Role of NADPH Oxidase in Retinal Vascular Inflammation

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PURPOSE. In another study, it was demonstrated that NADPH oxidase-derived reactive oxygen species (ROS) are important for ischemia-induced increases in vascular endothelial growth factor (VEGF) and retinal neovascularization. Diabetes-induced increases in retinal ROS, VEGF expression, and vascular permeability are accompanied by increases in the NADPH oxidase catalytic subunit NOX2 within the retinal vessels. The goal of this study was to evaluate the potential role of NOX2 and NADPH oxidase activity in the development of retinal vascular inflammation.

METHODS. Studies were performed in wild-type mice, mice lacking NOX2, and mice treated with the NADPH oxidase inhibitor apocynin in models of endotoxemia and streptozotocin-induced diabetes. Intracellular adhesion molecule (ICAM)-1 expression was determined by Western blot analysis. Leukocyte adhesion was assessed by labeling adherent leukocytes with concanavalin A. Vascular permeability was assessed by extravasation of FITC-conjugated albumin. ROS production was determined by dichlorofluorescein imaging.

RESULTS. Both endotoxemia- and diabetes-induced increases in ICAM-1 expression and leukostasis were significantly inhibited by deletion of NOX2, indicating that this enzyme is critically involved in both conditions. Moreover, apocynin treatment and deletion of NOX2 were equally effective in preventing diabetes-induced increases in ICAM-1, leukostasis, and breakdown of the blood–retinal barrier, suggesting that NOX2 is primarily responsible for these early signs of diabetic retinopathy.

CONCLUSIONS. These data suggest that NOX2 activity has a primary role in retinal vascular inflammation during acute and chronic conditions associated with retinal vascular inflammatory reactions. Targeting this enzyme could be a novel therapeutic strategy for treatment of the retinopathies associated with vascular inflammation. (Invest Ophthalmol Vis Sci. 2008; 49:3239–3244) DOI:10.1167/iovs.08-1755

Vascular inflammation is a common feature of a variety of potentially blinding retinal diseases associated with retinal vascular hyperpermeability and vitreoretinal neovascularization. The early stages of the inflammatory reaction are characterized by leukocyte adhesion to the vessel wall. The adhesion causes retinal capillary plugging and nonperfusion, which is followed by vascular injury and hyperpermeability.1–5 Increased expression of the adhesion molecule intracellular adhesion molecule (ICAM)-1 has been found to play a major role in mediating leukocyte adhesion to vascular endothelial cells, and this has been linked to vascular injury associated with ischemic retinopathy, diabetic retinopathy, and uveitis.6–7 Various reports have shown that ROS are obligatory mediators of vascular inflammation induced by growth factors and cytokines.6,8,9 Furthermore, ROS activate the transcription factors NF-kB and AP-1, which play a central and crucial role in inducing the expression of inflammatory cytokines and ICAM-1.10–13 Previous work has shown that diabetes-induced increases in VEGF expression, leukocyte adhesion, and breakdown of the blood–retinal barrier (BRB) all correlate with increases in oxidative stress.14–17 The specific role of oxidative stress in triggering retinal vascular inflammatory processes is not yet known. However, studies in models of atherosclerosis and other forms of peripheral vascular disease have implicated NADPH oxidase in the inflammatory reaction.18

In phagocytic cells NADPH oxidase is a multiprotein complex consisting of membrane-bound cytochrome b558, which is composed of the catalytic subunit NOX2 (formerly known as gp91phox), p22phox, the cytoplasmic subunits p47phox and p67phox, and the small Rho GTPase Rac. Vascular endothelial cells express the same subunits as well as two NOX2 homologues, NOX1, and NOX4 (for review, see Ref. 18). Our previous studies in animal and tissue culture models have shown that NOX2 is expressed at low levels in normal retinas and in retinal endothelial cells maintained in control conditions, but is substantially increased in retinal vessels of animals with diabetic or ischemic retinopathy and in retinal endothelial cells exposed to high glucose or hypoxia. Moreover, we have found that inhibiting NADPH oxidase blocks the upregulation of VEGF expression during diabetic and ischemic retinopathy19,20 and prevents vitreoretinal neovascularization during ischemic retinopathy.20 The goal of the present study was to determine the specific role of NADPH oxidase and NOX2 in retinal vascular inflammation related to acute and chronic vascular inflammatory reactions in endotoxemia and diabetic retinopathy, respectively.

