The Role of Supplemental Oxygen and JAK/STAT Signaling in Intravitreous Neovascularization in a ROP Rat Model

Grace Byfield, Steve Budd, and M. Elizabeth Hartnett

Purpose. To investigate whether oxygen stresses experienced in retinopathy of prematurity (ROP) trigger signaling through reactive oxygen species (ROS) and whether the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway lead to intravitreous neovascularization (IVNV) in an oxygen-induced retinopathy (OIR) rat model.

Methods. Newborn rat pups exposed to repeated fluctuations in oxygen and rescued in supplemental oxygen (28% O_2, 50/10 OIR+SO) were treated with apocynin, an NADPH oxidase and ROS inhibitor (10 mg/kg/d), AG490, a JAK2 inhibitor (5 mg/kg/d), or phosphate-buffered saline. Intraperitoneal injections were given from postnatal day (P)12 to P17 (apocynin), or from P3 to P17 (AG490). Outcomes were intravitreous neovascularization and avascular/total retinal areas, vascular endothelial growth factor, phosphorylated JAK2, and phosphorylated STAT3.

Results. Apocynin significantly reduced phosphorylated STAT3 in 50/10 OIR+SO ($P = 0.04$), in association with previously reported inhibition of the IVNV area. Inhibition of JAK with AG490 significantly reduced phosphorylated JAK2 ($P < 0.001$), phosphorylated STAT3 ($P = 0.002$), and IVNV area ($P = 0.033$) in the 50/10 OIR+SO model compared with control.

Conclusions. Activation of NADPH oxidase from supplemental oxygen works through activated STAT3 to lead to IVNV. In addition, inhibition of the JAK/STAT pathway reduces IVNV. Further studies are needed to determine the effects and relationships of oxygen stresses on JAK/STAT and NAPDH oxidase signaling. (*Invest Ophthalmol Vis Sci.* 2009;50:3360–3365) DOI:10.1167/iovs.08-3256

Retinopathy of prematurity (ROP) is a leading cause of nonreversible childhood blindness worldwide. First described in the 1940s and 1950s,[1] it was thought that ROP likely developed as a result of unregulated high-inspired oxygen at birth. Inspired oxygen and infant oxygen saturation are since monitored, and high oxygen at birth[2,3] rarely occurs in preterm infants in the United States. However, fluctuations in oxygen and supplemental inspired oxygen later in the course of prematurity are now reported to be associated with increased risk for severe ROP.[4–8]

In a model of ROP that treats newborn rat pups to repeated fluctuations in oxygen, we found that reactive oxygen species (ROS) were increased in the retina and that NADPH oxidase was activated to trigger the apoptosis of endothelial cells, which contributed to avascular retina.[9] When pups subjected to oxygen fluctuations were placed in supplemental oxygen instead of room air, NADPH oxidase activation was exacerbated, contributing to angiogenic blood vessel growth into the vitreous.[10] This intravitreous neovascularization (IVNV) appeared, in part, to be independent of vascular endothelial growth factor (VEGF).[11]

The Janus kinase-signal transducer and activator of transcription (JAK/STAT) signaling pathway can lead to angiogenesis through ROS either by triggering angiogenic factors, such as VEGF,[12,13] or through alternative pathways.[14,15] We wanted to determine the role of JAK/STAT signaling in IVNV and used models of ROP that expose pups to oxygen stresses similar to those experienced by preterm infants at risk for severe ROP (repeated fluctuations in oxygen and supplemental oxygen). Our hypothesis was that oxygen stresses experienced by preterm infants trigger signaling through JAK/STAT pathway to contribute to IVNV. To address this, we used a JAK2 inhibitor (AG490 [tyrphostin]), which is a chemical compound that potently inhibits JAK2 protein tyrosine kinase and blocks the constitutive activation of STAT3 to inhibit DNA synthesis and cell growth and to induce apoptosis.[15] We measured IVNV area and avascular retina in our models. Because NADPH oxidase could also increase IVNV when supplemental oxygen was an added stress, we studied its potential role in JAK/STAT signaling of angiogenesis.

