oxygen stresses activate NAD(P)H oxidase to varying degrees to trigger disparate pathways (angiogenesis or apoptosis). The oxygen stresses and outcomes used in this study are relevant to human ROP and may explain some of the complexity in the pathophysiology of ROP resulting from oxygen exposure. (*Invest Ophthalmol Vis Sci.* 2008;49:1591–1598) DOI:10.1167/iovs.07-1356

Oxygen level has been recognized as important in the development of retinopathy of prematurity (ROP). In the 1940s, high levels of unregulated oxygen at birth likely accounted for early cases.[1,2] Several animal models of oxygen-induced retinopathy (OIR) were created,[3–9] and, from these, it appeared a period of constant hyperoxia caused vaso-obliteration of newly formed capillaries. The ensuing relative hypoxia[6] within the avascular retina that occurred when animals were returned to room air led to abnormal neovascularization into the vitreous (intravitreous neovascularization [IVNV]).

Subsequently, it was shown that supplemental oxygen reduced the severity of OIR.[10] Mice raised in sustained hyperoxia beyond postnatal day (P)12 had less vaso-obliteration and neovascularization than mice exposed to the standard OIR model.[11] Rats that were exposed to oxygen fluctuations and that recovered in supplemental oxygen (28%) rather than room air had reduced IVNV levels at P20 (though not at P26[12]). Despite these data showing some benefit from supplemental oxygen, the Supplemental Therapeutic Oxygen for Prethreshold ROP (STOP-ROP) multicenter clinical trial did not find an overall significant benefit from supplemental oxygen given to infants with prethreshold ROP.[13] In addition, current clinical studies show that fluctuations in oxygen and increased inspired oxygen of infants are associated with higher risk for severe ROP.[14–18] Because reducing oxygen levels is of concern for brain development in preterm infants and because supplemental oxygen is common during preterm infant examinations and procedures in neonatal intensive care units (NICUs), knowledge of specific effects of supplemental oxygen (SO) on ROP would be helpful in designing future clinical trials.

The size of the avascular zone of the retina is associated directly with poor outcomes from severe ROP.[19,20] Based on studies of the avascular retina in animal models,[1,2] the ischemic microenvironment created by these avascular regions was theorized to produce an angiogenic substance responsible for later neovascularization.[1,21] Studies support the concept that increased apoptosis, such as through inflammatory leukostasis[22,23] or seen in bcl-2 knockout mice, which have a defect in protection against apoptosis,[24] leads to increased avascular zone size. Furthermore, the apoptosis of newly formed capillaries after hyperoxia-induced vaso-obliteration can be prevented by giving growth factors[25–28] or nutritional supplements[29,30] before the hyperoxic insult. We recently reported that the avascular peripheral retina in the rat model of ROP occurred in part from nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] oxidase–dependent retinal apoptosis.[31]

NAD(P)H oxidase is a major enzyme responsible for the release of superoxide radicals from macrophages to fight invading microorganisms.[32] It is activated by a number of stimuli relevant to ROP, including hypoxia,[33] hyperoxia,[34] and reactive oxygen species (ROS),[33,35–37] and its activation can trigger disparate signaling pathways from endothelial apoptosis[38] to endothelial proliferation and angiogenesis,[33] outcomes relevant to human ROP. One study provides evidence that the concentration of ROS produced by NAD(P)H oxidase activation may affect what signaling pathways are triggered.[39] To further study the effects of NAD(P)H oxidase under oxygen stresses, we used an animal model relevant to ROP[8] that exposed newborn rat pups to repeated fluctuations in oxygen and returned the pups to room air or supplemental oxygen (28% O_2). Analogous fluctuations and supplemental

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oxygen conditions are commonly experienced by preterm infants in NICUs today. Our goal was to determine the effect of supplemental oxygen on NAD(P)H oxidase activation and signaling pertinent to the pathogenesis in ROP, specifically the size of avascular retinal regions and the development of IVNV. Knowledge of the effects of supplemental oxygen on ROP would be helpful in designing future studies that address oxygen concentrations or fluctuations on outcomes in premature infants, not only those involving ROP but also those involving the preterm infant's pulmonary and central nervous system status.

Materials and Methods

All animals were cared for in accordance with the University of North Carolina's Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

50/10 Oxygen-Induced Retinopathy and Supplemental Oxygen Models

A bioactive gas controller (Oxycycler; BioSpherix, Redfield, NY), which regulates the atmosphere inside an incubator by injecting either nitrogen or oxygen, was used to induce oxygen-induced retinopathy in newborn Sprague–Dawley rats (Charles River, Wilmington, MA), as reported.[8] Within 4 hours of birth, pups and their mothers were placed in the incubator, which cycled oxygen between 50% and 10% every 24 hours for 14 days. At that point, pups were returned to room air (21% O_2) for 4 additional days (50/10 OIR) or to supplemental oxygen (28% O_2) for 4 days (50/10 OIR+SO). Carbon dioxide in the cage was monitored and flushed from the system by maintaining sufficient gas flow and using soda lime. Litters of 12 to 14 pups were used in all experiments. Pup body weights were measured at the time of intervention, and, because body weight can affect outcomes, only those litters in which pups had mean body weights ±2 g of one another were used in experiments.[40] There was no significant difference in mean body weights among groups of all litters at the time of assays at P18.

