

# Involvement of Nox2 NADPH Oxidase in Retinal Neovascularization

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**PURPOSE.** The proliferation of new blood vessels in the retina is a leading cause of vision impairment. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) is involved in cell signaling for ischemia-induced angiogenesis, but its role in retinal neovascularization is unclear. We have analyzed the dependence of retinal neovascularization on the Nox2 isoform in oxygen-induced retinopathy (OIR) in mice.

**METHODS.** Neonatal C57BL/6 mice aged 7 days (P7) were placed in a hyperoxic chamber (75% O<sub>2</sub>) for 5 days, followed by 5 days of exposure to room air. Eyes were harvested on P8 and P17 for the quantification of retinal vaso-obliteration and neovascularization, respectively. The retinal expression of Nox2 and VEGF-A were measured by RT-PCR, while superoxide generation was detected by in situ dihydroethidium (DHE) staining of fresh frozen sections.

**RESULTS.** In wild type (WT) mice, OIR was characterized by central retinal vaso-obliteration at P8 and neovascularization at P17, which was associated with increases in Nox2 and VEGF-A gene expression, superoxide generation, and accumulation of Iba-1 positive cells in the inner retina. In contrast, Nox2 knockout mice exhibited markedly less retinal neovascularization and VEGF-A mRNA expression at P17, despite showing comparable vaso-obliteration at P8. These changes were accompanied by reductions in DHE fluorescence and Iba-1-positive cell accumulation in the hypoxic retina.

**CONCLUSIONS.** The Nox2-generated reactive oxygen species (ROS) facilitate the retinal expression of VEGF-A and neovascularization in this mouse model of OIR. Therapies targeting Nox2 could be of value to reduce aberrant retinal neovascularization in retinopathy of prematurity, diabetes, and other disease processes driven by VEGF.

**Keywords:** NADPH oxidase, retinal neovascularization, mouse OIR

Retinal neovascularization remains a leading cause of serious loss of vision in conditions, such as retinopathy of prematurity and diabetic retinopathy. Pathologic retinal neovascularization typically occurs in the context of retinal hypoxia that is a consequence of the loss of preexisting vessels, or the cessation of retinal vascular development. The new vessels formed in these processes are prone to hemorrhage and are hyperpermeable, leading to retinal edema, as well as lipid and protein exudation.<sup>1</sup> The VEGF has been identified as a key mediator of these processes: it has long been known that levels of VEGF are significantly higher in the ocular fluids and tissues of patients with proliferative retinopathy than in those of unaffected individuals,<sup>2,3</sup> and the advent of intravitreally administered inhibitors of VEGF has revolutionized the clinical management of these disorders. However, current approaches that target VEGF remain far from ideal for several reasons, including the need for repeated intravitreal injections, often for indefinite periods, the potential for deleterious effects on the choroidal vasculature, and the emergence of treatment resistance.<sup>2,3</sup> There remains a strong case for better elucidation of the molecular pathways of retinal neovascularization, and

the development of alternative or adjunctive therapies to tackle these destructive process.

One of the animal models used to study the mechanisms underlying retinal neovascularization is oxygen-induced retinopathy (OIR) in the mouse, where VEGF has a significant role.<sup>4,5</sup> Using an OIR model, Al-Shabrawey et al.<sup>6</sup> demonstrated that neovascularization in the mouse was associated with increases in VEGF expression and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox)-generated reactive oxygen species (ROS). The antioxidant apocynin, which also blocks Nox activation, suppressed ROS generation, retinal VEGF expression, and neovascularization in this OIR model.<sup>6</sup> Knockdown of *Nox4* gene expression with *Nox4* RNAi also has been demonstrated to suppress VEGF-associated retinal leakage in diabetic mice.<sup>7</sup> These and other studies suggest that Nox-generated ROS signaling may be crucial for VEGF-driven angiogenesis and vascular leakage. Several isoforms of the Nox catalytic subunit have been identified, and the prototypical Nox2 is composed of membrane-bound Nox2 and p22phox subunits, as well as the cytosolic subunits p40phox, p47phox, p67phox, and small GTPase Rac (see Supplementary Fig.

S1). In addition, VEGF has been shown to stimulate an increase in ROS generation via an activation of the catalytic domain of Nox2 to regulate angiogenesis in the ischemic hind limb, in sponge models of angiogenesis, and in an in vivo tissue engineering chamber that we have used to grow new tissues.<sup>8-10</sup> Thus, ROS appear to act as downstream mediators of VEGF-mediated signaling in angiogenesis in a range of tissues. The precise roles played by the different Nox isoforms in angiogenesis, especially in the retina, remains to be defined. Nox2 is expressed in inflammatory and endothelial cells, and both cell types have been shown to have a role in angiogenic responses including neovascularization in mouse OIR.<sup>6,11</sup> Therefore, we hypothesized that Nox2 has an important role in retinal neovascularization in OIR. A better understanding of the molecular pathways of retinal neovascularization may pave the way to new therapies for blinding eye diseases.