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 Fluctuations and Supplemental Oxygen Rat Model

A bioactive gas controller (Oxycycler; BioSpherix, New York, NY) that 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 previously reported.[16] 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. Thereafter, pups were placed in supplemental oxygen (28% O_2) for 4 days (50/10 OIR+SO). Carbon dioxide in the chambers was monitored and flushed from the system by maintaining sufficient gas flow and using soda lime. Litter numbers were between 12 and 14 pups for each experiment to ensure consistency in outcomes. All animals were weighed, and mean body weights

From the Department of Ophthalmology, University of North Carolina, Chapel Hill, North Carolina.

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Corresponding author: M. Elizabeth Hartnett, Department of Ophthalmology, University of North Carolina, 6135 Neuroscience Research Building, Chapel Hill, NC 27599-7041; hartnet@med.unc.edu.

of litters were found to be within 2 g of each other at the time of treatment.

Treatment with AG490 and Apocynin

AG490 (LC Laboratories, Woburn, MA) is a synthetic protein tyrosine kinase inhibitor that inhibits JAK2[17] and JAK3 activation. It selectively blocks cell survival and proliferation by inducing programmed cell death and was used to inhibit the JAK/STAT pathway in rats.[18] To test whether JAK2 inhibition affects signaling through STAT to cause apoptosis or angiogenesis and ultimately lead to IVNV in the 50/10 OIR model, we treated rat pups with 5 mg/kg AG490 5% dimethyl sulfoxide (MP Biomedicals, Solon, OH) in phosphate-buffered saline (PBS; Sigma, St. Louis, MO). Intraperitoneal injections (5 μL/g body weight) were administered daily from postnatal day (P)3 to P6, P3 to P13, or P3 to P17. Control animals received intraperitoneal injections of sterile 5% dimethyl sulfoxide in PBS. Separate litters received intraperitoneal injections of apocynin (4-acetovanillione; Sigma) in sterile PBS at 10 mg/kg, (5 μL/g body weight) once a day from P12 to P17, as previously reported.[10] Controls received intraperitoneal injections of sterile PBS at 5 μL/g. Pups were removed from cycling for less than 20 minutes for treatments.

Dissecting Retinal Tissue

Rat pups were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylazine (18 mg/kg) and were perfused with intracardiac paraformaldehyde (PFA; 1.0 mL, 0.5%) before euthanatization by intracardiac pentobarbital (50 μL, 80 mg/kg). Eyes were enucleated and prefixed in 2% PFA for 2 hours before anterior segments, hyaloidal vessels, and vitreous were removed. Retinas with intact ora serratas were dissected, placed into PBS, and flattened onto microscope slides by four right-angle incisions. For fresh tissue, animals were euthanatized with pentobarbital (80 mg/kg intraperitoneally), and retinas were isolated without ora serratas. Tissue was frozen in radioimmunoprecipitation assay (RIPA) buffer with protease cocktail inhibitor (1:100; Sigma) and orthovanadate (1 mM; Sigma) for protein or tissue storage reagent (RNAlater; Ambion, Austin, TX) for RNA and were stored at −80°C until analysis.

Staining Retinal Flatmounts

Flattened retinas were permeabilized in ice-cold ethanol (70% vol/vol) for 20 minutes, then in PBS/1% nonionic surfactant (Triton X-100; Sigma) for 30 minutes. Retinas were incubated with Alexa Fluor 568–conjugated *Griffonia simplicifolia* (*Bandeiraea simplicifolia*) isolectin B4 (5 μg/mL; Molecular Probes, Eugene, OR) in PBS overnight at 4°C, then rinsed three times in PBS and mounted in PBS/glycerol (2:1) with Vectashield (Vector Laboratories, Burlington, CA). Slides were secured with coverslips and sealed with nail varnish. Images of the retinal blood vessels were captured with an inverted microscope (TE2000U; Nikon, Tokyo, Japan [Michael-Hooker Microscopy Facility, University of North Carolina, Chapel Hill, NC]) and were digitally stored for analysis.

