The present study compared susceptibilities of Sprague Dawley (SD) and Brown Norway (BN) rats with ischemia-induced retinal neovascularization. An exposure to constant hyperoxia followed by normoxia induced significant retinal neovascularization in BN rats but not in SD rats, as demonstrated by fluorescein retinal angiography, measurement of avascular area, and count of preretinal vascular cells. These results indicate a rat strain difference in susceptibility to retinal neovascularization. To understand the molecular basis responsible for the strain difference, we have measured the levels of pigment epithelium–derived factor (PEDF), an angiogenic inhibitor, and vascular endothelial growth factor (VEGF), a major angiogenic stimulator in the retina. The hyperoxia-treated BN rats showed a significant reduction in retinal PEDF, but they showed a substantial increase of VEGF at both the protein and RNA levels, resulting in an increased VEGF-to-PEDF ratio. Hyperoxia-treated SD rats showed changes in PEDF and VEGF levels that were less in magnitude and of shorter duration than in BN rats. In age-matched normal BN and SD rats, however, there was no detectable difference in the basal VEGF-to-PEDF ratio between the strains. These observations support the idea that different regulation of angiogenic inhibitors and stimulators under ischemia are responsible for the differences in susceptibility to ischemia-induced retinal neovascularization in SD and BN rats. *Diabetes* 51:1218–1225, 2002

Proliferative diabetic retinopathy is characterized by retinal neovascularization, which is the abnormal growth of retinal blood vessels (1,2). Neovascularization also occurs in sickle cell retinopathy, branch vein occlusion retinopathy, retinopathy of prematurity (ROP), and some forms of age-related macular degeneration (2,3). Neovascularization can lead to fibrosis, retinal detachment, and eventual loss of vision. It is a major cause of blindness in industrialized countries (1,2,4,5).

In the retina, angiogenesis is regulated by two counter-balancing systems: angiogenic stimulators, such as vascular endothelial growth factor (VEGF), and angiogenic inhibitors, such as angiostatin and pigment epithelium–derived factor (PEDF) (6–8). Under pathological conditions, such as diabetic retinopathy and ROP, regions of the retina become ischemic. Local ischemia increases the production of angiogenic stimulators and decreases the production of angiogenic inhibitors, breaking the balance between the positive and negative regulators of angiogenesis (2). As a result, endothelial cells overproliferate, leading to retinal neovascularization (2,9).

Most diabetic mouse and rat models do not develop typical retinal neovascularization (10). It has been shown that exposure to hyperoxia followed by normoxia can result in retinal ischemia, which induces retinal neovascularization in neonatal cats (11), mice (12,13), dogs (14), and rats (15–19). This ischemia-induced retinal neovascularization model closely resembles ROP. It is also an accepted model for other neovascular diseases because these diseases have similar pathological mechanisms (6).

A number of previous studies have shown that when compared with mice, rats are less susceptible to ischemia-induced retinal neovascularization and require cycling between hyperoxia and normoxia in order to develop retinal neovascularization (19,20). However, these previous studies used albino Sprague Dawley (SD) rats (20,21). We have recently observed that pigmented Brown Norway (BN) rats are more susceptible to ischemia-induced retinal neovascularization than SD rats (22). Similar to the mouse model, BN rats only require an exposure to constant hyperoxia for 5 days, followed by constant normoxia, to develop significant retinal neovascularization (22). The mechanism responsible for the strain-dependent susceptibility to retinal neovascularization has not been demonstrated.

To better understand the strain difference in susceptibility to ischemia-induced retinopathy, we have compared the time course of retinal neovascularization and the regulation of angiogenic stimulators and inhibitors in ischemic SD and BN rats. Our results showed that BN rats...
developed more severe retinal neovascularization, which also correlated with a greater increase of the VEGF-to-PEDF ratio in BN than in SD rats.

**RESEARCH DESIGN AND METHODS**

**Animals.** BN and SD rats were purchased from Harlan (Indianapolis, IN). Care, use, and treatment of all animals in this study were in strict agreement with the Statement for the Use of Animals in Ophthalmic and Vision Research from the Association for Research in Vision and Ophthalmology, as well as the guidelines set forth in the care and use of laboratory animals by the Medical University of South Carolina.

**Oxygen-induced retinopathy.** Induction of retinal neovascularization was performed as described by Smith et al. (13), with minor modifications. Briefly, newborn rats were randomly assigned to experimental and control groups. At postnatal day 7, rats in the experimental group were exposed to hyperoxia (75% O₂) for 5 days (postnatal days 7-12) and then returned to normoxia (room air) to induce retinal neovascularization. Control rats were kept in room air.

**Retinal angiography with high-molecular-weight fluorescent.** Rats at postnatal days 12, 14, 18, and 22 were anesthetized with 10 mg/kg xylazine plus 75 mg/kg ketamine i.p. and perfused with fluorescein via intraventricle injection of 50 mg/ml of 2 × 10⁻⁷–molecular-weight fluorescein isothiocyanate-dextran (Sigma, St. Louis, MO) as described by Smith et al. (13). The animals were immediately killed. The eyes were enucleated and fixed with 4% paraformaldehyde in PBS for 10 min. The retina was then separated from the eyecup and fixed with 4% paraformaldehyde for 3 h. Several incisions were made to the retina, which was flat-mounted on a gelatin-coated slide. The vasculature was then examined under a fluorescent microscope (Axioplan2 Imaging, Carl Zeiss, Jena, Germany). Both the total retinal area and the area of the avascular regions were measured using a computerized image-analysis system (Scion, Frederick, MD), averaged within each group and analyzed using Student’s t test.

