

# Early Intervention of Tyrosine Nitration Prevents Vaso-Obliteration and Neovascularization in Ischemic Retinopathy

Mohammed A. Abdelsaid, Bindu A. Pillai, Suraporn Matragoon, Roshini Prakash, Mohamed Al-Shabrawey, and Azza B. El-Remessy

*Program in Clinical and Experimental Therapeutics, University of Georgia, Augusta, Georgia (M.A.A., B.A.P., S.M., R.P., A.B.E.); Departments of Oral Biology (M.A.), Ophthalmology (M.A., A.B.E.), and Pharmacology and Toxicology (A.B.E.), Medical College of Georgia, Augusta, Georgia; and Veterans Affairs Medical Center, Augusta, Georgia (M.A.A., B.A.P., S.M., A.B.E.)*

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## ABSTRACT

Diabetic retinopathy and retinopathy of prematurity are blinding disorders that follow a pathological pattern of ischemic retinopathy and affect premature infants and working-age adults. Yet, the treatment options are limited to laser photocoagulation. The goal of this study is to elucidate the molecular mechanism and examine the therapeutic effects of inhibiting tyrosine nitration on protecting early retinal vascular cell death and late neovascularization in the ischemic retinopathy model. Ischemic retinopathy was developed by exposing neonatal mice to 75% oxygen [postnatal day (p) 7–p12] followed by normoxia (21% oxygen) (p12–p17). Peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTPPS) (1 mg/kg), the nitration inhibitor epicatechin (10 mg/kg) or the thiol donor *N*-acetylcysteine (NAC, 150 mg/kg) were administered (p7–p12) or (p7–p17). Vascular endothelial cells were incubated at hyperoxia (40% oxygen) or normoxia (21% oxygen) for 48 h. Vascular density was determined in retinal flat mounts labeled with

isolectin B4. Expression of vascular endothelial growth factor, caspase-3, and poly(ADP ribose) polymerase (PARP), activation of Akt and p38 mitogen-activated protein kinase (MAPK), and tyrosine nitration of the phosphatidylinositol (PI) 3-kinase p85 subunit were analyzed by Western blot. Hyperoxia-induced peroxynitrite caused endothelial cell apoptosis as indicated by expression of cleaved caspase-3 and PARP leading to vaso-obliteration. These effects were associated with significant tyrosine nitration of the p85 subunit of PI 3-kinase, decreased Akt activation, and enhanced p38 MAPK activation. Blocking tyrosine nitration of PI 3-kinase with epicatechin or NAC restored Akt phosphorylation, and inhibited vaso-obliteration at p12 and neovascularization at p17 comparable with FeTPPS. Early inhibition of tyrosine nitration with use of epicatechin or NAC can represent safe and effective vascular-protective agents in ischemic retinopathy.

Retinopathy of prematurity (ROP) and diabetic retinopathy (DR) are potentially blinding disorders that affect premature infants and working-age adults, respectively, in the United States (Aiello et al., 1998; Chen and Smith, 2007). ROP and DR follow a pathological progression pattern characteristic of ischemic retinopathy, where the loss of retinal capillary is an early initiating event, leading to a poorly controlled process of retinal neovascularization and the development of proliferative retinopathy (for review see, Caldwell et al., 2003). So far, the standard treatment for retinal

neovascularization is limited to laser photocoagulation. Although successful, this treatment is invasive and results in loss of peripheral vision (for review see, Ali and El-Remessy, 2009). The lack of approved pharmacological treatment for DR and ROP creates a great need for finding new effective therapeutic modalities to treat these devastating diseases. The mechanisms that control the process of retinal neovascularization are therefore of major clinical importance.

Peroxynitrite formed by reaction of nitric oxide and superoxide anion mediates a variety of biological processes including inhibition of key metabolic enzymes, lipid peroxidation, nitration of the protein tyrosine residue, and reduction of cellular antioxidant defenses by oxidation of thiol pools (Pacher et al., 2007). A critical role of increased oxidative stress and, in particular, the peroxynitrite is supported by previous studies showing that increases in peroxynitrite formation cause capillary endothelial cell apoptosis leading to

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**ABBREVIATIONS:** ROP, retinopathy of prematurity; DR, diabetic retinopathy; BRE, bovine retinal endothelial; VEGF, vascular endothelial growth factor; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride; PARP, poly(ADP ribose) polymerase; p, postnatal day; PI, phosphatidylinositol; NAC, *N*-acetylcysteine; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; GSH, reduced glutathione; GSSG, oxidized glutathione.

vascular cell loss in DR and ROP models (Brooks et al., 2001; Gu et al., 2002; Sennlaub et al., 2002; Beauchamp et al., 2004; Kowluru and Odenbach, 2004; Kowluru et al., 2007). Moreover, clinical studies showed that infants with active ROP have low serum levels of reduced glutathione (GSH), suggesting compromised antioxidant defense (Papp et al., 1999; Wright et al., 2006). We and others have shown that increased peroxynitrite formation correlates with capillary endothelial cell apoptosis (Du et al., 2002; Sennlaub et al., 2002; Zou et al., 2002; Gu et al., 2003; el-Remessy et al., 2005; Drel et al., 2008). However, the molecular mechanism for peroxynitrite-induced vascular cell death and the therapeutic role of blocking tyrosine nitration were not investigated in models of ischemic retinopathy.

In the present study, we took advantage of using the ischemic retinopathy mouse model because it has two distinguished stages: initial stage of hyperoxia (75% oxygen) characterized with excessive peroxynitrite formation and capillary dropout, followed by a later stage of hypoxia (21% oxygen) characterized with mild oxidative insult and retinal neovascularization (Al-Shabrawey et al., 2005; El-Remessy et al., 2007). We compared the effects of selectively blocking the tyrosine nitration by use of epicatechin, one of the green tea extracts that has no antioxidant properties but selectively inhibits tyrosine nitration process, versus 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron III chloride (FeTPPS) the specific peroxynitrite decomposition catalyst or *N*-acetylcysteine (NAC), which is a dietary supplement, general antioxidant, and a thiol donor. Although we showed previously that blocking tyrosine nitration by use of the selective nitration inhibitor epicatechin can restore VEGF survival signal (el-Remessy et al., 2005), it enhances VEGF's angiogenic signal in vitro and retinal neovascularization in vivo (El-Remessy et al., 2007).

Our previous studies showed that high levels of peroxynitrite inhibit the prosurvival signal of VEGF and basic fibroblast growth factor and cause endothelial cell death in culture models of diabetic retinopathy and retinopathy of prematurity (Gu et al., 2003; el-Remessy et al., 2005). Here, we elucidate the molecular mechanism by which peroxynitrite-mediated tyrosine nitration can inhibit p85 subunit of PI 3-kinase and trigger vascular cell death in vivo. We also investigate the effects of the early intervention of tyrosine nitration on preventing retinal vascular cell death and vaso-obliteration during hyperoxic stage [postnatal day (p) 7 to p12] and the continuous intervention (p7–p17) on preventing retinal neovascularization. Our results suggest that early blocking tyrosine nitration and peroxynitrite formation could be considered as a new effective therapeutic target for a possible control of common ischemic proliferative retinopathy diseases.

## Materials and Methods

**Cell Culture.** Primary cultures of bovine retinal endothelial (BRE) cells were prepared as described previously (El-Remessy et al., 2007). Cells from passages 4 to 8 were used in all experiments. Cells were maintained in M199 supplemented with 10% fetal bovine serum, 10% CS-C complete medium, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified CO<sub>2</sub> incubator.