Measuring IVNV and Avascular Areas

To measure IVNV, digitized retinal image sections were assembled with methods that maintain original image dimensions (PhotoFit Premium, version 1.44; Tekmate Inc., Anchorage, AK). Total retinal area, summed peripheral avascular retinal area, and IVNV area[19] were computed in pixels (Image Tool, version 3; University of Texas, San Antonio, TX) and were converted to square millimeters (using a calibration bar on each image). IVNV has been defined as neovascularization growing into the vitreous at the junction of vascular and avascular retina.[20] Avascular areas were expressed as a percentage of total retinal area for each eye. Measurements were performed by two independent masked reviewers. A third reviewer was used to rectify discrepancies in measurements, and a final consensus was determined.

Real-Time PCR

Samples were removed from tissue storage reagent (RNAlater; Ambion), and total RNA was extracted (RNeasy Mini kit; Qiagen, Valencia, CA). DNA contamination was removed with a kit (DNA-free; Ambion, Austin, TX), and RNA quantity was determined spectrophotometrically. RNA (1 μg) from each sample was reverse transcribed (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). Approximately 200 ng cDNA was analyzed per well with one-step real time PCR (TaqMan MasterMix with reverse transcriptase; 3.7 U/reaction, Applied Biosystems) and primers specific to rat STAT3 (forward, CTAGACAATATCATCATCGACCTTG; reverse, TCCCGCTC-CTTGCTGATGA; annealing temperature, 60°C). Rat β-actin was used as the control gene and was amplified with forward and reverse primers TGCCTGACGGTCAGGTCA and CAGGAAGGAAGGCTGGAAG, respectively. Duplicate reactions in addition to controls were run for each sample in a total volume of 16 μL. Cycle threshold (CT) values were normalized to β-actin and were expressed as a fold increase over mRNA expressed at birth (P0).

Protein Extraction and Western Blotting

Retinal samples frozen in modified RIPA buffer (20 mM Tris base, 120 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol) with protease cocktail inhibitor (1:100; Sigma) and orthovanadate (1 mM; Sigma) were homogenized and centrifuged at 13,000 rpm for 10 minutes at 4°C. Total protein in the supernatant was quantified by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL), and 50 μg total protein for each sample was separated by 7.5% or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride membrane, and incubated with one or more of the following primary antibodies overnight at 4°C: phospho-JAK2, JAK2, phospho-STAT3, STAT3 (all at 1:1000; Cell Signaling Technology, Beverly, MA). Membranes were washed three times in Tris-buffered saline/0.1% Tween-20 (TBST), probed with horseradish peroxidase-linked secondary antibody, and visualized by enhanced chemiluminescence (Millipore Corp., Billerica, MA). After probing with phospho-antibody, membranes were stripped and reprobed to determine the ratio of phospho/total protein. All membranes were reprobed with β-actin (1:20,000; Abcam, Cambridge, MA) to ensure equal protein loading. Densitometry analysis was performed on exposed films using the software (UN-SCAN-IT, version 6.1; Silk Scientific, Orem, UT).

VEGF ELISA

Aliquots of total protein, previously quantified by BCA protein assay (Pierce), were assayed in duplicate without dilution, using a commercially available ELISA kit, raised against rat VEGF (R&D Systems, MN).

Statistical Analysis

Data were analyzed with commercial software (SPSS, version 14.0; Chicago, IL). Individual Student's *t*-tests were used to analyze parametric data, including avascular area/total retinal area, IVNV area, and normalized densitometric values. Ratios from densitometry analyses are expressed as percentage of control (PBS taken as 100%). For all real-time PCR and protein analyses, $n \geq 5$ was used. For measurement of IVNV or areas of avascular/total retina, 14 to 20 retinas were analyzed for each group. In all experiments, at least two separate litters were used. For all comparisons, $P < 0.05$ was determined to be significant.