Treatment with Pimonidazole

For determination of retinal hypoxia, pups received intraperitoneal (IP) injections of the chemical analogue 2-nitroimadazole (pimonidazole; Hypoxyprobe [HP]; Chemicon, Temecula, CA) at 60 mg/kg 90 minutes before kill at P4 and P18 in room air and at P18 in 50/10 OIR or 50/10 OIR+SO. HP binds to protein thiol groups and forms adducts when oxygen levels drop below 10 mm Hg, and these can be quantified by Western blot or visualized using a fluorescence-labeled antibody. Eyes were promptly processed to reduce error in outcomes and were analyzed by immunostaining of flatmounts or cryosections or with Western blot.

Dissecting Retinal Tissue for Flatmounts and Cryosections

Pups were anesthetized by IP injection of ketamine (20 mg/kg) and xylazine (6 mg/kg). Paraformaldehyde (PFA; 1 mL, 0.5%) was directly perfused into the left ventricle before humane killing by intracardiac injection of pentobarbital (80 mg/kg). Both eyes were enucleated and fixed in 2% PFA for 2 hours. Anterior segments were removed, and retinas with intact ora serratas were carefully dissected and placed in phosphate-buffered saline (PBS), with care taken to remove the hyaloidal vessels and any remaining vitreous. For flatmounts, each retina was placed onto a microscope slide and flattened by making four incisions, each 90° apart, beginning at the ora serrata and extending centrally from the equator, stopping short of the optic nerve opening. For cryosections, retinas were put in 30% sucrose/PBS overnight. Each retina was dried with filter paper, soaked in optimal cutting temperature (OCT) compound (Tissue Tek; product no. 4583; EMS, Hatfield, PA), and kept at −80°C for future analysis. In most analyses, one eye of each pup was used for either flatmount or fresh tissue to reduce the effect of variability between pups.

Tissue Staining for Flatmounts

Flattened retinas were permeabilized in ice-cold 70% ethanol for 20 minutes and then in PBS/1% Triton X-100 for 30 minutes. The retinas were incubated with fluorescent dye (Alexa Fluor 594; Invitrogen-Molecular Probes, Carlsbad, CA)–conjugated *Griffonia simplicifolia* (*Bandeiraea*) isolectin B4 (5 μg/mL) in PBS overnight at 4°C, washed with PBS, and incubated in 5% normal goat serum to block nonspecific binding of the primary antibody for 60 minutes. For visualization of the HP-protein adducts, anti–HP mouse antibody (1:100; Chemicon) was added overnight at 4°C, then washed with PBS and incubated (1:500) with a goat anti–mouse antibody labeled with fluorescent dye (Alexa 488; Invitrogen-Molecular Probes). The retinas were rinsed in PBS, mounted in PBS-glycerol (2:1; Vectashield, Vector Laboratories, Burlingame, CA), and placed on slides with coverslips sealed with nail polish. Images of the superficial blood vessel layers were captured with an inverted microscope (TE2000U; Nikon, Tokyo Japan; Michael-Hooker Microscopy Facility, University of North Carolina, Chapel Hill) and digitally stored for analysis. Image sections were stitched with the commercial image-management software (Adobe Photoshop 7.0) and assembled using methods that maintain the original image dimensions and do not induce image distortion (Tekmate PhotoFit Premium version 1.44 or Adobe Photoshop).

Tissue Staining for Cryosections

Retinas cut into 10-μM sections (2800 Frigocut N; Reichert-Jung, Nussloch, Germany) were incubated in PBS/1% Triton X-100 for 30 minutes, then incubated with Alexa Fluor 594-conjugated *G. simplicifolia* (*Bandeiraea*) isolectin B4 (5 μg/mL; Invitrogen-Molecular Probes) in PBS for 30 minutes at room temperature. After 3 washes with PBS, retinas were incubated in 5% normal goat serum for 10 minutes to block nonspecific binding of the primary antibody. For visualization of the HP-protein adducts, anti-HP mouse antibody (1:100; Chemicon) was added for 60 minutes at room temperature, then washed with PBS, incubated (1:500) with a goat anti-mouse antibody labeled with Alexa-488 (Invitrogen-Molecular Probes) for 20 minutes. The retinas were rinsed in PBS and mounted in PBS-glycerol (2:1; Vectashield; Vector Laboratories). Images were captured with an inverted microscope (Nikon TE2000U Michael-Hooker Microscopy Facility, University of North Carolina, Chapel Hill) and digitally stored for analysis.

Fresh Tissue Preparation

Animals were humanely killed with overdoses of pentobarbital (80 mg/kg intraperitoneally). Eyes were enucleated, and retinas were isolated under a dissecting microscope in a fashion similar to that used for flatmounting except that each ora serrata was carefully removed. The tissue was placed in RIPA buffer (20 mM Tris base, 120 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol) with a protease inhibitor cocktail (1:100; Sigma, St. Louis, MO) and 1 M orthovanadate (1:100; Sigma) and was stored for Western blot.