**Treatment during Hyperoxia and Normoxia.** BRE cells were grown to 80% confluence and then switched to serum-free medium

and placed in a hyperoxic (40% O<sub>2</sub>, 5% CO<sub>2</sub>) or normoxic (21% O<sub>2</sub>, 5% CO<sub>2</sub>) environment for 48 h unless otherwise indicated. The 40% O<sub>2</sub> level was chosen for hyperoxia (40% oxygen) exposure based on previous research showing that 40% O<sub>2</sub> generates significant peroxynitrite without inducing toxicity in retinal cells (Gu et al., 2003). The hyperoxia (40% oxygen) exposure was performed in a humidified incubator modified by installing the PROOX model 110 oxygen regulator (Biospherix, Redfield, NY). The oxygen level was monitored continuously by use of the PROOX oxygen sensor.

**Animals.** All experiments were performed with use of C57Bl/6 mice and were approved by the institutional Committee for Animal Use in Research and Education at the Veterans Affairs Medical Center and conformed to the Statement for the Use of Animals in Ophthalmic and Vision Research of The Association for Research in Vision and Ophthalmology.

**Ischemic Retinopathy Mouse Model.** Following the protocol of Smith et al. (1994), on p7 newborn mice were placed along with their dams into a custom-built chamber in which the partial pressure of oxygen was maintained at 75% (Biospherix). Mice were maintained in 75% oxygen for up to 5 days (p12), after which they were transferred back to room air (relative hypoxia 21% oxygen). Room temperature was maintained at 20°C, and rooms were illuminated with standard fluorescent lighting on a 12-h light/dark cycle. Newborn mice were nursed by the dams that were given food (standard mouse chow) and water.

**Treatment during Hyperoxia and Hypoxia Periods.** Animals were treated by daily intraperitoneal injections with the following inhibitors: peroxynitrite decomposition catalyst FeTPPS (1 mg/kg; Calbiochem, San Diego, CA); the nitration inhibitor, (–)-*cis*-3,3',4',5,7-pentahydroxyflavone, (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-1(2*H*)-benzopyran-3,5,7-triol (epicatechin, 10 mg/kg; Sigma-Aldrich, St. Louis, MO); or the general antioxidant, NAC (150 mg/kg; Sigma-Aldrich). Animals were treated either during hyperoxia stage (75% oxygen) only (p7–p12) or during hyperoxia (75% oxygen) and hypoxia stage (21% oxygen) (p7–p17). Control animals were injected with the vehicle. The treatment regimen was optimized and described previously (El-Remessy et al., 2007).

**Dissecting Retinal Tissue.** Pups were deeply anesthetized by an intraperitoneal injection of 40 mg/kg Avertin 2. One eye was enucleated and fixed in 2% paraformaldehyde overnight to be flat-mounted. For the other eye, retinas were isolated and snap frozen for biochemical assays.

**Analysis of Vaso-Obliteration and Neovascularization.** Retinal vascular distribution was analyzed by use of retinal flat mounts labeled with biotinylated *Griffonia simplicifolia* lectin B4 and Texas Red-conjugated Avidin D (Vector Laboratories, Burlingame, CA). Retinas were viewed and imaged with fluorescence AxioObserver Zeiss Microscope (Carl Zeiss GmbH, Jena, Germany). Vaso-obliteration was assessed on p12 and p17 as described previously (Al-Shabrawey et al., 2005). The areas of retinal neovascularization were assessed on p17 as described previously (El-Remessy et al., 2007).

**Oxidized and Reduced Glutathione Ratio.** Total glutathione including GSH and oxidized glutathione (GSSG) were measured by use of a kit (Northwest Life Science, Vancouver, WA) according to manufacturer's protocol. For total glutathione, retinas were lysed in phosphate buffer (100 mM potassium phosphate and 1 mM EDTA) and were mixed with an equal amount of 10 mM 5,5'-dithiobis (2-nitrobenzoic acid) in the presence of glutathione reductase and NADPH producing a measurable yellow color. The color was measured at a wavelength of 412 nm. To detect GSSG, samples were treated with 10 mM 2-vinylpyridine (Sigma-Aldrich) in ethanol to sequester all the reduced GSH then measured using the same protocol of the glutathione. GSH was calculated as the difference between total glutathione and GSSG.

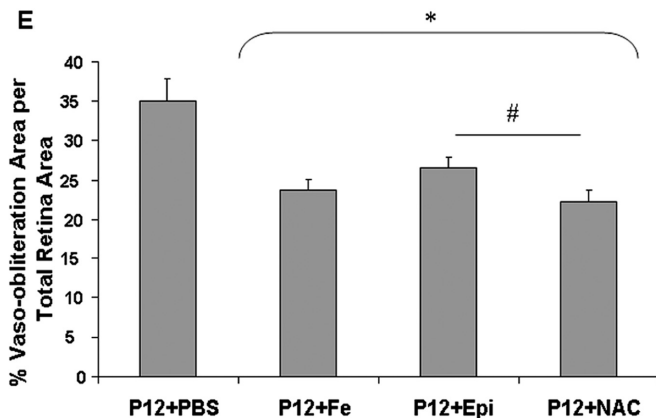
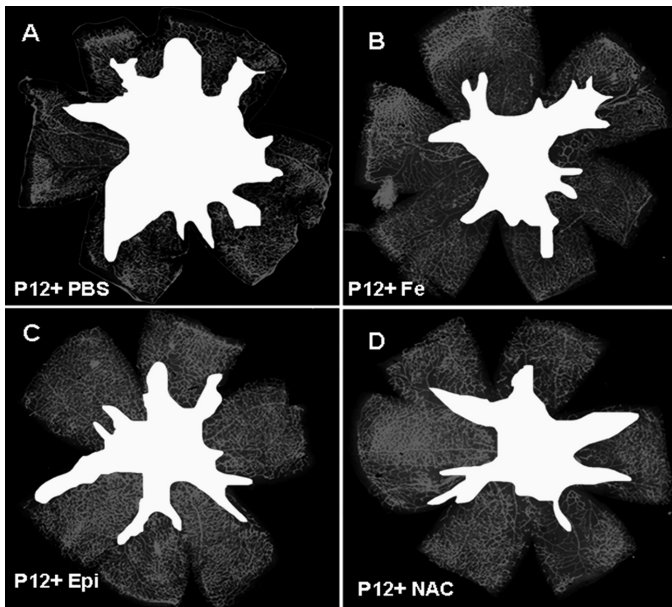
**Determination of Retinal Lipid Peroxides.** The assay was performed on retinal lysates as described previously (Ali et al., 2008). In brief, retinal lysate is reacted with 20% acetic acid, 8% SDS, and thiobarbituric acid at 95°C for 60 min, and the reaction was cooled

down on ice. The samples were centrifuged and the supernatant was extracted with *n*-butanol and pyridine (15:1, respectively) and the absorbance of the organic solvent layer measured at 532 nm. The results were compared with an external standard (tetramethoxypropane). The Bradford assay (Bio-Rad Laboratories, Hercules, CA) was performed to determine the protein concentration of the retinal lysate. Lipid peroxide level was expressed in nanomoles of malondialdehyde per milligram of total protein.