Results

Expression of STAT3 in Development and under Repeated Oxygen Fluctuations

We first determined the expression of STAT3 at time points in room air development and under oxygen fluctuations through

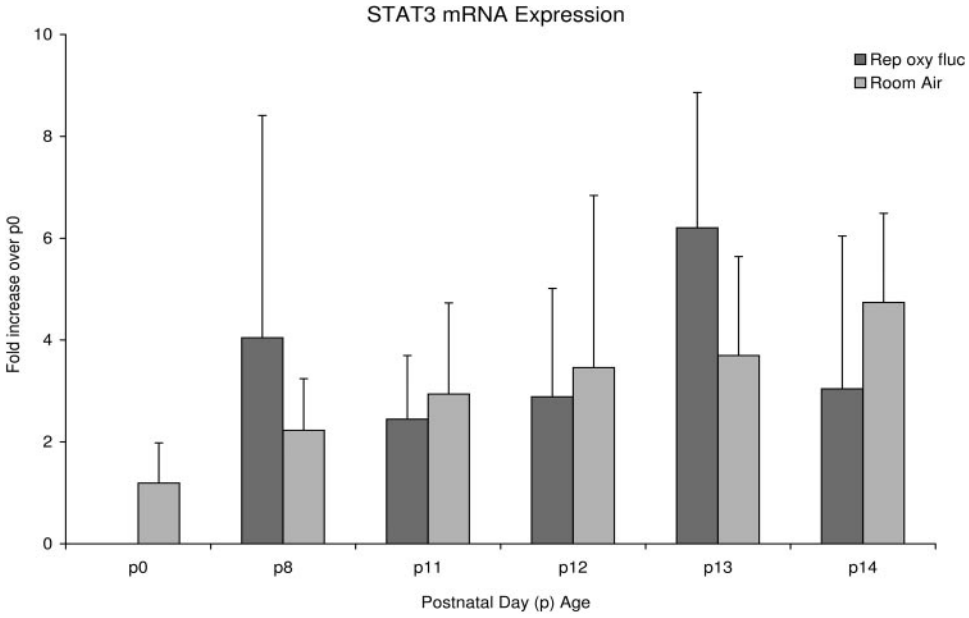

FIGURE 1. STAT3 mRNA expression measured at time points in retinas of pups from room air development and under repeated oxygen fluctuations (rep oxy fluc) through P14. STAT3 mRNA expression was analyzed by real-time PCR in four or five retinas at each time point, and data were expressed as fold change over P0. β-Actin was run as the internal control gene. There was only a modest increase in STAT3 mRNA expression under the condition of oxygen fluctuations compared with room air at several time points.

P14. We found only a modest increase in expression of STAT3 mRNA under the condition of oxygen fluctuations compared with room air at several time points (Fig. 1).

JAK/STAT Activation under Repeated Oxygen Fluctuations

AG490 was previously reported to inhibit the JAK/STAT pathway in adult male Sprague-Dawley rats with streptozotocin-induced diabetes mellitus.[18] To test the effect of AG490 on JAK2 and STAT3 activation, we measured the levels of each of these phosphorylated proteins by Western blot, normalized to β-actin, in pups treated with either AG490 or control. We found that JAK2 activation was significantly reduced at P7 ($P = 0.03$) and P14 ($P = 0.02$) in treated rats compared with controls (Figs. 2A, 2B). Phospho/total STAT3 was also significantly lower at P7 ($P = 0.033$) and P14 ($P = 0.007$) compared with control (Figs. 2C, 2D).

Intravitreous Neovascularization Induced by JAK/STAT Signaling in Supplemental Oxygen

We then determined whether IVNV would also be reduced by inhibition of JAK2, which is upstream of STAT3. Therefore, we treated pups in the 50/10 OIR+SO model with daily intraperitoneal AG490 injections and measured avascular/total retinal areas, IVNV area, phospho/total JAK2, and phospho/total STAT3. At P18, IVNV was significantly reduced by treatment with AG490 compared with control ($P = 0.033$; Figs. 3A, 3B). In addition, there was a significant decrease in retinal phosphorylated JAK2 ($P < 0.001$; Fig. 4A) and phosphorylated STAT3 ($P = 0.002$; Fig. 4B), both normalized to β-actin, in AG490-treated animals compared with controls. An analysis of VEGF protein showed no significant difference between the groups, and there were also no significant differences in total retinal or avascular/total retinal areas (data not shown). These