Protein Extraction, Immunoprecipitation, and Western Blot Analysis

Freshly dissected unfixed retinal tissue immersed in RIPA buffer was homogenized, and lysates were centrifuged at 13000*g* for 15 minutes at 4°C. Supernatants were collected, and total protein in the cell lysate was determined by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Fifty micrograms of protein for each sample was diluted in 2× sample buffer and was separated by 7.5% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membrane, and reacted to anti–HP rabbit monoclonal antibody (1:1000; gift from James Raleigh, University of North Carolina, Chapel Hill) overnight, followed by goat anti–rabbit

HRP–conjugated secondary antibody (1:200,000; R&D Systems, Minneapolis, MN). As a measure of NAD(P)H oxidase activation, the ratio of phospho $p47^{phox}$ to total was determined by immunoprecipitating 50 μg protein for each sample with $p47^{phox}$ antibody (1 μg; catalog no. sc-14015; Santa Cruz Biotechnology, Santa Cruz, CA) and probing with phosphoserine (1:300; catalog no. AB1603; Chemicon) to determine activated $p47^{phox}$, a cytoplasmic subunit that is phosphorylated and then joins with membrane-bound subunits to produce active NAD(P)H oxidase. Total $p47^{phox}$ was determined after membranes were stripped (Restore Plus Western Blot Stripping Buffer; catalog no. 46430; Pierce) according to manufacturer's protocol and were reprobed with $p47^{phox}$ antibody (1:500). All membranes were also probed with beta actin (1:20,000; catalog no. ab20272; Abcam, Cambridge, MA) as a control. Visualization was performed with enhanced chemiluminescence (SuperSignal Pico Luminol; Pierce). The signal intensity was quantified from exposed films with analysis software (UN-SCAN-IT, version 6.1; Silk Scientific, Orem, UT).

Treatment with Apocynin

Apocynin (4-acetovanillone) is a specific inhibitor of NAD(P)H oxidase, the enzyme mainly responsible for the release of superoxide radical in neutrophils[32] and important in endothelial cell signaling.[33] To test its effects with oxygen stresses, we treated half of each litter with IP injections of apocynin (Sigma-Aldrich, St. Louis, MO) in sterile PBS at 10 mg/kg (5 μL/g body weight) once a day from P12 to P17. Controls received IP injections of sterile PBS at 5 μL/g.

IVNV Area and Avascular/Total Retina Area

Digitized retinal images were randomized, labeled, and analyzed by two masked reviewers. Total retinal area and peripheral or summed avascular areas (computed in pixels and converted to square millimeters based on a calibration bar within each image) were made (ImageTool version 3; University of Texas, San Antonio, TX) and expressed as the percentage of avascular to total retinal area for each eye.[41] Vascular area was the area with lectin-stained retinal vessels. IVNV has been defined as intravitreous neovascularization occurring at the junction of vascular and avascular retina.[41] IVNV areas[42] were measured similarly to the manner used for avascular areas, from retinal images randomized and labeled to maintain masking and using Image Tool version 3 freeware (University of Texas). Significant differences between masked reviewers were resolved by an independent third masked reviewer.

In Vitro Studies

Human retinal microvascular endothelial cells (RMVECs; Cell/Systems, Kirkland, WA) at passage 2 were grown to 70% confluence in DMEM/low glucose supplemented with 120 μg/mL endothelial cell growth supplement (Sigma) and heparin (100 μg/mL; Sigma) in 5% CO_2. RMVECs were treated with either 16 μL apocynin (final concentration, 30 μg/mL) or PBS. Immediately thereafter, cells were placed in 1% O_2 in a hypoxia incubator (Biospherix) or in room air (21% O_2). After 6 hours, cells were removed and placed in RIPA (20 mM Tris base, 120 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol) with protease inhibitor cocktail (1:100; Sigma) and 1 M orthovanadate (1:100; Sigma) and were stored for ELISA and Western blot testing.

ELISA

Frozen protein samples processed from retinas or cells were thawed, or fresh samples were prepared and centrifuged (16,000*g*, 10 minutes, 4°C). RIPA was removed, and lysis buffer (0.15 M NaCl, 0.02 M Tris-HCl [pH 8.0], 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.01% proteinase inhibitor cocktail; Sigma) was added. Samples were homogenized and centrifuged at 16,000*g* for 10 minutes at 4°C, and total protein was quantified by BCA protein assay (Pierce). Supernatants from samples prepared from rat retinas were assayed without dilution in duplicate using commercially available ELISA kits raised against rat $VEGF_{164}$ isoform (R&D Systems).

It is believed that differential activation of NAD(P)H oxidase can trigger different signaling pathways.[39] To test effects of oxygen stress of NAD(P)H oxidase activated signaling pathways, we used RMVECs exposed to hypoxia (1% O_2) or room air and repeated with apocynin or control. Supernatants from samples were assayed without dilution in duplicate using a commercially available ELISA (DuoSet; R&D Systems) for phosphorylated Janus kinase (JNK), phosphorylated ERK, or phosphorylated Akt according to the manufacturer's specifications.