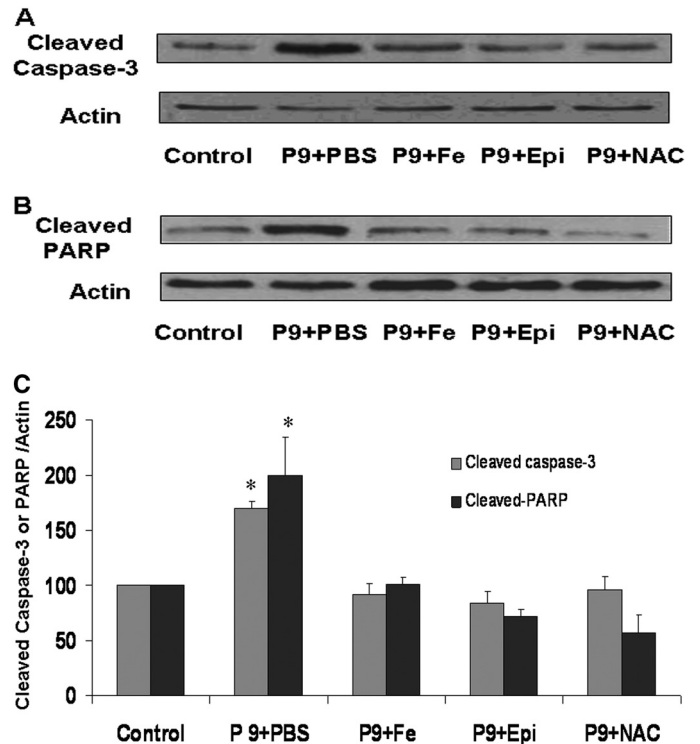
**Western Blotting Analysis.** Retinas and BRE cells were harvested after various treatments and lysed in modified radioimmunoprecipitation assay buffer (Millipore Corporation, Billerica, MA) 30 min on ice. Insoluble material was removed by centrifugation at 14,000g at 4°C for 30 min. Fifty micrograms of total protein was boiled in 6× Laemmli sample buffer, separated on a 10 to 12% SDS-polyacrylamide gel by electrophoresis, transferred

to nitrocellulose, and reacted with specific antibody. The primary antibodies for cleaved caspase-3, phospho-p38, p38, phospho-Akt, or Akt were obtained from (Cell Signaling Technology Inc., Danvers, MA) and were detected by use of a horseradish peroxidase-conjugated antibody and enhanced chemiluminescence (GE Healthcare, Piscataway, NJ). The films were subsequently scanned, and band intensity was quantified by use densitometry software ( $\alpha$  Innotech, San Leandro, CA). For PI 3-kinase tyrosine nitration, retinal lysates were incubated with p85 antibody and A/G agarose beads overnight. The precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis and blotted with nitrotyrosine antibody or p85 for equal loading as described above. Antibodies for p85 subunit of the PI 3-kinase and nitrotyrosine were obtained from (Millipore Corporation). For VEGF, retinal lysates were subjected to heparin beads (Sigma-Aldrich) as described previously (Platt et al., 2005). In brief, the beads were pelleted at 5000g for 1 min, washed in 400 mM NaCl and 20 mM Tris, and loaded onto a 4 to 20% gradient Tris glycine precast gel (Bio-Rad Laboratories). After blocking, the membrane was incubated with VEGF primary antibody (Calbiochem, Gibbstown, NJ). The band was visualized and quantified as described above.

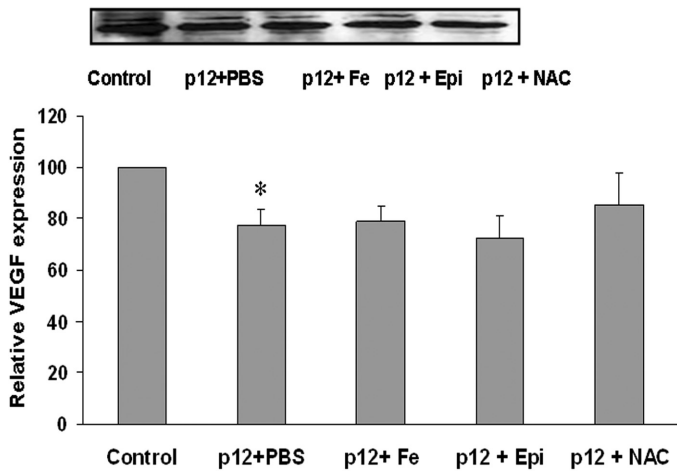
**Detection of Nitrotyrosine.** Relative amounts of proteins nitrated on tyrosine were measured by use of slot-blot techniques as described previously (El-Remessy et al., 2003). In brief, radioimmunoprecipitation assay lysate was immobilized onto nitrocellulose membrane by use of a slot-blot microfiltration unit (Bio-Rad Laboratories). A dilution series of peroxynitrite-modified bovine serum



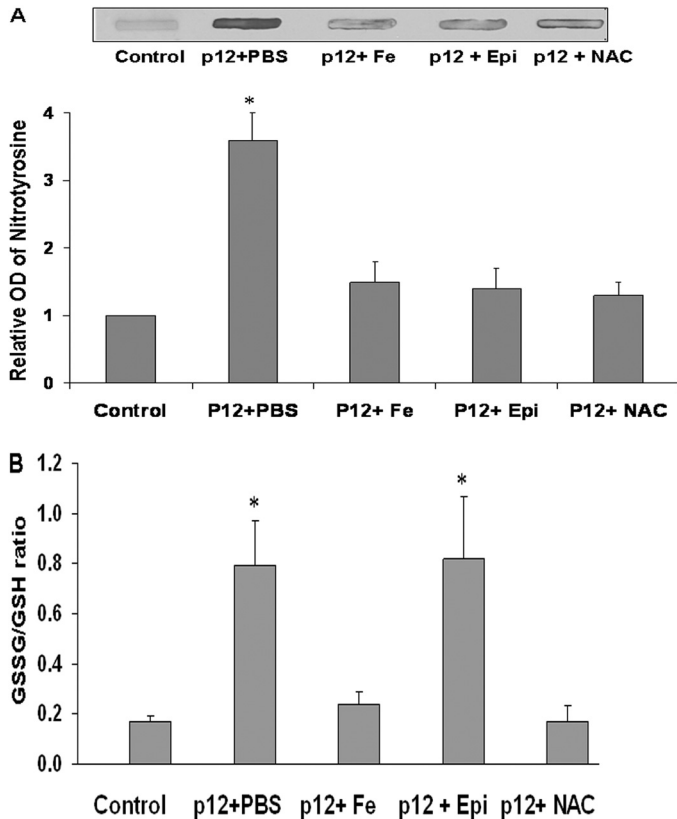
**Fig. 1.** Blocking peroxynitrite formation or tyrosine nitration decreases retinal vaso-obliteration. A–D, representative images of flat-mounted retinas labeled with *G. simplicifolia* lectin to examine vaso-obliteration. Mice pups were maintained in hyperoxia (75% oxygen) (p7–p12) and treated with FeTPPS (1 mg/kg per day i.p.), epicatechin (Epi; 10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.). Exposing the developing retina to high oxygen resulted in a 35% central capillary dropout area (white areas). Treatment with FeTPPS, epicatechin, or NAC significantly reduced the capillary dropout areas. E, statistical analysis of the ratio of central capillary dropout areas to the total retinal area in PBS-treated and other treated retinas showing the protective effects of blocking peroxynitrite formation and tyrosine nitration on retinal vaso-obliteration ( $n = 10–12$ ; \*,  $P < 0.05$ , versus PBS-treated p12; #,  $P < 0.05$ , versus NAC).



**Fig. 2.** Blocking peroxynitrite formation or tyrosine nitration prevents retinal apoptotic markers. Representative image of Western blot analysis of retinal lysates (50  $\mu$ g) shows the following: A, exposing retinas to hyperoxia (75% oxygen) (p7–p9) significantly increased cleaved caspase-3 expression compared with normoxic control. Mice pups were treated with FeTPPS (1 mg/kg per day i.p.) or epicatechin (Epi; 10 mg/kg per day i.p.). B, exposing retina to hyperoxia (75% oxygen) (p7–p9) resulted in significant increases in apoptosis as indicated by the 2-fold increase in cleaved PARP expression compared with normoxic control. Treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.) normalized cleaved PARP expression. C, statistical analysis of the ratio of cleaved caspase-3 or PARP expression to actin ( $n = 4$ ; \*,  $P < 0.05$ , versus control).