**Quantification of neovascularization.** Retinal neovascularization was quantified as previously described (22). Briefly, the eyes of eight rats from each group at postnatal day 18 were enucleated, fixed with 10% formaldehyde, sectioned, and then stained with hematoxylin and eosin. The nuclei of vascular cells on the vitreal side of the retina were counted under a light microscope in a double-blind study. Ten sagittal sections from each eye were examined, and cell numbers were averaged in each group of animals. The average number of preretal vascular nuclei was compared with the control group using Student’s t test.

**Western blot analysis.** The retinas were dissected from rats at postnatal day 12, 14, and 16 and homogenized by sonication. The insoluble pellet was removed by centrifugation, and the protein concentration of the supernatant was measured with the BioRad protein assay (BioRad Laboratories, Hercules, CA). Then, 100 μg soluble protein was resolved by SDS-PAGE (12% polyacrylamide gel for PEDF, 15% polyacrylamide gel for VEGF) and electro-transferred to a Hybond ECL nitrocellulose membrane (Amersham International, Piscataway, NJ). The membranes were blocked with a glycolenic acid anti-PEDF antibody (23) or an anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The retinas from four animals at postnatal day 12, 14, 16, and 18 were homogenized by sonication. The insoluble pellet was isolated from the retina using Trizol reagents (Gibco-BRL Life Technologies, Gaithersburg, MD) according to the protocol recommended by the manufacturer. Then, 15 μg total RNA from each sample was loaded for Northern blot analysis using a rat PEDF cDNA probe as described previously (23). The RNA blot was stripped and rebonded sequentially with a VEGF cDNA probe and a labeled oligonucleotide probe specific to the 18S ribosomal RNA (22). The RNA levels were semiquantitated by densitometry and normalized by 18S RNA levels. For each analysis, two independent experiments were performed, and the results averaged.

**RESULTS**

**Differences in ischemia-induced retinal neovascularization in BN and SD rats.** As shown by fluorescein angiography in flat-mounted retinas, both BN and SD rats maintained under normoxia developed superficial and deep vascular layers in the retina that extended from the optic disc to the periphery. The vessels formed a fine radial branching pattern in the superficial retinal layer and a polygonal reticular pattern in the deep retinal layer (Figs. 1 and 2). Small avascular areas were observed only at the earliest stage analyzed (postnatal day 12); at later stages (postnatal days 18–22), the avascular areas disappeared, and the entire retina was covered by a mature capillary network (Fig. 1). There was no difference in the time courses of retinal vascular development between age-matched BN and SD rats from postnatal days 12 to 22 under normoxia. These time courses are also similar to that observed in mice (13).

In contrast, the hyperoxia-treated BN and SD rats showed clearly different time courses in the development of retinal neovascularization. In hyperoxia-treated BN rats at postnatal day 12 (the first day after the return of the animals from hyperoxia to normoxia), retinal arteries were highly constricted, and large avascular regions in the retina were observed. Vascular networks were developed only in small areas in the peripheral retina (Fig. 1). Under the same conditions, SD rats at postnatal day 12 showed less constriction of the retinal arteries and larger areas in the retina covered by vascular networks (Fig. 1), suggesting that the retinal vascular development is inhibited more significantly by hyperoxia in pigmented BN rats than in SD rats.

From postnatal days 14 (2 days after the return from hyperoxia to normoxia) to 22 (10 days after the return), BN rat retinas showed tortuous and dilated radial vessels and the gradual appearance of neovascularization (Fig. 1). The most severe retinal neovascularization was found at postnatal day 18 (6 days after the rats were returned to normoxia), with a typical pattern of pathological retinal neovascularization, including neovascular tufts, avascular regions, microaneurism, and hemorrhage (Figs. 1 and 2A). Although the hyperoxia-treated SD rats showed tortuous and dilated blood vessels at postnatal day 14, they lacked the typical pattern of neovascular tufts, microaneurism, and hemorrhage (Figs. 1 and 2C). These results demonstrated that BN rats developed more severe retinal neovascularization than SD rats after the hyperoxia treatment.

**Quantification of retinal neovascularization in BN and SD rats.** The area of avascular regions in the retina has been shown to be a characteristic of ischemia-induced retinal neovascularization and to be correlated with the severity of retinal neovascularization in rat and mouse models (18,20,24). Therefore, the avascular area was measured and expressed as a percentage of the total retinal area in hyperoxia-treated BN and SD rats at postnatal day 18. Retinal flat-mounts showed a significantly larger avascular area in BN rats than in SD rats (P < 0.01, n = 5) (Fig. 3A–C).