Statistical Analysis

Data were analyzed with SPSS software (version 14.0; Chicago, IL). Individual Student's *t*-tests were used to analyze parametric data, including avascular area/total retinal area (% AVA), IVNV area/total retinal area, and normalized densitometric values. For all comparisons, $P < 0.05$ was used as the criterion of significance.

Results

Zones of Hypoxic and Oxygenated Retina throughout Vascularized Retina in ROP Model

We first sought to determine whether hypoxyprobe staining would delineate regions of abutting hypoxic and oxygenated retina visualized in flatmounts to determine regions possibly vulnerable to the generation of ROS. We anticipated that the junction of vascular and avascular retina would be such a region. In 50/10 OIR, we found staining for HP mainly in avascular retina and between retinal vessels in regions of vascularized retina with reduced capillary density (Fig. 1A), showing that the junction between vascular and avascular retina was not a unique region where abutting oxygenated and poorly oxygenated retina existed. Numerous regions within the vascular retina existed that might be vulnerable to the generation of ROS. In contrast, little to no HP staining was present in the vascularized or avascular areas in flatmounts from room air–raised rats at P4, in which there were peripheral avascular retinal areas (Fig. 1B). In cryosections of P18 50/10 OIR, HP staining was localized to the inner retinal layers (Fig. 1C).

Inhibition of NAD(P)H Oxidase Activation under Conditions of Supplemental Oxygen

To determine the activation of NAD(P)H oxidase, we measured the phosphorylation of a cytoplasmic subunit of NAD(P)H oxidase, $p47^{phox}$, ,which is phosphorylated before its assembly with cytoplasmic and membrane-bound subunits to create the active heterodimer.[33,43] We found that retinas of pups in the 50/10 OIR+SO had increased phosphorylation of $p47^{phox}$ at P18 compared with those in 50/10 OIR (Student's *t*-test, $P = 0.02$; Fig. 2), providing support that NAD(P)H oxidase was activated by supplemental oxygen after fluctuations in oxygen. Because $p47^{phox}$ phosphorylation occurs before the assembled active NAD(P)H oxidase heterodimer, apocynin, which inhibits its assembly,[33,43,44] did not inhibit $p47^{phox}$ phosphorylation.

NAD(P)H Oxidase–Dependent IVNV and Tissue Hypoxia in Supplemental Oxygen

Given that 50/10 OIR+SO had greater NAD(P)H oxidase activation, we sought to determine whether its activation had an effect on IVNV or avascular retina by treating pups exposed to 50/10 OIR+SO with IP injections of the specific NAD(P)H oxidase inhibitor apocynin or of PBS from P12 to P17. Although we found no statistical difference in avascular/total retina areas (Fig. 3) or cleaved caspase 3 protein (data not shown) at P18 in apocynin versus PBS-treated pups, we found that apocynin reduced the IVNV area significantly (Student's

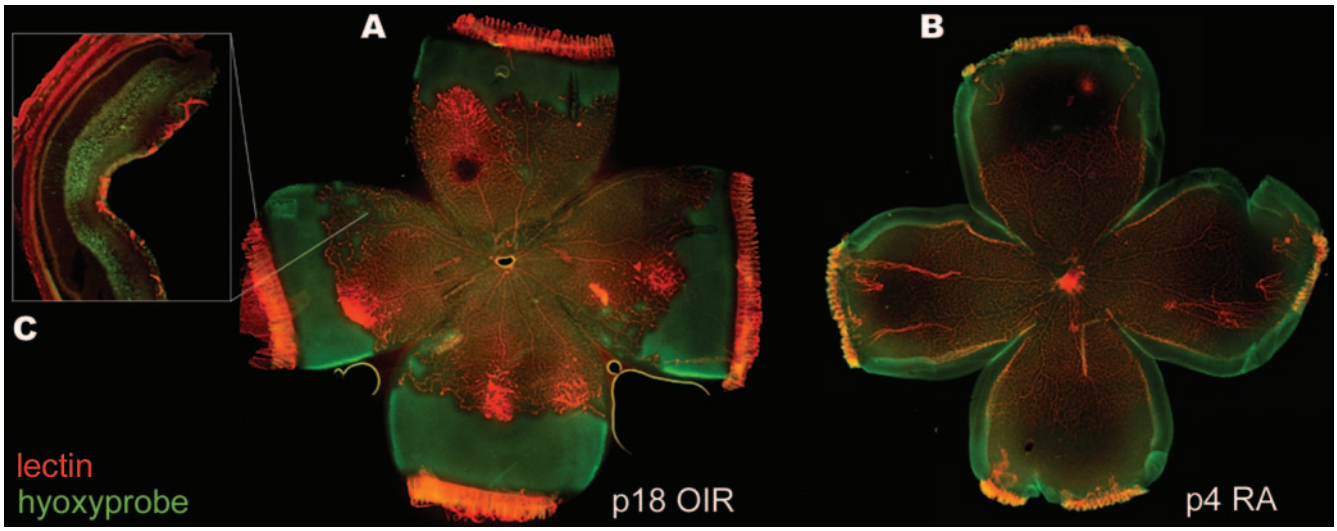

FIGURE 1. Lectin-stained (*red*) flatmounts and cryosections of retinas from 50/10 OIR and room air-raised pups given IP injections of pimonidazole (HP; *green*) 90 minutes before humane killing. (**A**) P18 50/10 OIR flatmount demonstrating hypoxic (*green*) retina in avascular zone and in regions surrounding lectin-stained blood vessels (*red*) in vascularized retina. (**B**) P4 room air-raised pup demonstrating little staining with HP, even though avascular retina is present approximately to the same extent as for P18 50/10 OIR. (**C**) Cryosection of eye from P18 50/10 OIR pup demonstrating inner retinal hypoxia (*green*) within avascular zone and some staining in ganglion cell layer in vascularized retina. Representative of eyes from three litters.