## MATERIALS AND METHODS

### Mouse Model of Oxygen Induced Retinopathy

Animal care guidelines according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were followed. All procedures were approved by the institutional animal care and use committee (St. Vincent's Animal Ethics Committee protocol No. 060/09). Wild type (WT) C57BL/6J mice and Nox2 knockout mice (Nox2 KO) on a C57BL/6J background were supplied by Mary Dinauer (Department of Pediatrics, Washington University of School of Medicine, St. Louis, MO),<sup>12</sup> and bred at the EMSU mouse facility (Fitzroy, Victoria, Australia). The Nox2 genotype was confirmed with conventional genotyping (see Supplementary Fig. S2; Nox2 WT forward primer, 5'AAGAGAACTCCTCTGCTGTGAA-3'; reverse primer, 5'CGCACTGGAACCCCTGAGAAAGG-3'; Nox2 KO forward primer, 5'AAGAGAACTCCTCTGCTGTGAA-3'; reverse primer, GTTCTAATTCCATCAGAAGCTTATCG-3'; Sigma-Aldrich, Sydney, New South Wales, Australia). The OIR was induced by exposing 7-day-old neonatal mice (P7) and their mothers to 75% oxygen for 5 days ( $74.91 \pm 0.05\%$  oxygen,  $n = 95$ , P7-P12) followed by a 5-day period in room air (P12-P17).<sup>4,13</sup> Oxygen levels were monitored with an external oxygen analyzer (Model No. 500-AE; Sensidyne, St. Petersburg, FL) and recordings were analyzed. Neonatal mice were sacrificed, and eyes were harvested at P8 and P17, time points corresponding with periods of peak vaso-obliteration and neovascularization, respectively.<sup>4,14</sup> Eyes were fixed in 4% paraformaldehyde (PFA; ProSciTech, Kirwan, Queensland, Australia) for 1 hour, and retinal flat mounts were prepared and stained with fluorophore-conjugated isolectin B4 (5 g/mL, Alexa Fluor 488 No. I21411; Life Technologies, Mulgrave, Victoria, Australia) overnight at 4°C.<sup>4,13</sup> Flat mounts then were washed in PBS and mounted in fluorescent mounting media (Dako, Glostrup, Denmark). Images of each retinal flat mount were captured with a fluorescence microscope ( $\times 4$  objective magnification, emission 495 nm, excitation 510 nm; Olympus, Victoria, Australia), and the degree of vaso-obliteration and neovascularization then was quantified as described previously.<sup>4</sup> Avascular areas and regions of neovascularization in images of retinal flat mounts were delineated and measured using ImageJ (National Institutes of Health, Bethesda, MD),<sup>15</sup> and expressed as a percentage of the total retinal area. Age-matched neonatal mice exposed to room air were used as normoxic controls. One eye was used for flat mount preparation, while the fellow eye was used either for gene expression analysis or in situ superoxide detection.

### Gene Expression Detected by Real-Time PCR

Total retinal RNA was purified using commercial kits in accordance with the manufacturer's instructions (RNeasy Mini Kit; Qiagen, Victoria, Australia). Briefly, a single retina was lysed and homogenized, and RNA in the lysate was purified using a column system. The RNA was reverse-transcribed to cDNA (100 ng) using a high capacity cDNA reverse transcription kit (catalogue No. 4374996; Life Technologies). Real-time PCR were performed (7300 real-time PCR systems; Life Technologies) using a TaqMan Universal PCR master mix, and commercially available TaqMan gene expression assays for mouse *Nox2* (Mm00432775\_m1; Life Technologies) and *VEGF-A* (Mm00437304\_m1; Life Technologies). Mouse *GAPDH* (catalogue No. 4352339E; Life Technologies) was used as a reference gene. Gene expression changes in the retinas of oxygen-exposed mice were normalized using values from room air-exposed mice.

### In Situ Superoxide Detection

Dihydroethidium (DHE, 5  $\mu$ M; Life Technologies) was used as described previously to detect superoxide in fresh frozen eye sections (10  $\mu$ m).<sup>16</sup> Sections preincubated with a superoxide dismutase mimetic MnTmPyP (10  $\mu$ M; Sapphire Bioscience, Waterloo, New South Wales, Australia) were used as background controls (data not shown). Sections were viewed under a fluorescence microscope (excitation 546 nm, detection 590 nm) and all images were taken with uniform exposure settings (100 ms). The fluorescence intensity of retinal sections was measured with ImageJ software (National Institutes of Health)<sup>15</sup> and data were normalized using values from room air-exposed mice.