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Supported by the Juvenile Diabetes Research Foundation (JDRF), Fight For Sight, and the American Heart Association Grant AHA000104 (MA); National Eye Institute Grants R01 EY04618 and R01 EY11766, and the Veterans Administration Career Development Award (CDA) and Merit Review Award (RBC).

Submitted for publication January 17, 2008; revised February 23, 2008; accepted May 12, 2008.

Disclosure: M. Al-Shabrawey, None; M. Rojas, None; T. Sanders, None; A. Behzadian, None; A. El-Remessy, None; M. Bartoli, None; A.K. Parpia, None; G. Liou, None; R.B. Caldwell, None.

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MATERIALS AND METHODS

Treatment of Animals
All procedures with animals were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional animal care and use committee (Animal Welfare Assurance no. A3507-01). Experiments were performed with C57Bl/6J mice and age-matched NOX2-deficient mice backcrossed on a C57Bl/6 background (Jackson Laboratories, Bar Harbor, ME). Genotyping of NOX2-deficient mice was performed before the experiment.

Mouse Model of Retinal Vascular Inflammation. Mice lacking NOX2 and age-matched wild-type mice (25 g) were injected with lipopolysaccharide from Salmonella typhimurium (LPS, 0.1 mg/kg in PBS, IP; Sigma-Aldrich, St. Louis, MO) as a model of acute retinal vascular inflammation. Age-matched control mice received vehicle alone. Mice were killed 24 hours later and prepared for quantification of leukocyte adhesion and ICAM-1 expression in the retina.

Mouse Model of Diabetic Retinopathy. Additional groups of age-matched wild-type and NOX2−/− mice (25 g) were made diabetic by intraperitoneal injections of 55 mg/kg streptozotocin (STZ; Sigma-Aldrich) dissolved in 0.1 M fresh citrate buffer (pH 4.5). The animals were considered diabetic when the plasma glucose level exceeded 250 mg/dL. Some diabetic mice were treated with apocynin (50 mg/kg/d, dissolved in drinking water) for the duration of the experiments. After 5 weeks of hyperglycemia diabetic and age-matched normoglycemic control mice were processed for quantification of permeability or leukocyte adhesion. Identical groups of animals were prepared, and retinas were processed separately for protein analysis or for frozen sectioning and immunohistochemical analysis.

Analysis of Leukocyte Adhesion
Adhesion of leukocytes to the wall of the retinal vessels was evaluated as described previously, with slight modification.5 After the induction of deep anesthesia, the chest cavity was carefully opened, and a 20-gauge perfusion cannula was introduced into the aorta. Drainage was achieved by opening the right atrium. The animals were then perfused with 10 mL of phosphate-buffered saline (PBS) to wash out nonadherent blood cells. Next, the animals were perfused with 10 mL of fluorescent isothiocyanate (FITC)-labeled concanavalin A (Con A) lectin (40 μg/mL in PBS, pH 7.4; Vector Laboratories, Burlingame, CA) to label the adherent leukocytes and vascular endothelial cells. Residual unbound Con A was removed by perfusion with PBS. The eyeballs were removed and fixed with 4% paraformaldehyde. The retinas were then observed by fluorescence microscopy, and the total number of adherent leukocytes per retina was determined. CD45 immunohistochemistry was performed with a specific anti-CD45 antibody (BD-PharMingen, San Diego, CA) to confirm that Con A-stained cells within the vasculature were leukocytes.