t-test, $P = 0.047$; Fig. 4). We also found that apocynin reduced whole retinal tissue hypoxia measured as reduced conjugated hypoxyprobe by Western blot analysis (Student's *t*-test, $P = 0.002$; Fig. 5).

Effect of NAD(P)H Oxidase Inhibition on VEGF under Supplemental Oxygen

Given that NAD(P)H oxidase activation can cause angiogenesis by triggering VEGF, we measured VEGF in 50/10 OIR+SO rats treated with apocynin or PBS control and found no difference in VEGF protein at P18 (Fig. 6). However, compared with 50/10 OIR, retinas from the 50/10 OIR+SO had reduced VEGF (Fig. 6; Student's *t*-tests; $P < 0.001$ for SO+50/10 OIR and 50/10 OIR). Furthermore, 50/10 OIR+SO did not have a reduced hypoxic retina, as measured by Western blot analysis of HP (Fig. 5). Therefore, supplemental oxygen reduced inner retinal VEGF but did not reduce hypoxic retina.

p-JNK, p-AKT, and p-ERK in Supplemental Oxygen versus Room Air

The activation of NAD(P)H oxidase can trigger disparate outcomes, such as apoptosis or endothelial proliferation. Because the concentration of ROS produced by NAD(P)H oxidase may affect what signaling pathway is activated,[39] we sought to determine whether hypoxia would activate NAD(P)H oxidase and trigger certain signaling pathways in retinal endothelial cells. We tested signaling pathways relevant to outcomes in the pathogenesis of ROP and those we found in vivo. Protein kinase B (Akt) is involved in cell survival, and extracellular signal-regulated kinase (ERK) is involved in proliferation, whereas JNK is involved in pathways of apoptosis. Activation of these proteins occurs through the phosphorylation of ty-

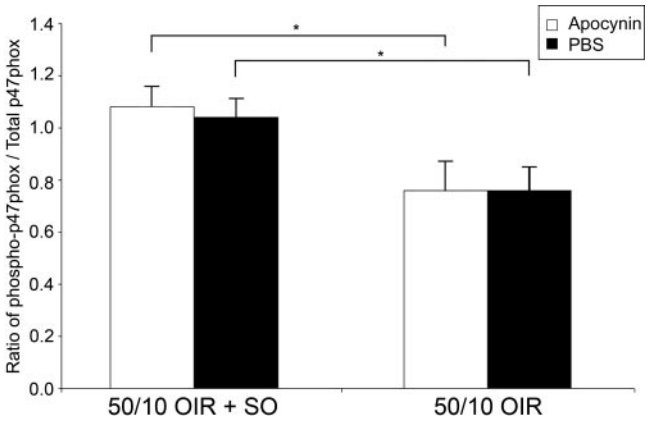

FIGURE 2. Western blot of retinas from P18 50/10 OIR+SO and P18 50/10 OIR immunoprecipitated for NAD(P)H oxidase cytoplasmic subunit $p47^{phox}$ blotted for phosphoserine to determine phosphorylated $p47^{phox}$/total $p47^{phox}$. Total $p47^{phox}$ was determined after membranes were stripped and reprobed with $p47^{phox}$ antibody ($^*P \leq 0.02$; Student's *t*-test). Retinas taken from at least four separate litters; at least five retinas were analyzed for each treatment group.

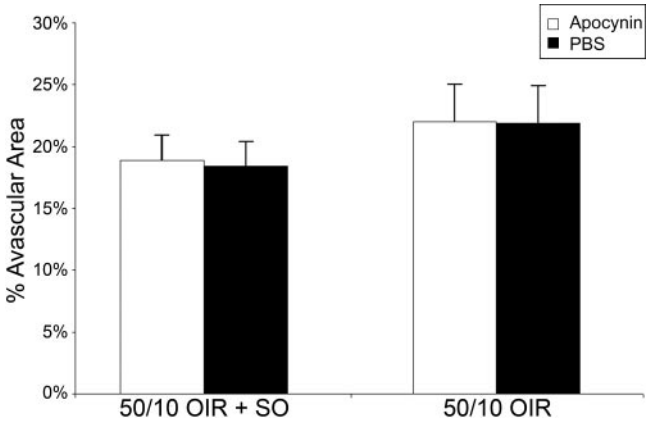

FIGURE 3. Avascular/total retina areas from pups in 50/10 OIR+SO and 50/10 OIR treated with IP injections of apocynin (10 mg/kg/d P12–P17) or an equivalent volume of PBS and assayed at P18 (P = NS). Retinas taken from at least four separate litters; at least five retinas were analyzed for each treatment group.