### Immunohistochemical Detection of Macrophages

The PFA (4%)-fixed flat mounts were blocked with 4% BSA (Sigma-Aldrich) with 0.4% Triton X-100 for 60 minutes at room temperature, and then coincubated overnight (4°C) with fluorophore-conjugated isolectin B4 (5  $\mu$ g/mL), and an antibody with specificity for ionized calcium binding adaptor molecule 1 (Iba-1) expressed by macrophages and microglia (rabbit anti-mouse polyclonal Iba-1 antibody, 0.5  $\mu$ g/mL, catalogue No. 019-19741; Wako Pure Chemical Industry, Osaka, Japan). Sections then were incubated with a secondary antibody (goat anti-rabbit Cy3 antibody, 1:300; Vector Laboratories, Burlingame, CA) for 1 hour and mounted in Dako fluorescent mounting media (Dako). Rabbit IgG (Dako) was used as a negative control. Six to eight images ( $\times 20$  magnification,  $680 \times 510 \mu$ m) were selected randomly to estimate the total number of Iba-1-positive (Iba-1+) cells in each retina. Average values were expressed as the number of Iba-1+ cells per field.

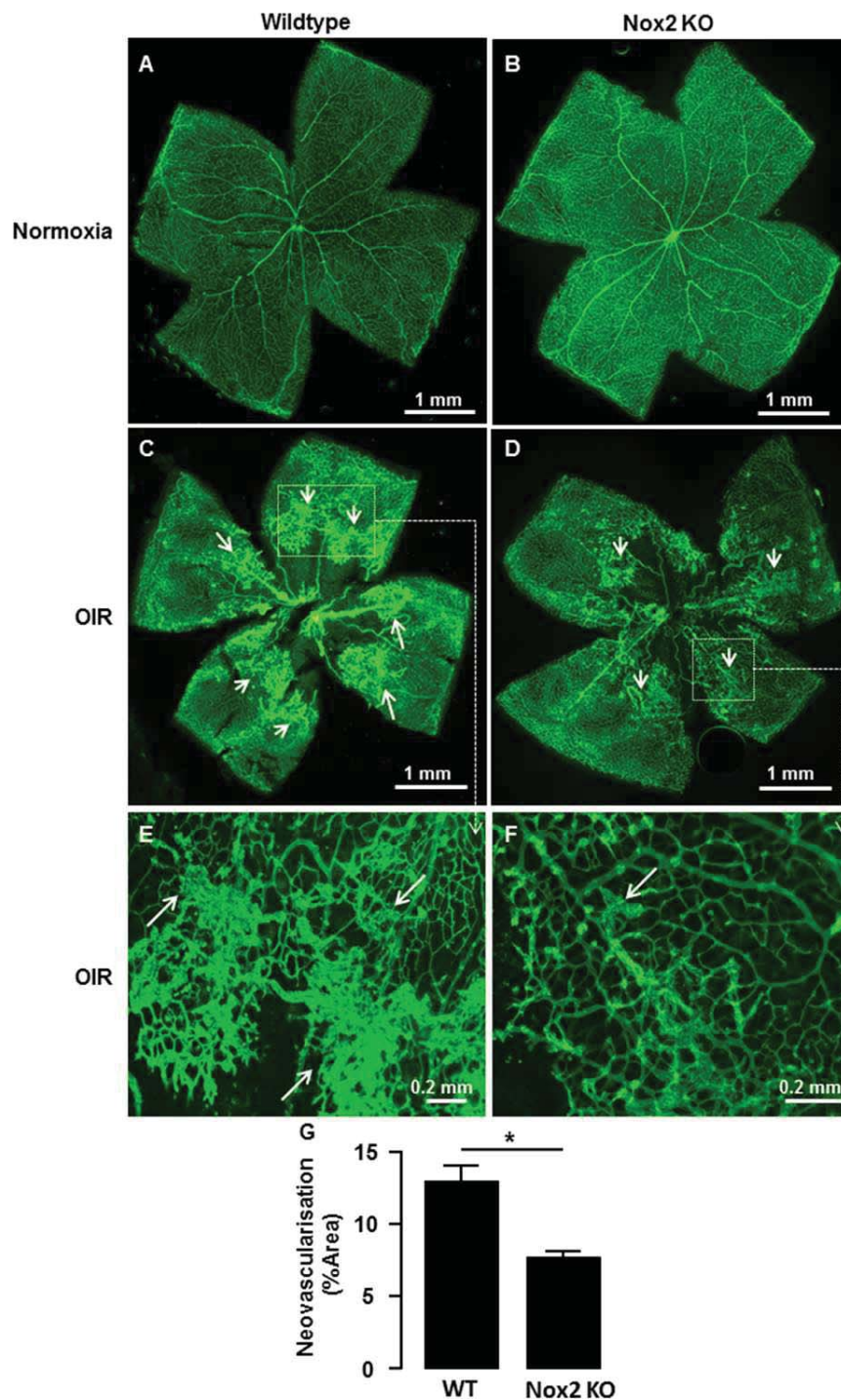
### Statistics

Mice were taken from at least three different litters for each experimental method. Between 6 and 20 mice were used for each experiment (see results for details). Data are expressed as mean  $\pm$  SEM. Mean data were analyzed with unpaired *t*-tests or 1-way ANOVA followed by post hoc Tukey analysis (GraphPad Prism 6.0). A value of  $P < 0.05$  was regarded as statistically significant.