Analysis of BRB Permeability
Retinal vascular permeability was measured as described previously.21 Both diabetic and control mice received external jugular vein injections of 10 mg/kg bovine serum albumin (BSA)-Alexa Fluor 488 conjugate (Invitrogen-Molecular Probes, Eugene, OR). After 30 minutes, the eyes were removed, embedded in optimal cutting temperature (OCT) embedding medium, and snap frozen in liquid nitrogen, and 12-μm-thick sections were prepared. Images (three per section) were collected at 60-μm intervals from 10 sections of each retina. Tracer extravasation was measured by morphometric analysis of fluorescein levels in nonvascular areas in the retinal images. Plasma was assayed for BSA-fluorescein concentration using a spectrofluorometer (model 4000; CytoFlour, Foster City, CA). Fluorescein intensity in the retinal sections was normalized according to the concentration of the tracer in the plasma of the same animal.

Western Blot Analysis
For analysis of ICAM-1 pooled retinas were homogenized in a modified RIPA buffer (20 mM Tris-HCl [pH 7.4], 2.5 mM EDTA, 50 mM NaF, 10 mM Na3PO4, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, and 1 mM phenyl methyl sulfonyl fluoride), and 30-μg protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, and reacted with anti-ICAM-1 (1:200 rabbit anti-human antibody; Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase–linked secondary antibody and enhanced chemiluminescence (GE Healthcare, San Francisco, CA). Membranes were stripped and reprobed for β-actin to demonstrate equal loading, and the results were analyzed using densitometry.

Measurement of NADPH Oxidase Activity
Activity of NADPH oxidase was assayed using dichlorofluorescein imaging of retinal frozen sections. Dichlorofluorescein (DCF), the oxidation product of 2,7-dichlorodihydro-fluorescein diacetate (DHDCF; Invitrogen) emits a green fluorescent signal and is a marker of cellular oxidation by hydrogen peroxide, peroxynitrite, and hydroxyl radicals.22 Retinal sections were incubated with NADPH (100 μM) and DHDCF (10 μM) for 20 minutes at 37°C. The specificity of the reaction was determined by incubating the retinal sections in buffer containing DHDCF with or without PEG-SOD, catalase, or apocynin (Sigma-Aldrich). DCF formation was measured with fluorescence microscopy, to collect the images, and computer-assisted morphometry, to determine fluorescence intensity.

Statistical Analysis
Group differences were evaluated by using ANOVA and post hoc comparisons. Results were considered significant at P < 0.05. Data are presented as the mean ± SE.

RESULTS

Abrogation of LPS-Induced Increases in Leukocyte Adhesion and ICAM-1 Expression in the Retinas of NOX2-Deficient Mice
To evaluate whether activity of NOX2-containing NADPH oxidase plays a role in retinal vascular inflammation, we analyzed leukocyte adhesion in the retinal vessels of LPS-treated wild-type and NOX2-deficient mice. The number of adherent leukocytes in LPS-injected mice was reduced by ~60% in the mice lacking NOX2 compared with wild-type (mean ± SE = 53 ± 17 vs. 127 ± 13, respectively; Fig. 1A). Immunoreactivity for CD45 confirmed that the adherent cells were leukocytes (Fig. 1B). Because ICAM-1 is a key mediator of leukocyte adhesion, we tested whether deletion of NOX2 affects its expression in the retina. Our Western blot analyses showed a significant increase in retinal expression of ICAM-1 after the LPS treatment. However, this increase was significantly reduced in mice lacking NOX2 (Fig. 2).