**Fig. 3.** Effect of inhibition of tyrosine nitration on VEGF expression. A representative image for Western blot analysis of retinal lysates (100  $\mu$ g) shows that exposing retina to hyperoxia (75% oxygen) (p7–p12) significantly decreased retinal VEGF expression compared with normal oxygen control. Treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.) did not alter VEGF expression ( $n = 4$ ; \*,  $P < 0.05$ , versus control).



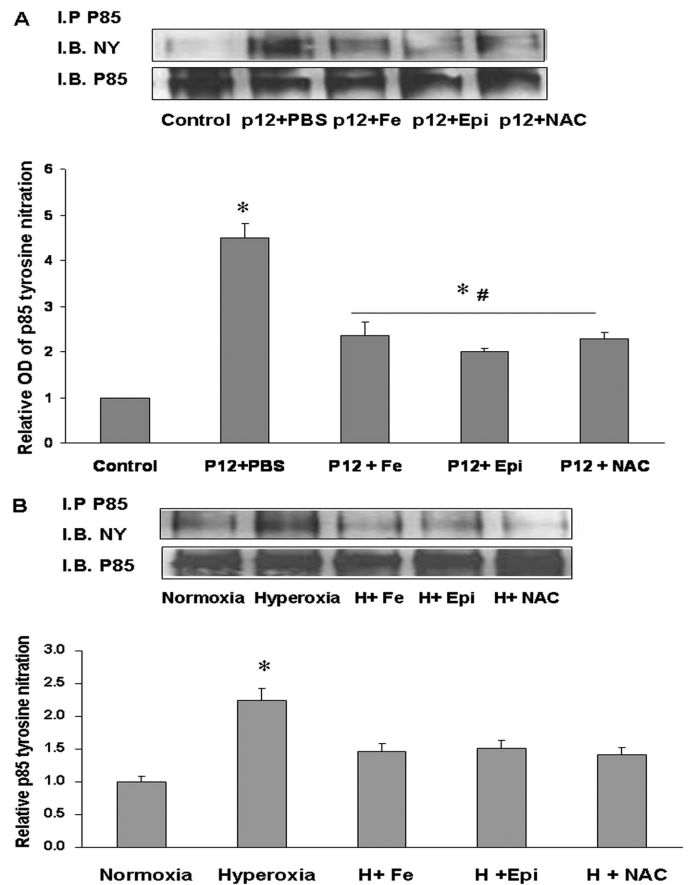
**Fig. 4.** Blocking tyrosine nitration selectively decreases nitrate stress but not antioxidant defense. A, a representative image for slot-blot analysis of retinal lysates (30  $\mu$ g) shows that exposing retina to hyperoxia (75% oxygen) (p7–p12) significantly increases retinal nitrotyrosine formations. Treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (Epi; 10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.) significantly decreased tyrosine nitration ( $n = 4$ ; \*,  $P < 0.05$ , versus control). B, statistical analysis of the ratio of oxidized to reduced glutathione (GSSG/GSH) in different retinal lysates. Hyperoxia (75% oxygen) (p7–p12) increased the GSSG/GSH ratio 4-fold compared with normoxia (21% oxygen). Treatment with FeTPPS (1 mg/kg/day i.p.) or NAC (150 mg/kg per day i.p.) restored the retinal antioxidant defense back to normal. Epicatechin (10 mg/kg per day i.p.) did not alter the GSSG/GSH ratio ( $n = 4$ ; \*,  $P < 0.05$ , versus control).

albumin (Cayman Chemical, Ann Harper, MI) was loaded to generate a standard curve, and nitrotyrosine was detected by use of a polyclonal antinitrotyrosine antibody (Millipore Corporation) followed by peroxidase-labeled goat anti-mouse IgG and enhanced chemiluminescence. Relative levels of nitrotyrosine immunoreactivity were determined by densitometry software (Alpa Innotech).

**Data Analysis.** The results were expressed as mean  $\pm$  S.E. Differences among experimental groups were evaluated by analysis of variance, and the significance of difference between groups was assessed by the post hoc test (Fisher’s protected least significant difference) when indicated. Significance was defined as  $P < 0.05$ .

## Results

**Blocking Tyrosine Nitration Decreases Retinal Vaso-Obliteration.** Previous studies showed a positive correlation between retinal vascular cell death, increases in peroxynitrite formation, and tyrosine nitration. Therefore, we screened the protective effects of selective inhibition of tyrosine nitration by



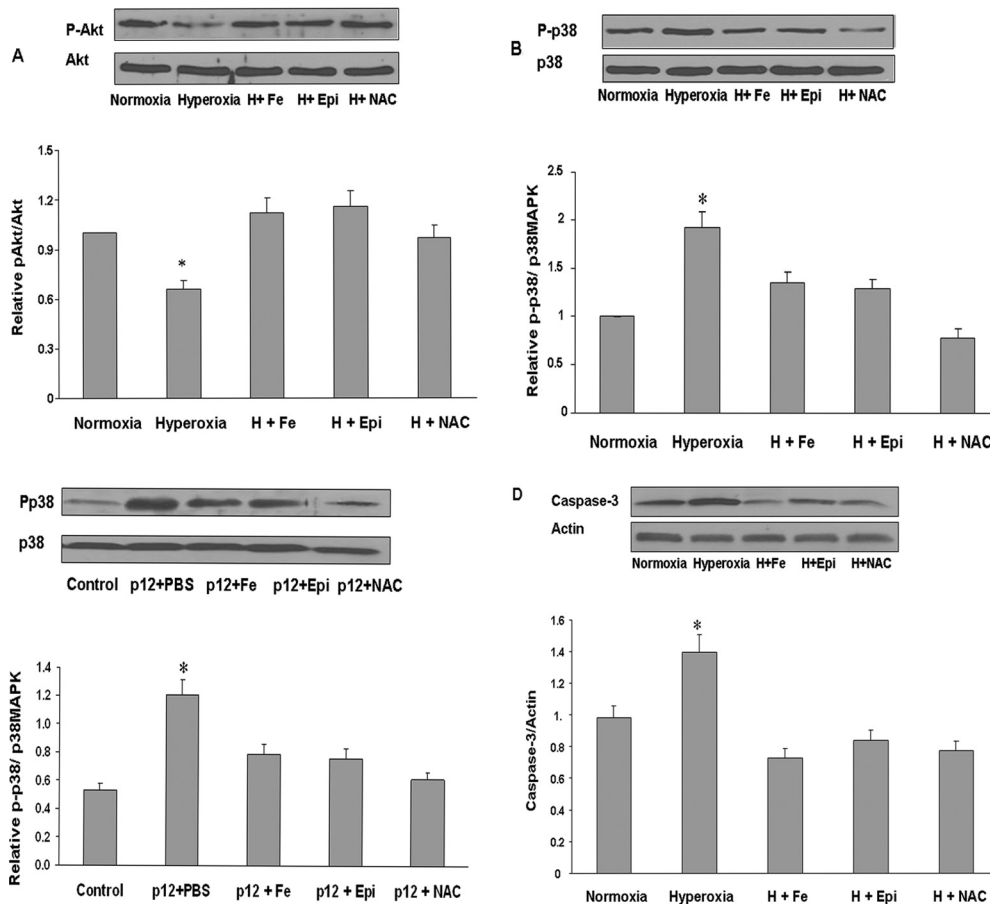
**Fig. 5.** Hyperoxia causes tyrosine nitration of p85 subunit of PI 3-kinase. A, immunoprecipitation with anti-p85 subunit of the PI 3-kinase and Western blot analysis with use of antinitrotyrosine antibody show that exposing retina to hyperoxia (75% oxygen) (p7–p12) significantly increased nitration on the regulatory p85 subunit compared with normal retinas. This effect was reduced by treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (Epi; 10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.) ( $n = 4$ ; \*,  $P < 0.05$ , versus control; #,  $P < 0.05$ , versus PBS-treated p12). B, immunoprecipitation with anti-p85 subunit of PI 3-kinase and Western blot analysis with use of antinitrotyrosine antibody showed that cells cultured in high oxygen conditions (40%), significantly increased nitration of the regulatory p85 subunit compared with cells cultured in normal oxygen. This effect was blocked by the specific peroxynitrite decomposition catalyst FeTPPS (2.5  $\mu$ M) and the specific nitration inhibitor epicatechin (100  $\mu$ M) and thiol donor NAC (1 mM) ( $n = 4$ ; \*,  $P < 0.05$ , versus normoxia 21% oxygen).