## RESULTS

### Retinal Neovascularization Was Suppressed in Nox2 KO Mice With OIR

To investigate the role of Nox2 in retinal neovascularization, we first compared the degree of oxygen-induced neovascular-

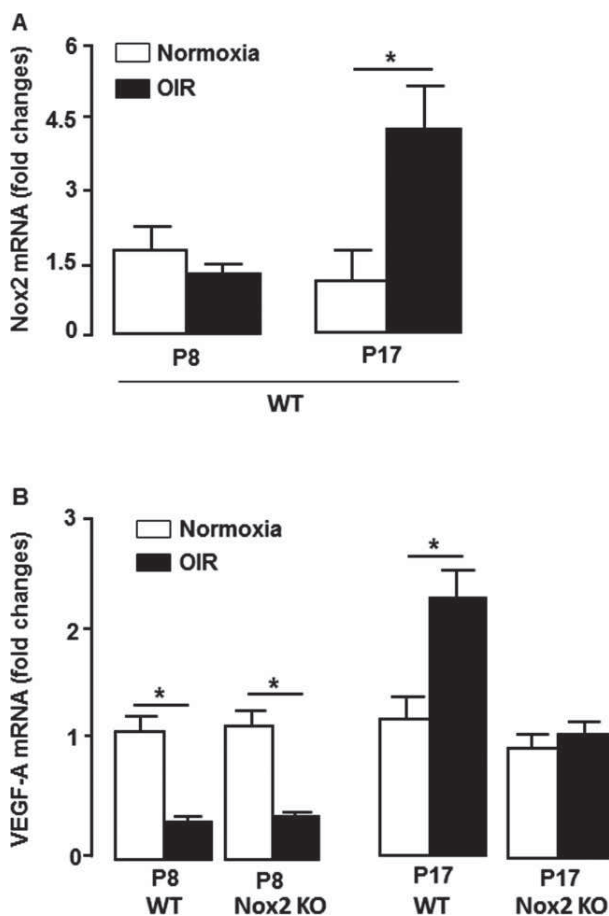


**FIGURE 1.** Representative images of retinal flat mounts from Nox2 KO and WT mice at P17. (A, B) Mice were raised in room air. (C, D) Mice were exposed to hyperoxia (75% O<sub>2</sub> from P7–P12) followed by 5 days in room air and developed OIR. Retinal vessels were identified with isolectin B4 conjugated with Alexa Fluor 488. Higher magnification images of the areas delineated by the boxes in (C, D) are shown in (E, F). Arrows mark neovascular tufts. Significantly less neovascularization is seen in the retinas of Nox2 KO mice with OIR ( $n = 20$  mice) than in WT mice ( $n = 16$ ) with OIR (G). \* $P < 0.0001$  when compared to WT.

ization between Nox2 KO and WT mice at P17 (Fig. 1). Retinal flat mounts were stained with a fluorophore-conjugated isolectin to identify blood vessels (Figs. 1A–F) and neovascular tufts (Figs. 1C–F). The extent of neovascularization was significantly lower in Nox2 KO mice than in WT mice (WT,  $12.95 \pm 1.04\%$ ,  $n = 16$  versus Nox2 KO,  $7.74 \pm 0.39\%$ ,  $n = 20$ ,

$P < 0.0001$ ; Figs. 1D, 1E, 1G), suggesting a role for Nox2 in OIR. By examining hematoxylin-eosin stained mouse eye sections, we confirmed that preretinal neovessels were more prominent in WT than in Nox2 KO mice (not quantified, see Supplementary Fig. S3). As hypoxia-induced neovascularization in OIR has been shown to vary with the extent of hyperoxia-





**FIGURE 2.** Retinal Nox2 and VEGF-A mRNA expression at P8 and P17. Retinal Nox2 mRNA expression was comparable in WT mice exposed to either room air or hyperoxia (P8). At P17, following a period of relative hypoxia, Nox2 mRNA expression was significantly higher in WT mice with OIR than in WT mice raised in room air (normoxia [A]). The VEGF-A mRNA expression was significantly and comparably reduced in WT mice and Nox2 KO mice exposed to hyperoxia (P8), relative to genotype-matched controls. Marked induction of VEGF-A mRNA expression was detected in WT mice with OIR, but not in Nox2 KO mice with OIR, relative to their genotype-matched controls raised in room air (B). \* $P < 0.05$ ,  $n = 6$  to 9 mice/group.