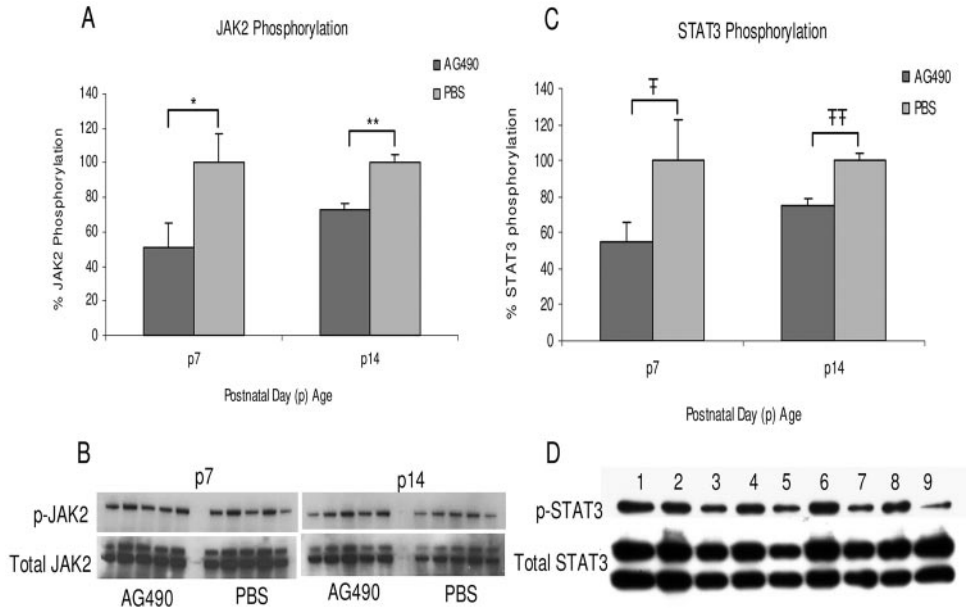

FIGURE 2. Densitometry results (**A**, **C**) and representative gel images (**B**, **D**) of phosphorylated/total JAK2 protein (**A**, **B**) and phosphorylated/total STAT3 protein (**C**, **D**) in 50/10 OIR retinas of AG490-treated rats and controls analyzed by Western blot at P7 and P14. Total protein (50 μg) separated by SDS-PAGE and probed with anti–phospho-JAK2 and JAK2 antibodies or anti–phospho-STAT3 and STAT3 antibodies. β-Actin was used as a loading control and to normalize values. Ratios are expressed as a percentage of control (PBS taken as 100%). Odd-numbered lanes are from AG490-treated retinas (P7), and even-numbered lanes show representative samples of PBS-treated retinas (P7). Data represent mean ± SE of $n = 5$ retinas for each analysis. *$P = 0.04$; **$P = 0.02$; †$P = 0.03$; ††$P = 0.01$. There were significant decreases in JAK2 and STAT3 phosphorylation after treatment with AG490 compared with controls.