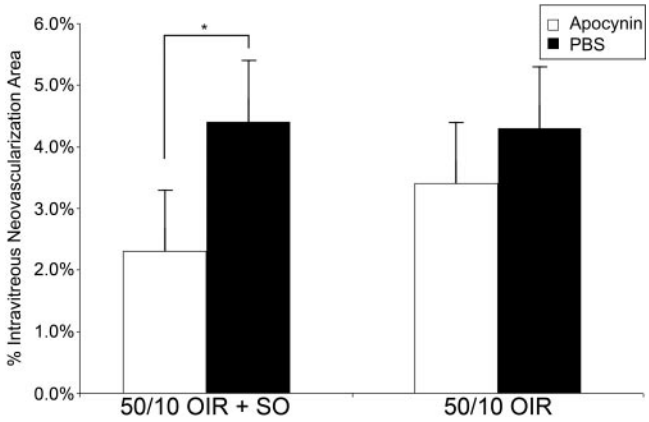

FIGURE 4. IVNV area from pups in 50/10 OIR+SO and 50/10 OIR treated with IP injections of apocynin (10 mg/kg/d P12–P17) or an equivalent volume of PBS and assayed at P18 (*$P = 0.047$; Student's *t*-test). Retinas taken from at least four separate litters; at least five retinas were analyzed for each treatment group.

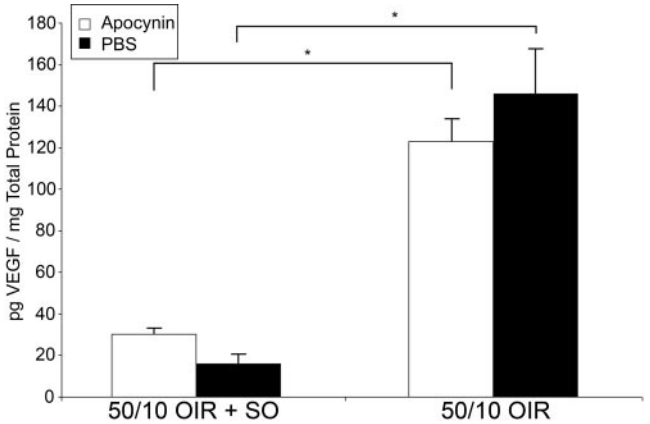

FIGURE 6. ELISA of VEGF from pups in 50/10 OIR or in 50/10 OIR+SO treated with either IP injections of apocynin (10 mg/kg/d) or an equivalent volume of PBS from P12–P17 and assayed at P18 (*$P < 0.001$; Student's *t*-test). Retinas taken from at least four separate litters; at least five retinas were analyzed for each treatment group.

rosine kinases. Therefore, we measured phosphorylated (p)-JNK, p-ERK, and p-Akt in RMVECs grown in 1% or 21% O_2 for 6 hours.[45] RMVECs exposed to 1% O_2 had increased phosphorylation of $p47^{phox}$ compared with room air–exposed cells (Fig. 7). There was undetectable p-ERK or p-Akt; however, p-JNK was increased in RMVECs in 1% O_2 compared with those exposed to room air (Fig. 8).

DISCUSSION

We previously found that one mechanism for the avascular retina in a rat model of ROP (Penn 50/10 OIR model) was NAD(P)H oxidase-dependent apoptosis.[31] In this study, we found pups exposed to repeated oxygen fluctuations followed by supplemental oxygen (i.e., 50/10 OIR+SO)[12] rather than room air (Penn 50/10 OIR) had a further increase in NAD(P)H oxidase activation, determined by increased phosphorylation of subunit $p47^{phox}$[46] (Fig. 2). In addition, the inhibition of NAD(P)H oxidase activity in 50/10 OIR+SO reduced IVNV (Fig. 4) but was not associated with reduced VEGF, suggesting there are other mechanisms involving angiogenic signaling through NAD(P)H oxidase activation.

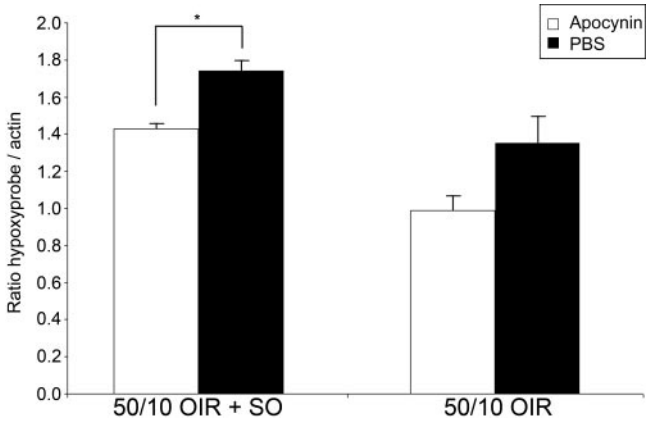

FIGURE 5. Ratio of hypoxyprobe/actin Western blot in 50/10 OIR+SO and 50/10 OIR treated with IP injections of apocynin (10 mg/kg/d P12–P17) or an equivalent volume of PBS and assayed at P18 (*$P = 0.002$; Student's *t*-test). Retinas taken from two separate litters; at least five retinas were analyzed for each treatment group.