induced vaso-obliteration,<sup>14</sup> we compared the extent of vaso-obliteration between Nox2 KO and WT mice at P8 (see Supplementary Fig. S4). Mice of both genotypes demonstrated comparable vaso-obliteration at P8 (WT,  $50.08 \pm 1.43\%$ ,  $n = 14$  versus Nox2 KO,  $49.16 \pm 1.76\%$ ;  $n = 19$ ;  $P = 0.70$ ), suggesting that differential vaso-obliteration did not account for the observed differences in neovascularization at P17. Each genotype showed similar body weights on tissue harvest at P8 (WT,  $5.07 \pm 0.36$  g,  $n = 14$  versus Nox2 KO,  $4.53 \pm 0.16$  g,  $n = 19$ ;  $P > 1.00$ ) and at P17 (WT,  $6.69 \pm 0.20$  g,  $n = 16$  versus Nox2 KO,  $6.65 \pm 0.15$  g;  $n = 20$ ;  $P = 0.47$ ).

#### Upregulation of Gene Expression of Nox2 and VEGF-A in OIR

Recently, Nox2 and VEGF have been shown to be involved in ischemia-induced angiogenesis in a mouse hind limb model,<sup>9</sup> and VEGF is well known as a key regulator of retinal neovascularization in OIR.<sup>5</sup> We, therefore, quantified the expression of Nox2 and VEGF-A mRNA in mouse retinas with

quantitative RT-PCR at P8 (hyperoxia) and P17 (relative hypoxia). There was no difference in the expression of Nox2 at P8 between WT mice raised in room air and those exposed to hyperoxia (OIR group, Fig. 2A). In contrast, Nox2 mRNA was significantly higher in OIR WT mice than in controls at P17, a time by which peak neovascularization had occurred (Fig. 2A). The VEGF-A gene expression was suppressed in hyperoxia (P8) and was induced after a period of relative hypoxia (P17, Fig. 2B), consistent with the known expression profile of VEGF-A in mouse OIR.<sup>5</sup> Importantly, Nox2 KO mice did not exhibit hypoxia-induced VEGF gene expression at P17, implicating Nox2 in the regulation of VEGF expression (Fig. 2B).

#### Superoxide Generation in Retinas Was Reduced in Nox2 KO Mice With OIR

Retinal superoxide accumulation was detected with DHE staining in fresh frozen eye sections to determine the enzyme activity of Nox (Fig. 3).

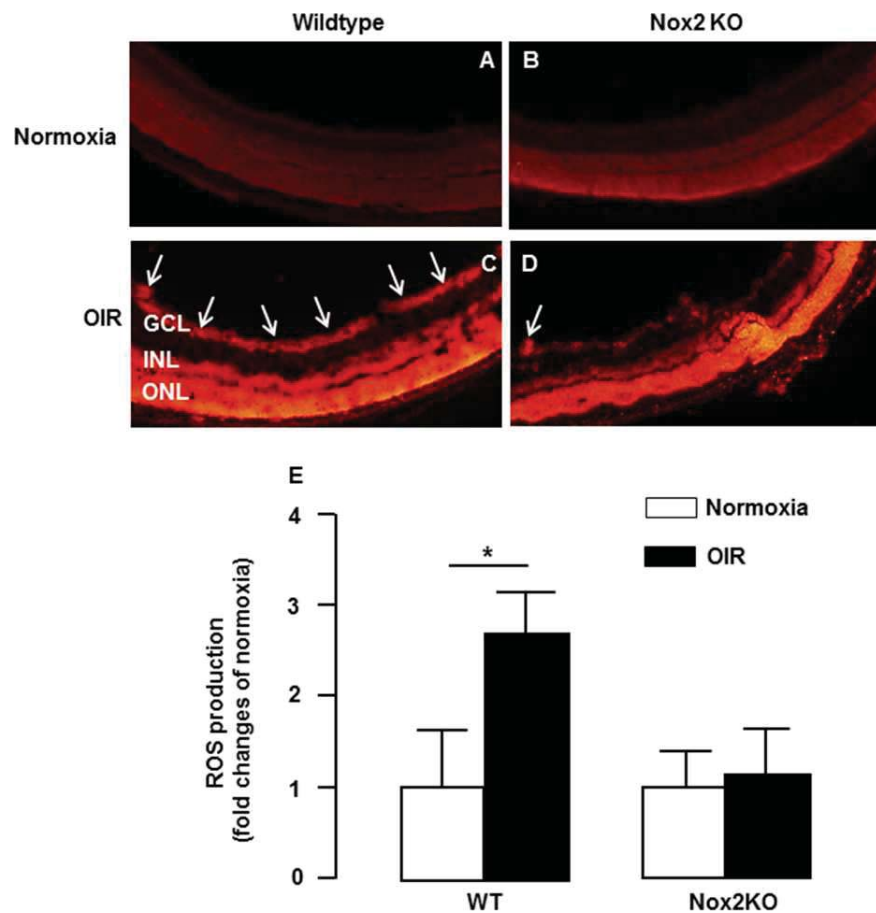
Minimal fluorescence was detected in the retinal sections of WT and Nox2 KO mice raised in room air (Figs. 3A, 3B). The DHE fluorescence was increased significantly in the retinas of WT mice with OIR when compared to those raised in room air (OIR,  $2.72 \pm 0.18$ -fold,  $n = 7$  versus normoxic,  $1.00 \pm 0.67$ -fold,  $n = 4$ ;  $P = 0.01$ ; Fig. 3E). The DHE fluorescence was most intense in the ganglion cell layer (GCL), in association with neovascular tufts, as well as in the inner (INL) and outer (ONL) nuclear layers (Fig. 3C). In contrast, OIR in Nox2 KO mice was not associated with an increase in DHE fluorescence (OIR,  $1.14 \pm 0.22$ -fold;  $n = 6$  versus normoxia,  $1.00 \pm 0.17$ -fold;  $n = 6$ ;  $P = 0.63$ ). Accordingly, DHE fluorescence appeared to be lower in the GCL of Nox2 KO mice with OIR than in WT mice with OIR (Fig. 3D).

