Diabetic eNOS-Knockout Mice Develop Accelerated Retinopathy

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PURPOSE. Dysfunction of endothelial nitric oxide synthase (eNOS) has been implicated in the pathogenesis of diabetic vascular complications. This study was undertaken to determine the role of eNOS in the development of diabetic retinopathy (DR), by investigating the functional consequences of its deficiency in the diabetic state.

METHODS. Diabetes was induced in eNOS-knockout (eNOS−/−) and C57BL/6 mice by streptozotocin (STZ) injection. Retinal vasculature was evaluated by albumin extravasation, to quantitatively measure vascular permeability, and by trypsin-digested retinal vascular preparations, to quantify acellular capillaries. Gliosis was evaluated by immunofluorescent techniques. Retinal capillary basement membrane thickness was assessed by transmission electron microscopy. Total retinal nitric oxide level was assessed by measuring nitrate/nitrite using a fluorometric-based assay, iNOS expression was examined by real-time PCR.

RESULTS. Diabetic eNOS−/− mice exhibit more severe retinal vascular permeability than age-matched diabetic C57BL/6 mice, detectable as early as 3 weeks after diabetes induction. Diabetic eNOS−/− mice also show earlier onset and an increased number of acellular capillaries, sustained gliosis, and increased capillary basement membrane thickness. Total nitric oxide (NO) level was also increased, concomitant with elevated iNOS expression in diabetic eNOS−/− retina.

CONCLUSIONS. Diabetic eNOS−/− mice exhibit a significantly wider range of advanced retinal vascular complications than the age-matched diabetic C57BL/6 mice, supporting the notion that eNOS-deficient NO plays an essential role in retinal vascular function. This mouse model also faithfully replicates many of the hallmarks of vascular changes associated with human retinopathy, thus providing a unique model to aid in understanding the pathologic mechanisms of and to develop effective therapeutic strategies for diabetic retinopathy. (Invest Ophthalmol Vis Sci. 2010;51:5240–5246) DOI:10.1167/iovs.09-5147

Diabetic retinopathy (DR) is the most common form of diabetic vascular complication and is the leading cause of severe vision loss in the working-age group.1 Vision loss in DR develops by slow and progressive alterations in the retinal vasculature, including increased capillary basement membrane thickening, increased vascular permeability, pericyte loss, and acellular capillary development, all of which contribute to breakdown of the blood-retinal barrier and subsequent pathologic angiogenesis.

Despite extensive research, the mechanisms underlying the pathogenesis of DR are still not fully understood. Endothelial cell-derived nitric oxide (NO), synthesized continuously in the endothelium from L-arginine by endothelial nitric oxide synthase (eNOS), plays an important role in modulating retinal vascular tone. Constitutive production and release of NO are critical in maintaining vascular homeostasis. Diabetes in both humans and animal models has been associated with reduced bioavailability of NO and impaired endothelium-dependent relaxation.2–4 Furthermore, decreased expression of eNOS concomitant with increased expression of iNOS and nitrotyrosine during diabetes progression in rats has been reported.5 Studies of mice genetically deficient in one of the constitutive NOS isoforms suggest that NO from both neuronal NOS (n)NOS and eNOS play a role in vascular function and disease.7–9 Diabetes is characterized by increased vascular permeability and increased levels of both NO and iNOS.8–10 Therefore, increased expression of iNOS may impair NO-mediated bioavailability and relaxation, contributing to further impairment of NO-mediated vascular function.7–9

To further investigate the pathogenic role of eNOS dysfunction in the development of DR, we induced diabetes in eNOS−/− and C57BL/6 control mice and evaluated their retinal vasculature. The diabetic eNOS−/− mice developed more severe DR symptoms than did the diabetic control mice and exhibited most of the pathologic vascular changes seen in human patients. This finding supports a role for the deficiency in eNOS-derived NO production in the pathogenesis of both diabetic nephropathy and retinopathy. Further understanding the underlying mechanisms of pathogenesis for these diabetic complications has important implications in designing effective therapies, and diabetic eNOS−/− mice also provide a good animal model for achieving that end.

MATERIALS AND METHODS

Animals and General Procedures

Wild-type C57B6/J mice (6–8 weeks old) and breeding pairs of eNOS−/− mice were purchased from Jackson Laboratories (Bar Harbor,
field from each quadrant of the retina were imaged. Acellular capillaries are counted from images for each retina and expressed as the number of acellular vessels per square millimeter.