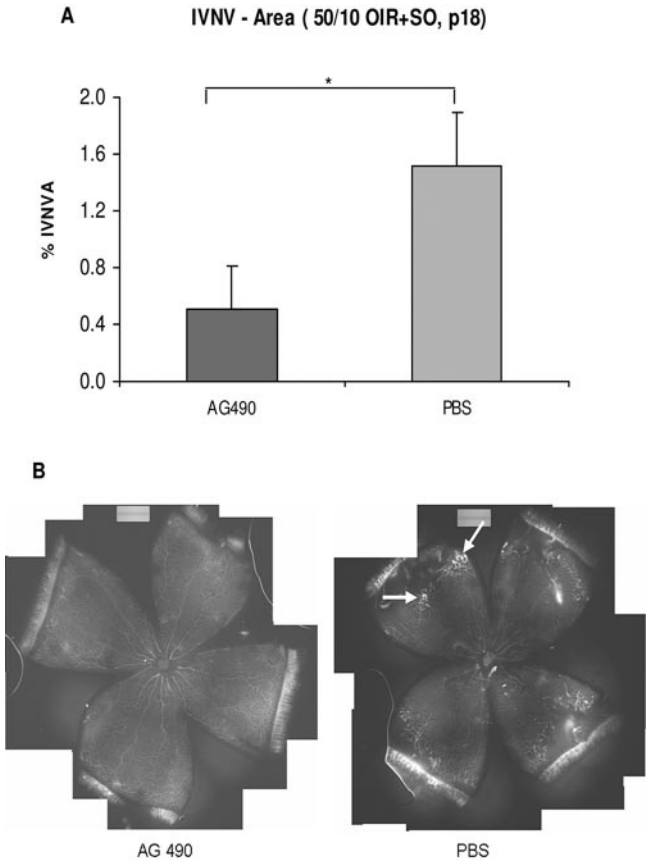

FIGURE 3. Areas of IVNV (*white arrows*) at P18 in retinas from the 50/10 OIR+SO model treated with daily intraperitoneal injections (5 mg/kg) of AG490 or PBS starting at P3 and continuing through P17. (**A**) Mean ± SE pixel density in 10 or more retinas analyzed per group; $^*P = 0.03$. (**B**) Flatmount images representative of each treatment group. AG490 treatment significantly reduced intravitreous neovascularization in the 50/10 OIR+SO model compared with control.

data provide evidence that inhibition of JAK/STAT signaling reduced IVNV.

Activated NADPH Oxidase and JAK/STAT Signaling in IVNV

JAK/STAT can be triggered by ROS,[21,22] and ROS are implicated in ROP.[23–26] We previously reported that NADPH oxidase, which when activated releases ROS, contributed to intravitreous neovascularization in the 50/10 OIR+SO model.[10] To determine whether ROS released from activation of NADPH oxidase triggered signaling through JAK/STAT to contribute to IVNV, pups were exposed to the ROP model (50/10 OIR+SO). All pups were treated with control or apocynin, a specific inhibitor of NADPH oxidase, at a dose previously shown to reduce IVNV at P18 in the 50/10 OIR+SO model.[10] Whereas apocynin significantly reduced the level of phospho/total STAT3 in the retina ($P = 0.04$; Fig. 5A), there was no difference in JAK2 phosphorylation between apocynin-treated and untreated 50/10 OIR+SO groups (Fig. 5B). These data provide evidence that activated NADPH oxidase during supplemental oxygen triggers STAT3 signaling.

DISCUSSION

The pathogenesis of severe ROP is complex and is influenced by several variables, including the effect of changes in oxygen concentration in developing retina and retinal vasculature[27,28]; the effects of nutrients and growth factors on fragile, newly formed capillaries[29–34]; and the effects of oxidative stress.[35,36] We are interested in the effects of oxygen stresses, similar to those experienced by preterm infants, on the development of severe ROP. In contrast to the more commonly used mouse model of extreme hyperoxia-induced capillary obliteration with subsequent relative hypoxia and endothelial budding (which mimicked ROP in the 1940s before more stringent monitoring of oxygen), we used a model of ROP that exposes pups to fluctuations in oxygen and supplemental oxygen, which are current risk factors for severe ROP (Hong PH, et al. *IOVS* 2002;43:ARVO E-Abstract 4011).[4,7,37,38] In addition, the extremes of the oxygen fluctuations used in this model cause arterial oxygen levels in rats[16] to be similar to the transcutaneous oxygen levels measured in human preterm infants with severe ROP.[4] In addition, the model develops characteristics similar to those of severe ROP with peripheral avascular retina,[16,20] followed by central vascular tortuosity,[39] and subsequently intravitreous neovascularization at the junction of vascular and avascular retina.[20,40] Finally, the model is highly reproducible in the measurements of characteristic features and the time points of their development.[16]

We previously reported that with an increasing number of fluctuations in oxygen, there was a trend toward an increase in oxidative compounds.[9] Oxidative stress can trigger signaling of angiogenic processes through factors including VEGF, and we and others have found that VEGF is important in the development of pathologic IVNV.[41,42] However, a reduction of VEGF alone does not totally prevent IVNV,[41,42] and VEGF is also important in normal development, which is ongoing in the preterm infant. Therefore, understanding of pathways independent of or downstream of VEGF could permit the development of effective and safer treatments.