NAD(P)H oxidase is the main source of ROS, particularly superoxide radical, released by neutrophils in response to invading organisms.[32] Besides neutrophils, macrophages, bone marrow-derived hematopoietic cells,[47] and endothelial cells[48] can activate NAD(P)H oxidase to release ROS that trigger disparate signaling events from endothelial cell survival, proliferation, and angiogenesis to endothelial apoptosis.[33] It is thought that the concentration and location of ROS released by different degrees of NAD(P)H oxidase activation may trigger different signaling pathways.[33,49,50] Our data and previous studies[31] provide evidence of this. Here we show that supplemental oxygen (50/10 OIR+SO) further increased NAD(P)H oxidase activation over that in 50/10 OIR and is implicated in angiogenesis (Figs. 2, 4), whereas previously we found NAD(P)H oxidase activation in the 50/10 OIR was a cause of peripheral avascular retina through increased apoptosis.[31] We also found that the activation of NAD(P)H oxidase and signaling of JNK

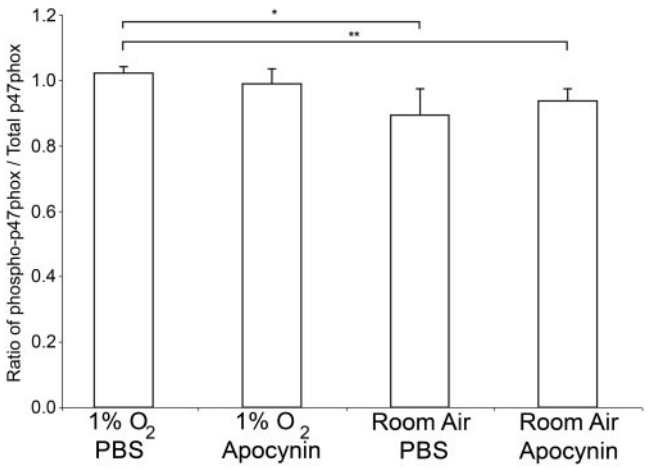

FIGURE 7. Western blot of human RMVECs treated with either apocynin (30 μg/mL final concentration) or PBS control and exposed to 1% O_2 or 21% O_2 for 6 hours, then immunoprecipitated for NAD(P)H oxidase cytoplasmic subunit $p47^{phox}$ blotted for phosphoserine to determine phosphorylated $p47^{phox}$/total $p47^{phox}$. Total $p47^{phox}$ was determined after membranes were stripped and reprobed with $p47^{phox}$ antibody. Individual Student's *t*-tests for 1% O_2 PBS versus 21% O_2 PBS (*$P = 0.02$) and for 1% O_2 PBS vs. 21% O_2 apocynin (**$P = 0.006$). $n = 4$ for each treatment group.

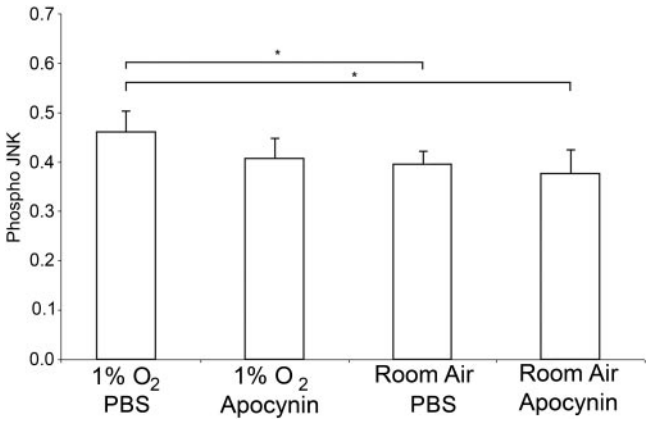

FIGURE 8. Human RMVECs treated with either apocynin (30 μg/mL final concentration) or PBS control and exposed to 1% O_2 or 21% O_2 for 6 hours, then undergoing ELISA testing for phospho-JNK. Individual Student's *t*-tests (*$P \leq 0.04$ for 1% O_2 PBS vs. 21% O_2 PBS and 1% O_2 PBS vs. 21% O_2 apocynin). ELISA was performed in duplicate for each treatment group.

was triggered in retinal microvascular endothelial cells exposed to hypoxia compared with room air (Figs. 7, 8). Previous studies reported that JNK signaling is implicated in H_2O_2 and stress-related apoptosis, whereas ERK activation occurred in VEGF-induced endothelial cell survival.[51]