Blockade of Diabetes-Induced Increases in Leukocyte Adhesion and ICAM-1 Expression by Deletion of NOX2 or Inhibition of NADPH Oxidase
To test whether NOX2 expression and NADPH oxidase activity have a role in vascular inflammatory processes associated with diabetic retinopathy, we examined leukocyte–endothelial cell attachment and expression of ICAM-1 in the retinas of diabetic mice lacking NOX2 or treated with apocynin. Analysis of leukocyte adhesion showed a significant increase in the number of adherent leukocytes in the diabetic retinas. This increase...
was substantially reduced by deletion of NOX2 or apocynin treatment (Fig. 3). Furthermore, Western blot analysis showed a significant increase in expression of ICAM-1 in the diabetic retina, which also was completely blocked by apocynin or deletion of NOX2 (Fig. 4). These results indicate that activity of NOX2-containing NADPH oxidase is critically involved in leukocyte–endothelial cell attachment in the diabetic retina.

**Effect of Deletion of NOX2 on ROS Formation in the Diabetic Retina**

To assess the potential contribution of NADPH oxidase activity to oxidative stress in the diabetic retina, we studied ROS generation by using real-time DCF imaging of flash-frozen retinas. Retinas of diabetic mice showed increased ROS production. Deletion of NOX2 or treatment of the mice with apocynin prevented the diabetes-induced increases in ROS production (Fig. 5), indicating the role of NOX2 and NADPH oxidase activity in diabetes-induced increases in oxidative stress in the retina.

**Effect of Deletion of NOX2 on the BRB in Diabetic Mice**

Because increases in ICAM-1 expression and leukocyte adhesion have been shown to correlate with the breakdown of BRB in diabetes, we also determined the effects of NOX2 deletion and apocynin treatment on retinal vascular permeability. Evaluation of BRB function by an assay for extravasation of BSA-Alexa-Fluor 488 conjugate showed that NOX2 deletion or apocynin treatment prevented diabetes-induced increases in retinal vascular permeability (Fig. 6), indicating that activity of NOX2-containing NADPH oxidase also plays a role in mediating the vascular permeability increase in diabetes.

**DISCUSSION**

These experiments showed that knocking out NOX2 or inhibiting NADPH oxidase activity by apocynin treatment completely blocks diabetes-induced increases in retinal ICAM-1 levels and leukocyte adhesion and preserves the BRB. Furthermore, the NOX2 deletion and apocynin treatment also substan-
tially reduced ROS formation, implying a causal role of NADPH oxidase–derived ROS in the inflammatory reaction. Because generation of ROS has been reported to be a major factor in triggering vascular injury during LPS-induced endotoxemia, we also determined the effect of deleting NOX2 on ICAM-1 expression and leukostasis in LPS-injected mice. These studies showed that both ICAM expression and leukostasis are markedly increased after LPS treatment and that this effect is abrogated in mice lacking NOX2. These findings confirm the importance of the activity of NOX2 NADPH oxidase in mediating retinal vascular inflammatory reactions in both acute and chronic disease conditions.

A potential link between NADPH oxidase activity and vascular inflammatory reactions has been suggested by previous studies showing that diabetes-induced increases in oxidative stress, expression of VEGF and ICAM-1, leukostasis, and breakdown of the BRB are all blocked by simvastatin, which is known to inhibit NADPH oxidase. Studies showing that diabetes-induced increases in systemic oxidative stress are blocked by apocynin also support the role of NADPH oxidase in diabetic tissue damage. Studies showing that retinal leukostasis induced by diabetes or intravitreous injection of angiotensin II is prevented by treatment with apocynin also support a role for NADPH oxidase activity in retinopathy. Further support for the role of NADPH oxidase in retinal vascular inflammatory reactions comes from studies showing that blockade of angiotensin II type 1 receptor signaling, a known stimulus for NADPH oxidase activation, blocks leukocyte adhesion to the retinal vessels and ICAM-1 expression in models of uveitis or diabetes. Studies of coronary arteries from diabetic pigs have shown that diabetes-induced increases in NADPH oxidase activity are accompanied by upregulation of
inflammatory cytokines (IL-6 and TNF-α), chemokines (MCP-1), and vascular cell adhesive molecules (VCAM-1), providing further support for the role of NADPH oxidase in vascular inflammation. Overall, these reports suggest that NADPH oxidase-derived ROS are critically involved in triggering leukocyte–endothelial cell adhesion and vascular injury during diabetes or other inflammatory conditions.