**Retinal Capillary Basement Membrane Evaluation**

The basement membrane thickness of retinal capillaries was evaluated by transmission electron microscopy (TEM). Anesthetized mice (two nondiabetic and two diabetic mice at 2 months after induced diabetes) were perfused with fixative containing 2% paraformaldehyde and 2% glutaraldehyde and the eyes were enucleated and immersed in the same fixative overnight, after the cornea and lens were removed. The eyes were then postfixed in 2% OsO₄, dehydrated in ethanol series, embedded in epoxy resin. Thin sections (0.5 μm) were stained with toluidine blue for orientation and identification of capillaries. Ultrathin sections (60 nm) were counterstained with uranyl acetate and lead citrate and examined by TEM. Retinal capillary basement membrane thickness (CBMT) was measured from TEM images captured from deep capillaries residing between the outer plexiform (OPL) and inner nuclear (INL) layers (see Fig. 5A, arrow). Minimally, 10 capillaries from the central, mid, and peripheral retina were measured for each eye, at least 30 measurements were taken per capillary.

**Measurement of Retinal NO Levels**

Nitric oxide (NO) production in the retina was examined by measuring the total level of NO. Since NO is a gaseous free radical with a short half-life in vivo of a few seconds or less, levels of the more stable NO metabolites nitrite (NO₂⁻) and nitrate (NO₃⁻) have been used in the indirect measurement of NO in biological fluids. A nitrate/nitrite fluorometric assay kit (Cayman Chemical, Ann Arbor, MI) was used for total nitrite/nitrate measurement in the retina, according to the manufacturer’s instructions.

**Tissue Process, Immune Fluorescence, and Histochemistry**

For immune-fluorescent staining, the eyes were fixed in 4% paraformaldehyde freshly made in phosphate-buffered saline (PBS) at 4°C overnight. The eye cups were cryoprotected in 30% sucrose/PBS for several hours or overnight before quick freezing in OCT compound. Then 12-μm-thick sections were cut at ~20 to ~22°C. A rabbit polyclonal antibody against GFAP (1:1000; Sigma-Aldrich) was used. Secondary antibodies conjugated with Alexa594 and Alexa488 were from Invitrogen-Molecular Probes (Carlsbad, CA) and were used according to the manufacturer’s instruction. The sections were coverslipped with antifade medium (Vectashield; Vector Laboratories), and examined with a microscope (AxioVision; Carl Zeiss Microimaging, Inc., Thornwood, NY) equipped with epifluorescence illumination and a high-resolution digital camera.

For RT-PCR, total RNA was isolated from freshly dissected retinas (Trizol Reagent; Invitrogen), according to the manufacturer’s instructions. RT-PCR was performed (Enhanced Avian HS RT-PCR kit; Sigma-Aldrich). Primer pairs for iNOS sequence were TCGCTTTGCCACG-TTACTG (forward) and TACTCTTCTACTATGCAGG (reverse), and primer pairs for β-actin were CAACAATGAGCTGCGTGTGG (forward) and CGGTGAGATCTTCATGAGG (reverse).

**Statistics**

All values are presented as the mean ± SD. A paired Student’s t-test was used to assess the significance of the difference between two groups. One-way ANOVA followed by the post hoc Tukey (Fisher’s protected least-significant difference) test was used to assess statistical significance between multiple groups. Differences were significant at \( P < 0.05 \).
RESULTS

Development of Diabetes in Wild-Type and eNOS<sup>−/−</sup> Mice: General Parameters

As originally reported, the eNOS<sup>−/−</sup> mice are viable and fertile, but have blood pressure that is approximately 20 mm Hg higher than that in their normal wild-type siblings (Table 1). In the eye, the retinal vasculature develops normally in eNOS<sup>−/−</sup> mice and is associated with increases in vascular-associated nNOS activity that compensate for the eNOS deficiency in the developing and adult mutant retina. Moreover, these mice demonstrate increased iNOS immunoreactivity in the retina. STZ treatment resulted in hyperglycemia in both C57B/6 and eNOS<sup>−/−</sup> mice (Table 1) and progressive loss of body weight. The weight loss in the diabetic eNOS<sup>−/−</sup> mice was more severe than that in the age-matched diabetic C57B/6 mice, as reported previously.

Progressive Vascular Changes and Increased Retinal Vascular Permeability in Diabetic eNOS<sup>−/−</sup> Mice

Abnormal vascular changes were clearly detectable on fundus fluorescein angiography in diabetic eNOS animals, including increased vessel tortuosity, whereas similar changes were not observed in C57B6 mice, even after longer periods of diabetes (Fig. 1). Retinal vascular permeability was evaluated by albumin extravasations and quantified by measuring the fluorescence intensity in serial sections from both nondiabetic and diabetic control C57B/6 and eNOS<sup>−/−</sup> mice. Diabetic eNOS<sup>−/−</sup> mouse retina showed increased vascular leakage of FITC-albumin compared with nondiabetic retina (Fig. 2A). Increased retinal vascular permeability was detectable at 2 weeks after STZ treatment in the eNOS<sup>−/−</sup> mice and numerous focal vascular lesions were present, as evidenced by intense albumin leakage, whereas similar permeability change occurred much later and less severely in diabetic C57/B6 mouse retinas (Fig. 2B).