#### Accumulation of Iba-1 Positive Macrophages and Microglia in P17 Retinas With OIR Was Reduced in Nox2 KO Mice

Retinal microglia and macrophages are known to have important roles in the neovascularization that occurs in the mouse model of OIR.<sup>11</sup> Therefore, we looked for macrophages and microglia in P17 retinal flat mounts using an Iba-1 antibody (Fig. 4). There was a significant increase in density of macrophages and microglia in the retinas of WT mice with OIR when compared to those of mice raised in room air (number of Iba-1+ cells, normoxia  $27 \pm 1$ ,  $n = 9$  versus OIR  $49 \pm 3$ ,  $n = 10$ ;  $P < 0.0001$ ; Fig. 4A). The Iba-1+ cells were colocalized predominantly with neovascular tufts (Figs. 4B-E), reflecting the known roles of these cells in neovascularization in OIR. In contrast, few Iba-1+ cells were seen in the retinas of Nox2 KO mice with OIR (number of Iba-1+ cells, normoxia  $30 \pm 1$ ,  $n = 9$  versus OIR  $33 \pm 2$ ,  $n = 14$ ;  $P = 0.31$ ; Fig. 4A).

#### DISCUSSION

The aberrant growth of new blood vessels in proliferative eye diseases is preceded by a period of hypoxia that triggers the release of growth factors, including VEGF.<sup>1</sup> The VEGF has angiogenic and proinflammatory actions, increasing vascular permeability, and it remains a key mediator of retinal neovascularization.<sup>2</sup> We have identified a mechanism by which Nox2-generated ROS facilitates VEGF-mediated retinal neovascularization in a mouse model of OIR. The reduction in retinal neovascularization in Nox2 KO mice with OIR was associated with a decrease in the accumulation of Iba-1+ macrophages and microglia, pointing to a role for these inflammatory cells in



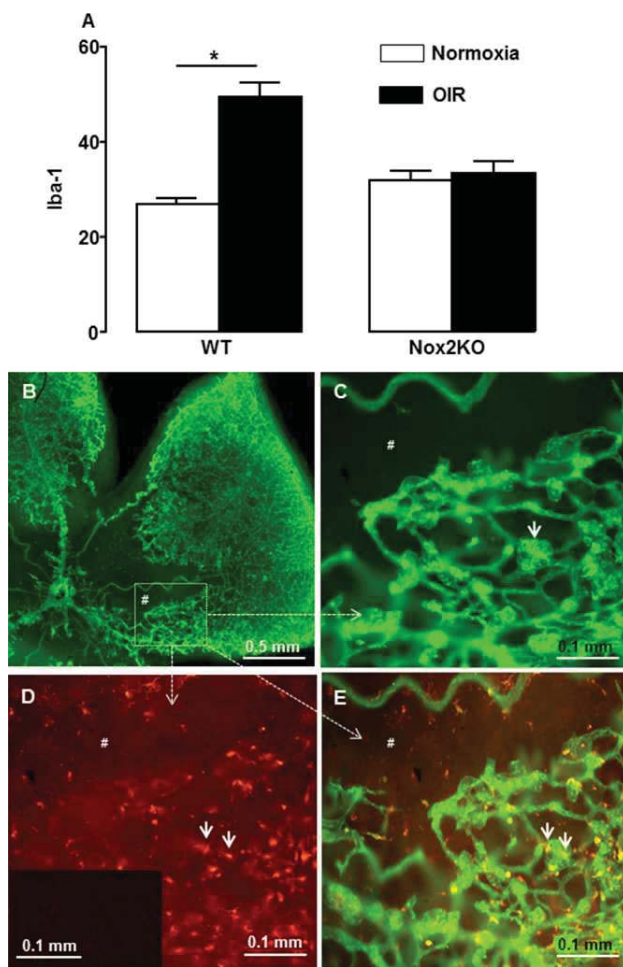
**FIGURE 3.** DHE imaging of superoxide accumulation in fresh frozen retinal sections at P17. Minimal DHE fluorescence was detected in Nox2 KO and WT mice raised in room air (normoxia [A, B]). The DHE fluorescence was increased in the GCL, INL, and ONL of WT mice with OIR (C). The DHE fluorescence appeared to be higher in WT mice with OIR (C) than in Nox2 KO mice with OIR (D). Fluorescence was particularly prominent in the GCL of WT mice with OIR (arrows in [C]). Quantitative analysis of DHE fluorescence intensity at P17 showed a marked increase in superoxide accumulation in retinas of WT mice with OIR, but not in the retinas of Nox2 KO mice with OIR (E). \* $P = 0.01$ ,  $n = 4$  to 7 mice/group.