use of epicatechin or the general antioxidant and the thiol donor NAC versus decomposing peroxynitrite using FeTPPS, a selective peroxynitrite decomposition catalyst. As shown in Fig. 1, A–D, exposing the developing retina of pups (p7–p12) to high oxygen concentration caused retinal vaso-obliteration as indicated by the capillary dropout area in the central retina. Treatment of pups with FeTPPS (1 mg/kg), epicatechin (10 mg/kg), or NAC (150 mg/kg) significantly reduced central capillary dropout by 34.2%, 25%, and 42%, respectively (Fig. 1E). NAC showed superior effect over epicatechin in protecting the retina from vascular cell death as shown (Fig. 1E). Treatment of control pups with FeTPPS, epicatechin, or NAC did not alter physiological retinal vascular density (data not shown). These results suggest that selective blocking of tyrosine nitration has a protective effect comparable with decomposing peroxynitrite or preventing thiol oxidation on retinal vascular cell death.

**Blocking Tyrosine Nitration Prevents Retinal Apoptosis.** Apoptosis has been postulated as the mechanism by which vascular cell death occurs in ischemic retinopathy. Therefore, we investigated the expression of apoptotic markers before p12. Exposing the retina (p7–p9) to high oxygen

resulted in increases in cleaved caspase-3 (Fig. 2A). Treatment with FeTPPS, epicatechin, or NAC significantly reduced cleaved caspase-3 by 46.5%, 51%, and 43.5% respectively (Fig. 2A). Apoptosis was further confirmed by detecting poly(ADP ribose) polymerase (PARP), a cleavage target of caspase-3 (Fig. 2B). Retinas exposed to hyperoxia (75% oxygen) showed an increase in PARP activation. Treatment with FeTPPS, epicatechin, or NAC reduced PARP activation by 50%, 64%, and 71.5%, respectively.

**Effect of Inhibition of Tyrosine Nitration on VEGF.** Down-regulation of VEGF expression has been postulated to cause vaso-obliteration of the newly formed capillaries (Alon et al., 1995). Therefore, we tested whether the protective effects of blocking tyrosine nitration on preventing retinal vaso-obliteration involve alteration of VEGF expression. As shown in Fig. 3, exposing the retina (p7–p12) to high oxygen resulted in modest but significant decreases in VEGF expression compared with retinas developed at normal oxygen. It is interesting that treatment with FeTPPS, epicatechin, or NAC did not affect VEGF expression compared with PBS-treated pups (Fig. 3).



**Fig. 6.** Hyperoxia-induced nitration inhibits Akt survival and activates p38 MAPK. Western blot analysis of lysates (50  $\mu$ g) from retina or cells that were cultured in high oxygen conditions (40%) and compared with normoxia (20%). A, hyperoxia (40% oxygen) decreased the Akt phosphorylation by 40%. Treating cells with FeTPPS (2.5  $\mu$ M), epicatechin (Epi; 100  $\mu$ M), or NAC (1 mM) for 48 h restored Akt activity ( $n = 4$ ; \*,  $P < 0.05$ , versus normoxia 21% oxygen). B, hyperoxia (40% oxygen) increased the p38 phosphorylation with 2-fold increase. Treating cells with FeTPPS (2.5  $\mu$ M), epicatechin (100  $\mu$ M), or NAC (1 mM) for 48 h decreased p38 phosphorylation ( $n = 4$ ; \*,  $P < 0.05$ , versus normoxia 21% oxygen). C, exposing retina to hyperoxia (75% oxygen) (p7–p12) significantly increases p38 MAPK phosphorylation compared with normoxia (21% oxygen). Retinas from PBS-treated pups showed a 2-fold increase p38 MAPK phosphorylation compared with normoxia (21% oxygen). Treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.) reduced p38 MAPK phosphorylation by 34.6%, 36.6%, and 49.1%, respectively ( $n = 4$ ; \*,  $P < 0.05$ , versus control). D, hyperoxia (40% oxygen) significantly increases cellular apoptosis as indicated by the 1.5-fold increase in cleaved caspase-3 expression compared with normoxic control. Treatment with FeTPPS (2.5  $\mu$ M), NAC (1 mM), or epicatechin (100  $\mu$ M) for 48 h reduced the cleaved caspase-3 expression to normal levels ( $n = 4$ ; \*,  $P < 0.05$ , versus normoxia 21% oxygen).

**Blocking Tyrosine Nitration Selectively Decreases Nitrotyrosine Stress.** The retina is believed to be vulnerable to nitrotyrosine and oxidative damage because of the abundance of polyunsaturated fatty acids. Hyperoxia (75% oxygen) showed a significant increase in the nitrotyrosine formation compared with normoxia (21% oxygen) (Fig. 4A). Treatment of pups with FeTPPS, epicatechin, or NAC significantly decreased nitrotyrosine formation (50%, 58%, and 60% respectively). In addition to tyrosine nitration, peroxyxynitrite can cause thiol oxidation, which compromises antioxidant defense. Measuring the ratio between the cellular oxidized to reduced glutathione is used as a marker for the retinal antioxidant defense. Normal tissue has a balanced ratio between oxidized and reduced glutathione. As shown in Fig. 4B, retinas from p12 pups exposed to high oxygen showed (4-fold) increases in the tissue GSSG/GSH ratio. Treatment with either FeTPPS or NAC significantly restored the GSSG/GSH ratio and increased the retinal antioxidant defense. In contrast, treatment with epicatechin has a modest but not significant effect on the GSSG/GSH ratio.