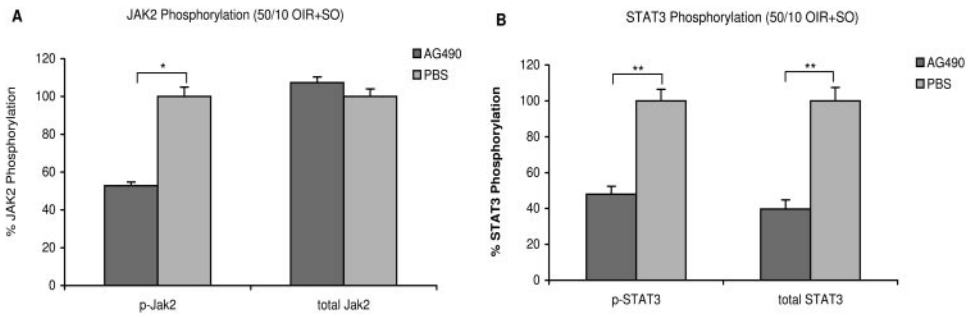

FIGURE 4. Densitometry results of JAK/STAT activation in 50/10 OIR+SO retinas from AG490-treated and control animals analyzed by Western blot at P18. Total protein (50 μg) separated by SDS-PAGE and probed with respective antibodies. (**A**) Anti–phospho-JAK2 and total JAK2. (**B**) Anti–phospho-STAT3 and total STAT3. β-Actin was used as a loading control and to normalize values. Ratios are expressed as a percentage of control (PBS taken as 100%). Data represent mean ± SE of $n = 5$ retinas each repeated from several litters. $^*P < 0.001$; $^{**}P = 0.002$. STAT3 and JAK2 activations were significantly reduced in retinas from AG490-treated animals compared with controls in the 50/10 OIR+SO model.

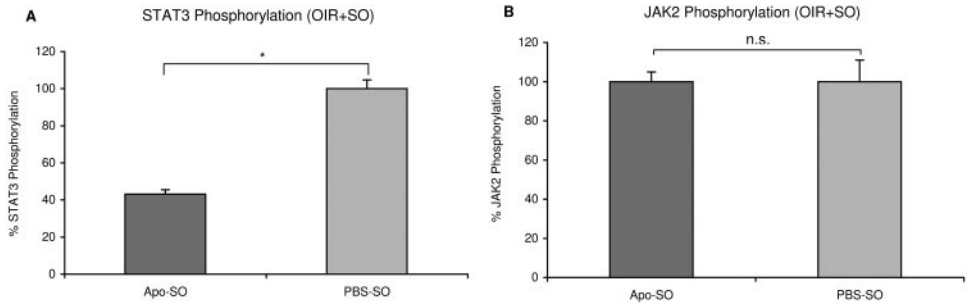

FIGURE 5. JAK/STAT activation in retinas from 50/10 OIR+SO model. Western blot of (**A**) phospho/total STAT3 and (**B**) phospho/total JAK2 after treatment with daily intraperitoneal injections of apocynin (10 mg/kg) or PBS from P12 to P17 and analyzed at P18. Six retinas were analyzed for each group, and data represent values after normalization to β-actin. $^*P = 0.04$. Ratios are expressed as a percentage of control (PBS taken as 100%). In the 50/10 OIR+SO model, apocynin significantly reduced STAT3 but not JAK2 phosphorylation compared with control. n.s., not significant.