the development of new vessels. Inhibitors of this pathway, therefore, may represent an additional means of suppressing neovascularization and inflammation associated with retinal eye disease.

Studies of inhibitors of Nox and antioxidants, including siRNA targeting Nox subunits p22phox,<sup>17</sup> as well as the antioxidant and a nonselective inhibitor apocynin,<sup>6</sup> have demonstrated a role for the Nox enzyme complex and ROS in mouse models of retinal and choroidal neovascularization. Al-Shabrawey et al.<sup>6</sup> showed that the antioxidant apocynin, which also blocks Nox activation, suppressed ROS generation, retinal VEGF expression, and neovascularization in OIR model. We extended their findings by showing that Nox2 activation is an important component of retinal neovascularization. While Al-Shabrawey et al.<sup>6</sup> demonstrated that vascular endothelial cells contributed to hypoxia-induced activation of Nox, we found reductions in superoxide production and Iba-1+ cell accumulation in the hypoxic retina in Nox2 KO, suggesting an association between Nox2-generated ROS from Iba-1+ macrophages and microglia, and neovessels in OIR. Our finding also is consistent with a recent study by Zhang et al.,<sup>18</sup> which demonstrated that suppressing the expression of Rac mRNA with shRNA reduced VEGF protein levels in hypoxic retinas. It also has been demonstrated that inactivation of Nox achieved by deletion of the Nox cytosolic subunit p47phox suppresses choroidal neovascularization in mice.<sup>19</sup> Five isoforms of Nox

(Nox1, Nox2, Nox3, Nox4, and Nox5) have been identified in different tissues, and each isoform requires different subunits for full activation.<sup>20</sup> The p22phox is an integral functional subunit for Nox1, Nox2, Nox3, and Nox4, while p47phox and Rac are required by Nox1 and Nox2 for effective ROS generation when the enzyme is activated, for example by growth factors, like VEGF or stimuli, such as hypoxia.<sup>20</sup> While all of these studies have firmly established a role for Nox in retinal neovascularization,<sup>6,17-19</sup> they did not identify the isoform of Nox that is responsible for these effects. Our study established that Nox2 is a key mediator of retinal neovascularization in mouse OIR.

The reduction in retinal neovascularization observed in Nox2 KO mice with OIR implicates Nox2 in hypoxia-induced neovascularization. The VEGF is a mediator of retinal neovascularization in OIR,<sup>4,5</sup> and ROS production has been shown to induce VEGF expression by retinal vascular endothelial cells in hypoxia<sup>6,7</sup> and in pulmonary vessels in a mouse model of hypoxic lung injury.<sup>21</sup> We have demonstrated that the expression of VEGF-A and Nox2 mRNA, as well as the production of superoxide were higher in the hypoxic mouse retina than in age-matched normoxic controls. In the absence of Nox2 gene expression, the stimulatory effect of hypoxia on VEGF-A mRNA expression and ROS production was attenuated significantly. These observations indicated that Nox2 is a major source of ROS production and a driver of VEGF expression in



**FIGURE 4.** Immunofluorescent detection of Iba-1+ macrophages and microglia in retinal flat mounts at P17. Significantly more cells stained with Iba-1 (macrophages and microglia) are seen to accumulate in the retinas of WT mice with OIR at P17 than in WT mice raised in room air. In contrast, similar numbers of Iba-1+ cells are seen in the retinas of Nox2 KO mice with OIR as in control Nox2 KO mice exposed to room air (A). Representative retinal flat mounts show neovascular tufts, labeled with isolectin B4 (B, C) and macrophages, and microglia stained with Iba-1. The inset box shows negative staining with the negative control rabbit IgG (D). The merged image (E) demonstrates the accumulation of macrophages and microglia in avascular areas (#) and adjacent to neovascular tufts (arrows). \* $P < 0.0001$ ,  $n = 9$  to 14 mice/group.

hypoxia. In keeping with these findings, *in vitro* studies have demonstrated that hypoxia enhances Nox2 protein expression in retinal vascular endothelial cells<sup>7</sup> and the inhibition of Nox2 activity suppresses hypoxia-induced VEGF expression.<sup>6</sup> Thus, it is apparent that Nox2-dependent ROS production is involved in the induction of retinal VEGF expression by hypoxia.