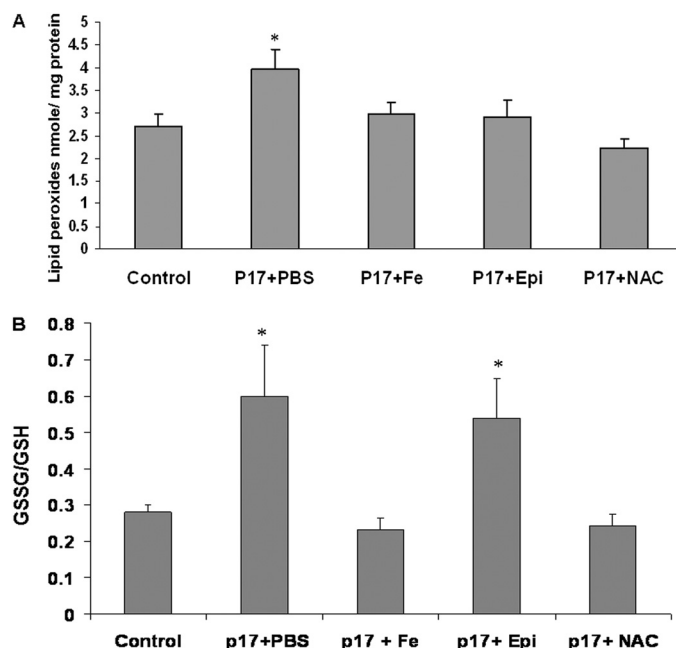
**Hyperoxia Causes Tyrosine Nitration of p85 Subunit of PI 3-Kinase.** Our previous work has shown that the p85 regulatory subunit of PI 3-kinase is a susceptible target for peroxyxynitrite-induced tyrosine nitration in endothelial cells (el-Remessy et al., 2005). As shown in Fig. 5A, retinas from p12 that were exposed to high oxygen showed a (4-fold) increase in tyrosine nitration of p85 compared with normoxia (21% oxygen). Treatment (p7–p12) with epicatechin or NAC significantly reduced the nitrating effect of peroxyxynitrite on tyrosine residues similar to decomposing peroxyxynitrite with FeTPPS.

**Hyperoxia-Induced Nitration Inhibits Akt Survival and Activates p38 MAPK.** Because the retina is composed of several cell types, we further examined the role of tyrosine nitration in hyperoxia-induced vascular cell death. BRE cells were cultured in high oxygen conditions (40%) and compared with normoxia (21%). BRE cells showed 2.3-fold increase in tyrosine nitration of the p85 subunit compared with normoxia (21% oxygen) (Fig. 5B). Treatment with FeTPPS (2.5  $\mu$ M), epicatechin (100  $\mu$ M), or NAC (1 mM) for 48 h significantly reduced hyperoxia-induced p85 tyrosine nitration (Fig. 5B). To confirm the inhibitory effect of tyrosine nitration on PI 3-kinase, we tested the effects of inhibitors on Akt phosphorylation. Akt, a downstream target of PI 3-kinase, mediates cell survival by inhibiting apoptotic processes. Hyperoxia (40% oxygen) significantly decreased Akt phosphorylation compared with normoxia (21% oxygen) by 40% (Fig. 6A). Treating cells with FeTPPS, epicatechin, or NAC for 48 h restored Akt activation. PI 3-kinase/Akt signaling promotes endothelial cell survival by inhibiting p38 MAPK-dependent apoptosis (Gratton et al., 2001). Therefore, blockade of PI 3-kinase/Akt via tyrosine nitration of p85 subunit can lead to enhanced activation of p38 MAPK and endothelial apoptosis. As shown in Fig. 6B, cells cultured in hyperoxia (40% oxygen) showed a 2-fold increase in p38 MAPK phosphorylation that was decreased by treatment with FeTPPS, epicatechin, or NAC. Increases in p38 MAPK phosphorylation were also observed in p12 retinas subjected to high oxygen compared with controls (Fig. 6C). Retinas from PBS-treated pups showed a 2-fold increase in p38 MAPK phosphorylation compared with normoxia (21% oxygen). Treatments with FeTPPS, epicatechin, or NAC reduced p38 MAPK phosphorylation by 34.6%, 36.6%, and 49.1% respectively. Next, we evaluated the effects of hyper-

oxia (40% oxygen) to induce endothelial cell apoptosis. As shown in Fig. 6D, hyperoxia (40% oxygen) induced endothelial cell apoptosis as indicated by increased expression of cleaved caspase-3. Blocking tyrosine nitration with FeTPPS, epicatechin, or NAC protected the cells against apoptosis by decreasing the level of cleaved caspase-3 by 50%, 43%, and 46% respectively.

**Early Intervention of Tyrosine Nitration Prevents Retinal Oxidative Injury.** Lipid peroxidation has been established as a general marker of oxidative stress and cellular injury. As shown in Fig. 7A, retinas from pups that underwent hyperoxia (75% oxygen) and hypoxia (21% oxygen) (p7–p17) showed high levels of lipid peroxides compared with the pups in normal air. Early intervention with FeTPPS, epicatechin, or the thiol donor NAC decreased lipid peroxidation, hence, the cellular injury. It is interesting that treatment with FeTPPS or the thiol donor NAC, but not epicatechin, restored the antioxidant defense as indicated by the ratio of GSSG to GSH (Fig. 7B). These results confirm the effect of epicatechin in selectively blocking tyrosine nitration without exerting antioxidant effect.

**Early Intervention of Tyrosine Nitration Prevents Retinal Neovascularization.** Our previous studies showed that blocking nitration after vaso-obliteration (p12–p17) did not prevent retinal neovascularization (El-Remessy et al., 2007). Here, we tested the long-term protective effects of blocking tyrosine nitration during both hyperoxia (75% oxygen) and hypoxia (21% oxygen) (p7–p17). Compared with PBS-treated pups, treatment with epicatechin significantly



**Fig. 7.** Early intervention of tyrosine nitration prevents retinal oxidative stress. A, statistical analysis of lipid peroxidation showing that exposing neonates to hyperoxia (75% oxygen) and then hypoxia (21% oxygen) (p7–p17) increased retinal cellular injury indicated by the significant increases in lipid peroxidation. Treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (Epi; 10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.) decreased it ( $n = 8$ ;  $*$ ,  $P < 0.05$ , versus control). B, statistical analysis of the ratio of oxidized to reduced glutathione (GSSG/GSH) showing that hyperoxia (75% oxygen) followed by hypoxia (21% oxygen) (p7–p17) significantly increased the retinal GSSG/GSH ratio (3-fold). Treatment with FeTPPS (1 mg/kg per day i.p.) or NAC (150 mg/kg per day i.p.) restored the normal antioxidant defense. Epicatechin (10 mg/kg per day i.p.) did not alter the GSSG/GSH ratio ( $n = 8$ ;  $*$ ,  $P < 0.05$ , versus control).