Gial Cell Changes

Müller gial cells play a vital role in maintaining normal retinal vascular and neuronal function. Glial activation (gliosis) is a common feature of many retinal diseases including diabetic retinopathy. A key feature of retinal gliosis is the upregulation of the intermediate filament, glial fibrillary acidic protein (GFAP). Increased GFAP expression has been shown in diabetic rats, as well as in humans with diabetic retinopathy. However, no change or transient change of GFAP activation has been detected in diabetic C57/B6 mouse retina.

Glial reactivity in diabetic eNOS<sup>−/−</sup> mouse retina was detected by immune fluorescence labeling with antibody against GFAP. In nondiabetic eNOS<sup>−/−</sup> retina, GFAP expression was detectable only in retinal astrocytes (Fig. 3D) as in wild-type C57/B6 retinas (Figs. 3A, 3B), but was highly elevated in diabetic eNOS<sup>−/−</sup> retina (Figs. 3E, 3F). The elevated GFAP expression can be detected as early as 3 weeks after diabetes induction (data not shown).

Increased Acellular Capillaries in Diabetic eNOS<sup>−/−</sup> Mouse Retina

Nondiabetic eNOS<sup>−/−</sup> mouse retinas showed normal vasculature indistinguishable from that of age-matched C57B/6 mice (Figs. 4A, 4B). Diabetic C57B/6 mouse retinas exhibited only a slight (nonsignificant) increase in the number of acellular capillaries per unit area over 6 months of diabetes. In contrast, diabetic eNOS<sup>−/−</sup> mouse retinas exhibited much earlier onset and increased the number of acellular capillaries (Fig. 4B), even more than that of diabetic C57B/6 mouse after 13 month of diabetes (data not shown). Because of the increased mortality...
Increased Basement Membrane Thickness in Retinal Vascular Capillaries

Thickening of microvascular basement membrane represents a histopathologic hallmark of diabetic complications and has been observed in human diabetes and in several different animal models of experimental diabetes. The basement membrane of retinal capillaries from the diabetic eNOS mice at 2 months after STZ induction of diabetes was significantly thicker than those from age-matched, nondiabetic animals (95.72 ± 20 nm in DM versus 71.29 ± 19 nm in NDM eNOS retina, *P < 0.01). Representative TEM micrographs of retinal capillaries from diabetic and nondiabetic eNOS mice are shown in Figure 5.

Retinal NO Levels

NO is constitutively produced by endothelial cells and maintained at physiological levels under normal conditions. Diabetes has been associated with reduced bioavailability of NO and impaired endothelium-dependent relaxation, both in patients and in animal models.\(^2\)\(^{-4}\) We measured the NO level with an indirect fluorometric-based assay to determine total nitrite/nitrate levels in retinas isolated from NDM and DM mice 2 months after diabetes induction. The total retinal NO levels in nondiabetic eNOS/−/− mouse retina were similar to those in age-matched C57B/6 mice (diabetic or nondiabetic), but were significantly increased in the diabetic retina (Fig. 6A), and this NO increase was associated with a concomitant increase in iNOS expression that was undetectable in nondiabetic conditions (Fig. 6B).

DISCUSSIONS

Animal models of diabetes have been valuable tools for shaping our understanding of the pathophysiological mechanisms of diabetic retinopathy and discovering and assessing new potential therapeutic agents. Although numerous diabetic animal models have been described (see a recent review and references therein\(^23\)), none of these models replicates the key features of the disease in humans, which is characterized by progressive alterations to the retinal vasculature, including capillary dilation and leakage, capillary occlusion, pericyte/capillary loss, and subsequent new vessel formation.\(^24\),\(^25\) Most of these models exhibit early features of retinal complications of diabetes, but none of the advanced changes.\(^26\)\(^{-32}\) In this study, we examined the role of eNOS in the development of diabetic retinopathy by inducing diabetes in the eNOS mice and characterized the ensuing retinal vascular changes. In the present study diabetic eNOS/−/− mice developed a significantly wider range of severe retinal vascular complications than did age-matched STZ-induced diabetic C57B6 mice. These complications resemble most of the major vascular hallmarks of DR.
including increased retinal vessel leakage, gliosis, an increased number of acellular retinal capillaries, and increased basement membrane thickening of retinal capillaries. Moreover, the increase in retinal vessel leakage, gliosis, and number of acellular capillaries occurred at an accelerated rate compared with wild-type STZ-treated diabetic mice. These observations strongly support the essential role of NO in retinal vascular function.