We have reported that rescuing pups exposed to oxygen fluctuations in supplemental oxygen (i.e., the 50/10 OIR +SO model) rather than room air led to a reduction in retinal VEGF levels of room air–raised pups at the same postnatal day ages.[10] We proposed that increased retinal vascular oxygen reduced the stimulus for VEGF overexpression within the retina. However, we also found that retinal hypoxia, determined by conjugated pimonidazole, was not reduced in the 50/10 OIR+SO model compared with the 50/10 OIR model (oxygen fluctuations followed by rescue in room air).[10] Overall retinal hypoxia may occur as a result of photoreceptor metabolism, which increases during development, as shown by other investigators.[43,44] Therefore, the activation of STAT3 in this model appears to be from increased oxygen in the retinal vasculature; however, there may also be an effect from retinal hypoxia. In the present study, we used the same model with rescue in supplemental oxygen (50/10 OIR+SO) to reduce the VEGF effect on IVNV to focus on other causative pathways. Because the JAK/STAT pathway can lead to angiogenesis through ROS, we wanted to study its role in the 50/10 OIR+SO model. We found that inhibiting JAK2 with AG490 significantly reduced IVNV with associated reduced phosphorylated JAK2 and STAT3.

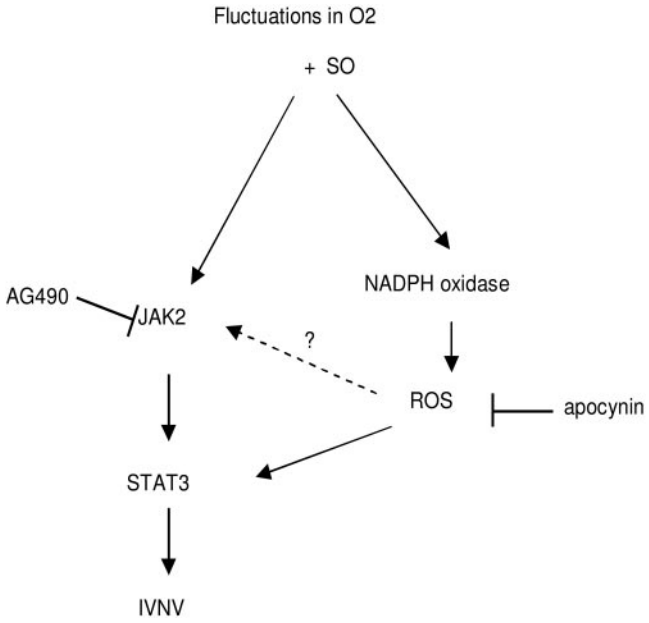

FIGURE 6. Proposed steps in signaling pathways in the 50/10 OIR+SO model that may be triggered to cause intravitreous neovascularization.

Activation of NAPDH oxidase can lead to release of ROS and, in this manner, has been reported to trigger signaling through STAT3 to increase VEGF expression in models of diabetic retinopathy.[45] We previously reported that NADPH oxidase activation in the 50/10 OIR+SO model contributed to IVNV through a pathway that appeared independent of VEGF.[10] In the present study, we found that the activation of NADPH oxidase contributed to IVNV through STAT3 signaling in the 50/10 OIR+SO model and that inhibiting NADPH oxidase activation and ROS with apocynin resulted in a reduction in phosphorylated STAT3 but not phosphorylated JAK2 (Fig. 6). Besides causing VEGF expression, the activation of STAT3 can lead to the transcription of antiapoptotic pathways and an increase in inflammatory mediators. Inflammation and apoptosis inhibition may be involved in angiogenesis.[45] Previous investigators have provided evidence of a direct involvement of ROS in activating STAT3 outside JAK2 activation.[13,14] Treatment with apocynin[10] or AG490 did not significantly affect the VEGF level in the retina, suggesting that other angiogenic factors may be involved in IVNV. Furthermore, given that VEGF and STAT3 have been reported to provide neuroprotection in some models,[45,46] a distinction among the contributions of VEGF, JAK2, and STAT3 to IVNV may provide a safer therapeutic strategy to treat severe ROP. Additional study is needed to understand the role of JAK/STAT signaling and pathways downstream of gP130 in OIR models and in the developing preterm infant at risk for ROP.

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