We also found that the inhibition of NAD(P)H oxidase significantly reduced tissue hypoxia after supplemental oxygen (Fig. 5) but was not associated with concomitant reductions in avascular/total retina area (a source of tissue hypoxia[6]) or of VEGF (Fig. 6). HP is given systemically 90 minutes before euthanatization and is soluble, quickly diffusing into tissues and cells, forming insoluble conjugates where oxygen tension is lower than 10 mm Hg.[52] Binding to oxygen is essential, and the question as to whether redox conditions overwhelm the binding of HP to proteins in low oxygen was disproved in several studies. In the pericentral region of the liver, which has a high concentration of nitroreductase enzymes, binding of HP was assessed during anterograde and retrograde perfusion of the liver. Retrograde perfusion shifted HP binding to the then hypoxic periportal region.[52] In addition, high concentrations of NADH or NADPH were shown not to overwhelm the binding of HP to hypoxic tissue.[53] HP has also been correlated with other tissue features associated with hypoxia, including spatial relation to perfused vessels and inversely to proliferation[54,55] and correlation with oxygen microelectrode measurements when tumor hypoxia was intentionally manipulated.[56] All experimental groups in our study were treated in the same manner, and all tissue was processed within the same time frame. Although excess HP is conjugated after death and gives some background signal, the 90-minute exposure time before euthanatization causes the amount of background staining to be less than 1% of the intensity of specific binding on euthanatization. That we did not find differences in avascular/total retina may indicate that measuring the percentage of the retinal avascular area is not as sensitive as measuring conjugated proteins within hypoxic retinal cells. We therefore cannot rule out that hypoxia was related to a reduction in vascular support to the retina.

The 50/10 OIR retinal flatmounts showed hypoxic retina in the avascular zones and in zones throughout the vascular retina, suggesting that all these regions may be vulnerable to the creation of ROS. In contrast, the P4 rat pup retinal flatmount, which had peripheral avascular retina, did not have HP staining. We suspect this is because at P4, the hyaloidal circulation supplies oxygen to the retina.

Even though the inhibition of NAD(P)H oxidase activation reduced hypoxic tissue in 50/10 OIR+ SO, we were unable to find evidence that VEGF was affected (Fig. 6). We might have missed the time point in our VEGF analysis, though previous studies showed that VEGF was elevated at P12, P14, and P18 in the 50/10 OIR model.[57,58] We suspected mechanisms other than VEGF signaling, such as signaling through ROS or inflammatory cytokines,[59,60] are involved in the NAD(P)H oxidase-dependent IVNV we found in 50/10 OIR+SO.

We found reduced VEGF in 50/10 OIR+ SO compared with 50/10 OIR but not reduced tissue hypoxia. Again, we took care to treat eyes equally, and our data include minimal background signal of HP after euthanatization. In the kitten OIR model, Ernest[6] reported that once avascular retina occurred from hyperoxia induced vaso-obliteration, breathing oxygen did not result in an increase in preretinal oxygen over the avascular retinal areas, though it did initially cause an increase in preretinal oxygen tension over the vascular areas.[6] Berkowitz et al.[12] found that supplemental oxygen (SO) given over 6 days after 50/10 OIR in the rat, led to a reduced ΔPo_2 or a difference in arterial oxygen levels after breathing 95% O_2, 5% CO_2 (carbogen), and 21% O_2. (Carbogen breathing can relax the autoregulatory effect of retinal vessels, preventing the constriction seen in hyperoxia or hypertension.) The reduced ΔPo_2 in the group rescued in supplemental oxygen suggested a failure in autoregulatory or perfusion reserve, and it can be interpreted as a dysfunction in constriction of retinal vessels in high oxygen or a failure of dilation in low oxygen. Because these pups also had high levels of systemic arterial Po_2, lower ΔPo_2 may indicate higher retinal vascular oxygen concentration during supplemental oxygen, which was also what Ernest found initially over the vascular retina in the kitten study.[6] Cells within the inner retina (Müller cells and ganglion cells) sense hypoxia and are the major producers of VEGF.[61] Increased oxygen to the retina would reduce the stimulus for VEGF expression. Meanwhile, as photoreceptor development occurs and the metabolic demand of the outer retina increases, more oxygen is required. Recent evidence implicates the photoreceptors in creating oxygen demand that results in features of severe retinopathy seen in models of ROP.[62,63] Furthermore, the choroid, which is a main supplier of oxygen in the areas of avascular retina, may not be able to meet the oxygen demand because, unlike the choroid in the adult rat, the choroid in the P15 rat was unable to support increased oxygen tension with supplemental oxygen.[64] Therefore, given that the metabolic demand of the photoreceptors is inadequately met by the choroidal and retinal vasculatures, it is possible that supplemental oxygen may increase oxygen tension in the retinal vasculature that has a dysfunctional autoregulatory capacity but that still leads to overall retinal hypoxia.

Thus, in the preterm infant, anatomic factors such as avascular retinal area, history of oxygen exposure, current oxygen exposure, and level of maturation of the photoreceptors may play roles in the retinal outcomes of delayed intraretinal vascularization and IVNV. Our data support that signaling events that lead to pathologic features of ROP, namely the avascular retina and IVNV, can depend on the degree of NAD(P)H oxidase activation from repeated oxygen fluctuations followed by room air or supplemental oxygen. Supplemental oxygen and fluctuations in oxygen can cause features that increase the severity of ROP. These studies help explain some of the complexity of oxygen effects in ROP. More study is required because a reduction in oxygen concentration or broad inhibition of ROS may be detrimental to the preterm infant, whose developing central nervous system requires oxygen and whose immune system and abilities to fight infection are limited.[65]

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