To the best of our knowledge, the present study is the first to show that NOX2 is critically involved in retinal vascular inflammation and diabetes-induced breakdown of the BRB. However, consistent with our finding that diabetes-induced ROS formation is reduced in the NOX2 knockout mouse retina, others have reported that deletion of NOX2 inhibits ROS production in various tissues, including aorta in DOCA (deoxycorticosterone acetate)-salt–induced hypertension, brain with intracerebral hemorrhage, intrapulmonary arteries with chronic hypoxia, and ischemic hindlimb. Furthermore, knocking out NOX2 has been shown to reduce leukocyte adhesion in a mouse model of hypercholesterolemia or ischemia–reperfusion injury in brain.

Various sources of oxidative stress have been identified in experimental models of diabetes. Studies in diabetic animals and retinal cells exposed to high-glucose conditions in vitro have shown that activation of the mitochondrial electron transport chain is a primary source of oxidative stress. Mitochondria-derived ROS trigger the activation of multiple pathways of hyperglycemic damage, including NADPH oxidase via protein kinase C. Moreover, studies in other models indicate that although mitochondrial-derived ROS are important for the initiation of oxidative stress responses, activity of NADPH oxidase is needed to sustain sufficient levels of ROS formation for the transduction of specific cellular responses. Further study is needed to determine the potential role of mitochondrial ROS in activating NADPH oxidase in models of diabetes and endotoxemia. However, our finding that increases in retinal expression of ICAM-1 and leukocyte adhesion to endothelial cells were substantially inhibited in the LPS model and completely blocked in mice with diabetes that lacked NOX2 or in diabetic mice treated with apocynin provides strong evidence of the critical role played by NOX2 and NADPH oxidase activity in mediating retinal vascular inflammatory reactions in both models.

Further work is also needed to identify the specific cellular source(s) of the NADPH oxidase activity responsible for the retinal vascular inflammatory reactions observed in our experiments. NOX2 is expressed by both phagocytic and vascular endothelial cells. During conditions of inflammation or host defense reactions, ROS are produced at high levels by phagocyte NADPH oxidase. In normal endothelial cells ROS are produced mainly by activity of the NOX4 enzyme. However, previous studies have shown that diabetes and high-glucose treatment promote significant increases in NOX2 levels in retinal endothelial cells. Thus, it seems likely that both phagocyte- and endothelial cell-derived ROS are involved in the pathologic response.

**Figure 5.** In situ production of ROS as measured by dichlorofluorescein (DCF) imaging. ROS production was increased in retinas of diabetic wild-type mice (D+/+) compared with the control wild-type mice (C). The increase was blocked in NOX2−/− diabetic mice (D−/−) and in diabetic mice treated with apocynin (D+/+ apocynin). *P < 0.05 vs. C; #P < 0.05 vs. D−/− (n = 7).

**Figure 6.** Quantification of BRB breakdown by measuring the extravasation of FITC-conjugated albumin. There was a significant increase in vascular permeability in diabetic mice (DM+/+) compared with the control wild-type (C) or NOX2 knockout (C−/−) mice. The permeability increase was blocked by treatment of the diabetic mice with apocynin (DM+apocynin) or by deletion of NOX2 (DM−/−). *P < 0.05 vs. C; #P < 0.05 vs. DM−/− (n = 7).
In summary, our present results suggest that reactive oxygen species produced by the NOX2 NADPH oxidase mediate retinal vascular inflammatory reactions associated with both acute and chronic models of retinal vascular disease. Inhibition of this enzyme may offer a novel therapeutic strategy for the amelioration of vascular injury during retinopathy.

References