Several pathologic pathways could contribute to accelerated retinopathy in this model: First, a deficiency in endothelial-derived NO, coupled with concomitant activation of iNOS, which can produce a larger amount of NO which, when encountering reactive oxygen species (ROS), can generate highly reactive nitrogen species, leading to oxidative stress, and accelerated retinopathy. Endothelial cell-derived NO plays an essential role in maintaining vascular homeostasis. Diabetes both in human and animal models has been associated with reduced NO bioavailability and impaired endothelium-dependent relaxation. Recent studies have shown that eNOS gene polymorphisms are associated with increased risk of developing DR. Furthermore, decreased expression of eNOS concomitant with increased expression of iNOS and nitrotyrosine during diabetes progression in rats have been reported. The nondiabetic eNOS−/− mice are viable, fertile, and exhibit no gross anatomic abnormalities, despite the absence of detectable eNOS mRNA, protein, or enzymatic activity. Further analysis showed that eNOS−/− mouse exhibit abnormalities in vascular relaxation, blood pressure regulation, and cardiac contractility, and increased propensity to form neointima in response to vessel injury. Studies of genetically deficient mice in one of the constitutive NOS isoforms suggest that the NO from both nNOS and eNOS provide mutually compensating pathways in normal conditions that probably account for the lack of gross anatomic abnormalities in these knockout mice. In the eye, the retinal vasculature develops normally in eNOS−/− mice, and that normal development is associated with increases in vascular-associated nNOS activity compensating for the eNOS deficiency in the developing and adult mutant retina. Leukocyte–endothelial cell interactions, which are normally modulated by the eNOS isoform, are also replaced by nNOS in eNOS−/− mice. However, nNOS cannot completely compensate for eNOS in oxidative stress conditions, where there is insufficient NO produced by nNOS to overcome leukocyte recruitment elicited by such stress. In our results, the total NO level in the nondiabetic eNOS−/− retina was similar to that in the wild-type C57/B6 retina, consistent with that reported in the literature. However, NO levels were highly increased during hyperglycemia, accompanied by increased iNOS expression, whereas no further increase in nNOS expression was evident in high-glucose conditions (data not shown). This finding would explain the increased levels of NO products reported in diabetic retinas. Increased expression of iNOS has been observed in retinas of both human and experimental animals, and such high NO concentrations produced by iNOS can be toxic. NO toxicity has been attributed to various mechanisms including peroxynitrite-mediated oxidative damage to macromolecules and cells and energy failure.

A second pathologic process, increased gliosis, as evidenced by increased GFAP expression in Müller cells in diabetic eNOS−/− retinas, may also contribute to the observed retinopathy in this model. Reactive changes in Müller cells, such as upregulation of GFAP occur early in the course of the disease and precede the onset of overt vascular changes in both human and STZ-treated rat retinas during early diabetes. However, increased GFAP expression is not...
FIGURE 6. Total nitrite/nitrate levels and iNOS expression in eNOS−/− retina. (A) Total NO level was measured from retinal protein extracts isolated from nondiabetic (NDM, n = 5) and diabetic (2 months after induced diabetes, n = 6) mice by using indirect fluorometric-based assays to measure total nitrite/nitrate levels in the retina. (B) iNOS mRNA detection in eNOS−/− retinas by RT-PCR (n = 3).

References


5. Nagareddy PR, Xia Z, McNeill JH, MacLeod KM. Increased expression of iNOS in early diabetic retinopathy. 45,52 Thus, the diabetic eNOS−/− mouse model also provides a valuable tool for further understanding the cellular and molecular mechanisms of glial responses in Müller cells; it should be valuable in developing therapeutic strategies for DR.

A third process involves the observation that eNOS−/− mice also develop hypertension, one of the major risk factors for type 2 diabetes53 and also an important independent risk factor for both the initial development and its subsequent progression of diabetic retinopathy.54,55 Control of hypertension in patients with type 2 diabetes has been shown to help prevent retinopathy and other microvascular complications.56 The possible mechanisms by which hypertension contributes to diabetic retinopathy are thought to be both direct hemodynamic processes including impaired autoregulation and hyperperfusion resulting in endothelial damage in the retinal vasculature57 and independently through increased expression of VEGF.58 It has been shown that hypertension independent of hyperglycemia increases VEGF expression in retinal endothelial cells and ocular fluids.59 In animal models, combined diabetes and hypertension by STZ treatment of spontaneous hypertensive rats exacerbates some of the earlier pathologic changes such as inflammation and increased oxidative stress60,61; however, detailed characterization of retinal vascular changes in this model has not been reported.

Finally, we note that in addition to retinopathy, eNOS−/− mice develop more severe diseases in other organs under stress conditions, including severe and early-onset diabetic nephropathy,7,8 consistent with clinical studies that overt nephropathy is strongly associated with proliferative diabetic retinopathy in both type 1 and 2 diabetic patients.82–84 Thus, diabetic eNOS−/− model provides a valuable tool to further investigate the pathophysiological mechanisms at multiple levels in both retina and kidney.