Hypoxia also is known to stimulate the expression of VEGF in developmental retinal vascularization and in pathologic neovascularization via stabilization of hypoxia-inducible factor (HIF-1 $\alpha$ ).<sup>22,23</sup> The inhibition of retinal HIF-1 $\alpha$  gene expression in mice is known to reduce retinal VEGF expression and neovascularization.<sup>23</sup> Further, ROS are known to induce the expression of HIF-1 $\alpha$  and stabilize HIF-1 $\alpha$  mRNA in hypoxia *in vivo*<sup>7</sup> and *in vitro*,<sup>21</sup> with concomitant upregulation of VEGF. Moreover, Diebold et al.<sup>24</sup> showed that the expression of HIF-1 $\alpha$  protein in a mouse model of subcutaneous angiogenesis was significantly lower in Nox2 KO mice than in WT controls,

consistent with the conclusion that Nox2 and ROS generation are involved in the expression of HIF-1 $\alpha$ . Furthermore, it is likely that Nox2 enhances VEGF-dependent retinal neovascularization by interfering downstream with protein tyrosine phosphatases (PTPs), which are negative modulators of the VEGF receptor VEGFR2 signaling.<sup>25</sup> This is achieved by oxidation of crucial cysteine residues in PTPs, thus, inactivating them, and promoting VEGFR2 phosphorylation and signaling.<sup>24</sup>

The prevention of hyperoxia-induced vaso-obliteration is known to attenuate hypoxia-induced neovascularization in mouse OIR.<sup>14</sup> We have demonstrated that Nox2 KO and WT mice are equally prone to hyperoxia-induced vaso-obliteration at P8, excluding this as a driver for the differences observed in the extent of retinal neovascularization at P17. Interestingly, Saito et al.<sup>26</sup> found that intraperitoneal administration of apocynin during and after the phase of hyperoxia in a rat model of OIR reduced the retinal avascular area, without affecting VEGF protein expression and intravitreal neovascularization at 4 days after the phase of hyperoxia. In a different study, Saito et al.<sup>27</sup> found that supplementary oxygen given for 4 days after the phase of hyperoxia enhanced the phosphorylation of p47phox, indicating an activation of Nox. Because apocynin blocks the activation of other Nox isoforms, the contribution of other isoforms cannot be ruled out.

We have demonstrated that the accumulation of Iba-1+ macrophages and microglia in the hypoxic retina of Nox2 KO mice was significantly lower than that seen in WT mice. As macrophages and microglia are known to be important drivers of retinal neovascularization in OIR,<sup>11</sup> this observation may account in part for the reduction in neovascularization seen in Nox2 KO mice. Expression of the macrophage chemoattractant protein CCL2 is known to be enhanced during the neovascular phase of OIR,<sup>11</sup> and Nox2 also might be involved in CCL2 expression. In support of this hypothesis, renal expression of CCL2 is lower in Nox2 KO mice than WT mice in the setting of diabetes.<sup>28</sup> Furthermore, endothelial overexpression of Nox2 following arterial injury increases macrophage adhesion.<sup>29</sup> Thus, the proinflammatory actions of Nox2 may well contribute to hypoxia-induced retinal neovascularization.<sup>11</sup>

We have found that superoxide generation, as evidenced by DHE fluorescence, was predominantly localized in the GCL, and the INL and ONL during the period of neovascularization in OIR. This expression pattern bears close resemblance to the distribution of macrophages and microglia in retinal cross sections from mice with OIR.<sup>11</sup> Other work has demonstrated colocalization of Nox2 with the endothelial cell marker CD31 in neovascular tufts in OIR, and Nox2 is known to be expressed in endothelial cells and inflammatory cells.<sup>30</sup> Therefore, it is likely that Nox2-generated ROS from endothelial<sup>6</sup> and inflammatory cells are involved intimately in the development of retinal neovascularization in OIR.

In conclusion, our findings demonstrated that Nox2-generated ROS are important mediators of hypoxia-induced retinal neovascularization. The Iba-1+ macrophages and microglia appear to be one of the key sources of Nox2-generated ROS. Therapies that target Nox2 directly might well prove to be valuable in the management of aberrant ocular neovascularization and inflammation.

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