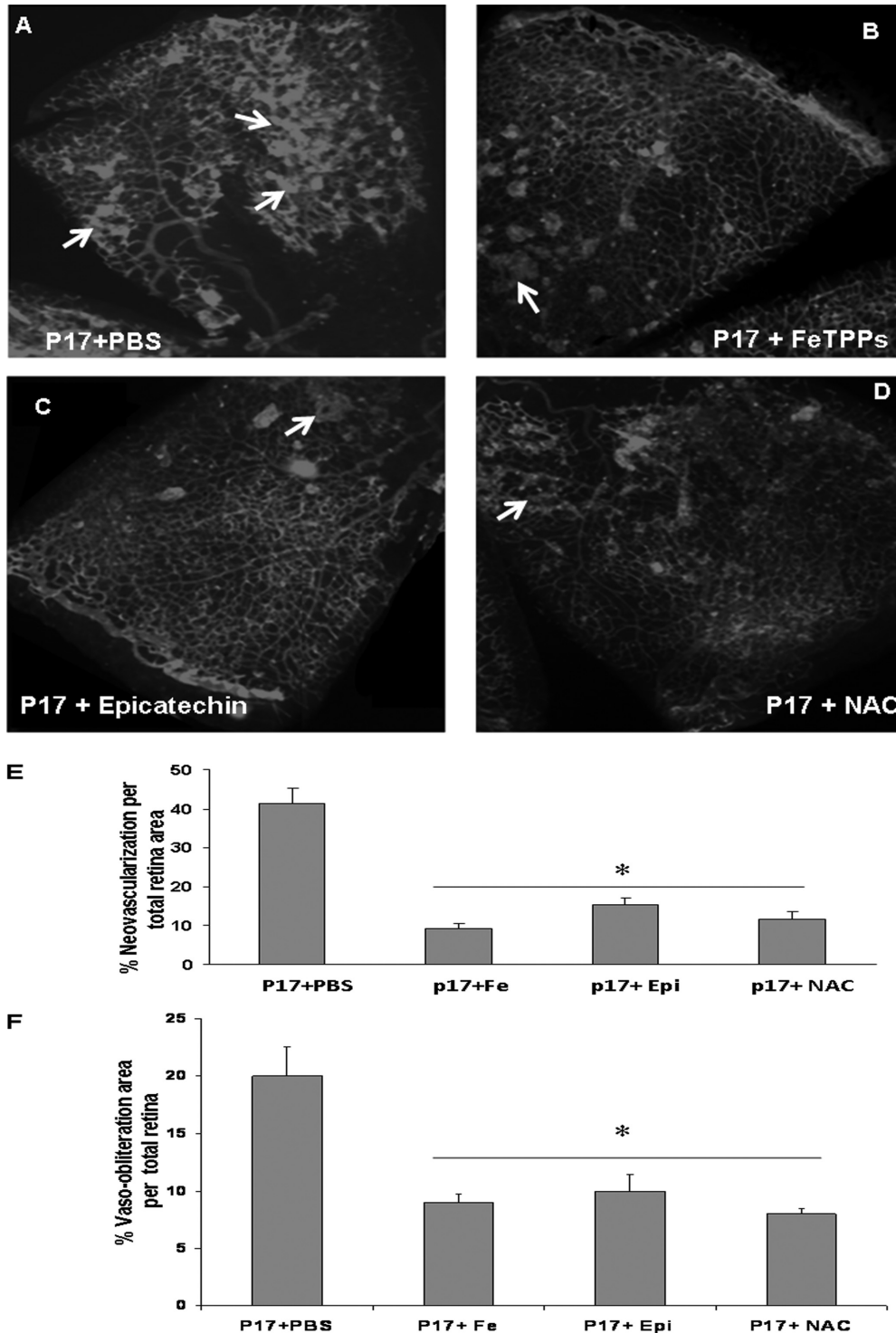
reduced (by 62.1%) retinal neovascularization. This effect was comparable with, but to a lesser extent, pups treated with FeTPPS or NAC that had a 76.8% and 71.5% reduction in neovascularization, respectively (Fig. 8, A–E).

Next, we measured the capillary dropout areas at the end of hypoxia (21% oxygen) (p17) to test the effect of treatment on the physiological revascularization in the central retina. As expected in this model, PBS-treated controls showed a 29% reduction in capillary dropout areas compared with the reduction at the end of hyperoxia (75% oxygen) (p12). Treat-

ment with FeTPPS, epicatechin, or NAC further reduced capillary dropout areas by 55%, 50%, and 60%, respectively, compared with p17 PBS-treated animals; suggesting that blocking peroxynitrite did not alter physiological revascularization; instead, it enhanced vascular regrowth and continued protection from vaso-obliteration (Fig. 8F).

## Discussion

The present study documents novel data suggesting that 1) peroxynitrite mediates retinal vaso-obliteration via ty-



**Fig. 8.** Early intervention prevents retinal neovascularization. A–D, representative images of flat-mounted retinas labeled with *G. simplicifolia* lectin to examine retinal neovascularization. Mice pups were maintained in hyperoxia (75% oxygen) followed by hypoxia (21% oxygen) (p7–p17), which resulted in neovascularization as indicated by tufts in the peripheral retinas. Treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (Epi; 10 mg/kg per day i.p.) or NAC (150 mg/kg per day i.p.) significantly reduced the neovascularization areas. E, statistical analysis of the ratio of neovascularization areas to total retinal showing the protective effects of blocking peroxynitrite formation and tyrosine nitration on retinal neovascularization ( $n = 8$ ; \*,  $P < 0.05$ , versus p17 PBS-treated). F, statistical analysis of the ratio of central capillary dropout areas to total retinal area in (p7–p17) treated retinas showing that treatment with FeTPPS (1 mg/kg per day i.p.), epicatechin (10 mg/kg per day i.p.), or NAC (150 mg/kg per day i.p.) significantly reduced capillary dropout by 54%, 50%, and 60% respectively ( $n = 8$ ; \*,  $P < 0.05$ , versus p17 PBS-treated).

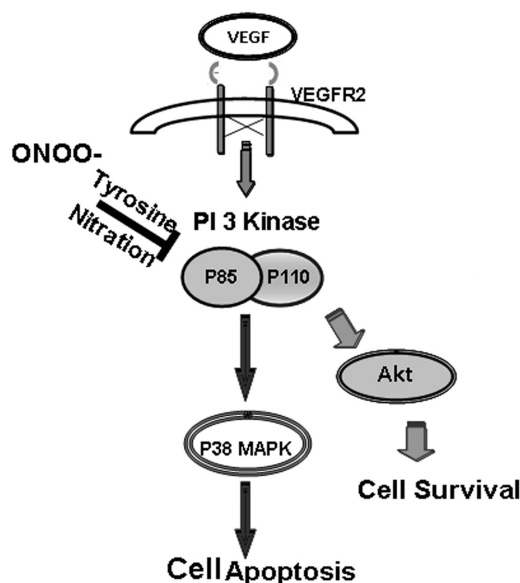
rosine nitration of p85 kinase and inhibition of PI 3-kinase/Akt survival pathway; 2) blocking tyrosine nitration restores survival signal and prevents endothelial cell apoptosis and retinal ischemia; and 3) early intervention with treatments that target peroxynitrite and tyrosine nitration prevents retinal neovascularization. To our knowledge this is the first *in vivo* study to elucidate the mechanism by which peroxynitrite-mediated tyrosine nitration and inhibition of PI 3-kinase survival pathway trigger vascular cell death in ischemic retinopathy model. Our results also demonstrate that targeting tyrosine nitration with dietary supplements such as epicatechin and NAC represent potentially safe and effective therapeutic strategy that could be translated to patients with ischemic-proliferative diseases.

The focus of the current study is to test the protective effects of blocking peroxynitrite and tyrosine nitration on retinal cell death *in vitro* and to prevent vaso-obliteration and neovascularization *in vivo*. Therefore, we took advantage of using the ischemic retinopathy mouse model because it has two distinguished stages, initial vascular cell death and vaso-obliteration, which are associated with excessive peroxynitrite formation followed by retinal neovascularization, which is associated with a mild oxidative insult (Al-Shabrawey et al., 2005; El-Remessy et al., 2007). We used epicatechin, a flavonoid, and one of the green tea extracts that selectively block peroxynitrite-mediated tyrosine nitration but not thiol oxidation (Schroeder et al., 2001; el-Remessy et al., 2005; El-Remessy et al., 2007) and compared its effects with blocking peroxynitrite by use of FeTPPS, the peroxynitrite decomposition catalyst, or blocking thiol oxidation by use of the dietary supplement and the thiol donor NAC (el-Remessy et al., 2005; El-Remessy et al., 2007). Our results showed that exposing the developing retina to high oxygen induces significant capillary dropout areas that was accompanied by nitrotyrosine formation and apoptosis as indicated by increased expression of cleaved caspase-3 expression and PARP. These results are in agreement with previous experimental and clinical studies showing that ischemic retinopathy is associated with increases in retinal oxidative damage and decreases in antioxidant defense (Papp et al., 1999; Brooks et al., 2001; Gu et al., 2002; Beauchamp et al., 2004; Wright et al., 2006; Bartoli et al., 2008). Our results showing that hyperoxia causes significant increases in cleaved PARP and the caspase-3 substrate *in vitro* and *in vivo* lend further support to the previous reports of the critical role of caspase-3 in executing peroxynitrite-induced apoptosis in endothelial cells (Kotamraju et al., 2001; Gu et al., 2003; Zhu et al., 2004; el-Remessy et al., 2005). Treatment with epicatechin blocked tyrosine nitration and significantly reduced capillary dropout areas and the expression of cleaved caspase-3 and PARP. However, epicatechin did not alter the GSSG/GSH ratio, confirming its selective properties in blocking tyrosine nitration but not thiol oxidation. However, treatment with FeTPPS or NAC not only blocked tyrosine nitration but also improved the GSSG/GSH ratio indicating restoration of retinal antioxidant defense, which explains the superior effects of NAC and FeTPPS in reducing vaso-obliteration over epicatechin. In contrast, NAC failed to show protective effects on reducing retinal avascularity and apoptosis in a rat model of ROP that could be attributed to the different nature of the model and the shorter duration of treatment (Saito et al., 2007). Our results establish a unique role for selective block-

ing of tyrosine nitration, apart from the antioxidant effect, in preventing retinal vaso-obliteration and support the notion of its therapeutic intervention for ischemic retinal diseases.

To elucidate the molecular mechanisms by which blocking tyrosine nitration protect the retina from apoptosis, we determined the expression of VEGF because its down-regulation has been postulated to cause vaso-obliteration of newly formed capillaries (Alon et al., 1995). In agreement, our results showed a modest but significant decrease in VEGF expression in hyperoxic retinas. However, treatment with FeTPPS, epicatechin, or NAC did not alter VEGF expression compared with PBS-treated ones. These results suggest that the protective effects of blocking peroxynitrite and tyrosine nitration in preventing retinal apoptosis and vaso-obliteration are not mediated by altering the VEGF expression level, but instead by modulating the VEGF signal.

VEGF's function as a survival factor for endothelial cells is well established (Duh and Aiello, 1999). VEGF activation of VEGFR2 transduces antiapoptotic signal via PI 3-kinase/Akt signaling pathway (Gerber et al., 1998; Fujio and Walsh, 1999). Tyrosine nitration and subsequent inhibition of the p85 regulatory subunit of PI 3-kinase have been documented in response to peroxynitrite (Hellberg et al., 1998; el-Remessy et al., 2005). Here, we show that retinas from pups exposed to hyperoxia (75% oxygen) showed significant increases (4-fold) in tyrosine nitration of p85 subunit compared with pups in normoxia (21% oxygen). A similar pattern was observed in retinal endothelial cultures maintained at hyperoxia (75% oxygen) compared with the ones maintained at normal oxygen. These effects were associated with impaired Akt phosphorylation, increased p38 MAPK phosphorylation, and increased expression of the apoptotic marker caspase-3. Treatment with epicatechin or NAC had effects comparable with FeTPPS, and prevented tyrosine nitration of p85, restored Akt survival pathway, and reduced the activation of the p38 MAPK apoptotic pathway. These results confirm the



**Fig. 9.** A schematic representation of the proposed mechanism by which high oxygen via peroxynitrite inactivates VEGF/PI 3-kinase/Akt-1 pro-survival pathway and stimulates cell death via activation of the p38 MAPK pathway. Nitration of PI 3-kinase is proposed as a mechanism by which peroxynitrite switches off the VEGF pro-survival and triggers the proapoptotic path.



relationship between nitration of p85, decreases in Akt activity, and the proapoptotic effects of hyperoxia-induced peroxynitrite. Our findings lend further support to previous reports of significant increases in oxidative and nitrative stress in the ischemic retinopathy model (Papp et al., 1999; Brooks et al., 2001; Gu et al., 2002, 2003; Beauchamp et al., 2004; Saito et al., 2007; Medina et al., 2008). Understanding of the molecular pathogenesis of ischemic retinopathy provides the basis for identifying novel therapeutic targets. The role of the hypoxia-induced factors VEGF and erythropoietin, as well as the maternally derived factor insulin-like growth factor-1, have begun to be elucidated (for review, see Heidary et al., 2009). However, our study is the first we know of that elucidates the molecular mechanism of the tyrosine nitration of p85 leading to inactivation of the PI 3-kinase/Akt survival signal and activation of the proapoptotic p38 MAPK signal in the ischemic retinopathy model. A scheme of the proposed mechanism is depicted in Fig. 9. These findings explain the protective effects of epicatechin in preventing vaso-obliteration, even though it does not completely restore retinal antioxidant defense.

Retinal neovascularization takes place because the initial stage of capillary loss leads to inner retinal ischemia that drives up-regulation of angiogenic growth factors (Mizutani et al., 1996). The combined vascular protection of reducing pathological neovascularization while allowing physiological revascularization of the retina is the ideal therapeutic modality for ischemic retinopathy diseases. Therefore, it is critical to evaluate the effects of early blocking of tyrosine nitration and capillary dropout on the prevention of subsequent neovascularization. Continuous intervention of tyrosine nitration during both hyperoxia (75% oxygen) and hypoxia (21% oxygen) (p7–p17) with epicatechin, FeTPPS, or NAC significantly reduced lipid peroxidation and retinal neovascularization, and further reduced central capillary dropout compared with PBS controls. The vascular protective effects of epicatechin in the current study are mainly due to its effects in preventing earlier vaso-obliteration, and hence the stimulus to neovascularization. This concept is further supported by our previous findings that late intervention with epicatechin after vaso-obliteration (p12–p17) did not prevent retinal neovascularization (el-Remessy et al., 2005; El-Remessy et al., 2007). The vascular protective effects of NAC and FeTPPS were associated with restoration of antioxidant defense as indicated by the GSSG/GSH ratio, but not the effects of epicatechin, confirming its selective effect of inhibiting tyrosine nitration apart from thiol oxidation. Although the vascular protective effects of FeTPPS are significant, the fact that it contains iron will limit its therapeutic use for chronic administration. The vascular effects of NAC were superior to epicatechin but did not reach significance and this could be attributed to the antioxidant of NAC over epicatechin. New studies showed that NAC also may have other mechanisms that can interfere with cell proliferation by regulating the cell cycle regulatory protein (Menon et al., 2007). However, both epicatechin and NAC proved effective, and the fact that they are already available as dietary supplements opens the door for therapeutic utility. Together, these results point out the importance of the timing and the molecular target of intervention. In support of these findings, studies using the same ischemic retinopathy model showed that early intervention with omega-3 polyunsaturated fatty acid

failed to rescue oxygen-induced vessel loss during hyperoxia (75% oxygen) but prevented late retinal neovascularization (Connor et al., 2007). However, intervention with statins effectively reduced retinal oxidative stress and capillary dropout, and prevented neovascularization (Bartoli et al., 2008; Medina et al., 2008).

In conclusion, our study is the first to elucidate the mechanism by which tyrosine nitration of p85 kinase and inhibition of the PI 3-kinase/Akt survival pathway cause vascular cell death in vivo and to demonstrate the vascular protective effects of the early and continuous intervention of peroxynitrite and tyrosine nitration on retinal vessel loss and neovascularization. Furthermore, our results provide compelling evidence that targeting tyrosine nitration with safe dietary supplements, such as epicatechin and NAC, in animal models should provide the rationale for testing these agents as a possible control of common ischemic proliferative retinopathies such as DR and ROP.

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**Address correspondence to:** Dr. Azza B. El-Remessy, Program in Clinical and Experimental Therapeutics, College of Pharmacy, University of Georgia, Augusta, GA 30912. E-mail: aelremessy@mcg.edu

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