Retinal neovascularization was also quantified by counting preretal vascular cells, as described by Smith et al. (13). At age postnatal day 18 (6 days after the rats were returned to normoxia), BN rats showed an average of 56 ± 11 (mean ± SE) preretal vascular cells/section (n = 8) (Fig. 3D). At the same time point (postnatal day 18), SD rats showed less neovascularization, with 17 ± 4 (n = 8) preretal cells per section (Fig. 3E). Statistical analysis
demonstrated that BN rats developed significantly more preretinal neovascular cells than albino SD rats under the same conditions \((P < 0.01, n = 8)\) (Fig. 3F). Taken together, Figs. 1, 2, and 3 demonstrated that pigmented BN rats are more susceptible to ischemia-induced retinal neovascularization than albino SD rats.

### Different changes in retinal PEDF levels in response to ischemia in BN and SD rats.

PEDF was used as a representative angiogenic inhibitor. In BN rats, after exposure to 75% \(O_2\) for 5 days (postnatal day 12), retinal PEDF levels were elevated by 3.8-fold over that in age-matched normal controls, which correlated with the enlarged avascular areas in the retina at this time point (Fig. 1). After the return from hyperoxia to normoxia for 4 days (postnatal day 16), PEDF was decreased to \(-50\%\) of the control level. The decreased PEDF levels correlated with the progression of retinal neovascularization (Figs. 1 and 4).

In hyperoxia-treated SD rats, however, retinal PEDF levels were not increased at postnatal day 12 when compared with levels in the control animals, which may explain why the avascular area was not significantly enlarged (Figs. 1 and 4). Retinal PEDF levels showed only a slight decrease at postnatal day 12 that recovered to control levels by postnatal day 16, when PEDF reached its lowest levels in BN rats (Fig. 4).

### Different changes in VEGF levels in response to ischemia in BN and SD rats.

In the same hyperoxia-treated BN rats at postnatal day 12, VEGF was reduced by threefold, when compared with the age-matched normal controls (Fig. 5). At postnatal day 14 (2 days after the return to normoxia), VEGF started to increase, and by postnatal day 16, it reached a level that was fivefold more than the control (Fig. 5).

In hyperoxia-treated SD rats, however, VEGF levels...
were increased by only 1.9-fold at postnatal day 12 (Fig. 5). The VEGF levels decreased to the normal control level by postnatal day 14 and remained at the normal level thereafter (Fig. 5), indicating that the induction of VEGF expression by ischemia occurs and disappears earlier and is less significant in SD rats than in BN rats.

Different VEGF-to-PEDF ratios in hyperoxia-treated BN and SD rats. The VEGF-to-PEDF ratio was calculated as a measure of the balance between angiogenic stimulators and inhibitors (Fig. 6). In BN rats, the VEGF-to-PEDF ratio progressively increased from a level that was less than normal to 10-fold higher than the controls in the period between postnatal days 12 and 16. Consistently, the most aggressive progression of neovascularization was observed in BN rats during this period (Fig. 1). In contrast, hyperoxia-treated SD rats at postnatal day 12 showed a 2.6-fold increase in the VEGF-to-PEDF ratio, which may account for the slight retinal neovascularization at the early stage. This ratio decreased to the control level by postnatal day 16 (Fig. 6).

The RNA levels of PEDF and VEGF in hyperoxia-treated BN and SD rats. PEDF and VEGF mRNA levels were examined in the retina by Northern blot analysis. In hyperoxia-treated BN rats, the decreased PEDF mRNA and increased VEGF mRNA levels relative to respective controls were detected at postnatal day 12 (Fig. 7). At postnatal day 14, the PEDF mRNA was decreased by 2-fold to the lowest level, whereas the VEGF mRNA level peaked at 2.5-fold over the control level at the same time point (Fig. 7). Consistent with our previous findings, maximal changes in the VEGF and PEDF mRNA appeared 48 h before their protein changes (23)

In SD rats, only slightly decreased PEDF mRNA levels were observed at postnatal days 12 and 14 (25 and 14% of control levels, respectively). By postnatal day 16, the mRNA level of PEDF recovered to the control level (Fig. 7). The VEGF mRNA change was also less significant than that in BN rats, with only a 40% increase over the control at postnatal day 12, followed by a decrease to control levels by postnatal day 14 (Fig. 7). The smaller changes in
PEDF and VEGF mRNA levels in SD rats, when compared with BN rats, were consistent with their protein level (Figs. 4 and 5).

**Basal levels of PEDF and VEGF in BN and SD rats under normoxia.** To determine whether SD rats have higher basal levels of angiogenic inhibitors and lower levels of angiogenic stimulators than BN rats, the basal levels of PEDF and VEGF in the retina were measured in normal BN and SD rats using Western blot analysis. Rats maintained under normoxia at postnatal day 16 were selected for this analysis because the most significant differences in PEDF and VEGF levels between hyperoxia-treated SD and BN rats were observed at this time point (Figs. 5 and 6). After normalization by β-actin levels, both PEDF and VEGF levels in SD rats were ~50% of those in BN rats (Fig. 8). Lower levels of both PEDF and VEGF in SD rats results in no change in the VEGF-to-PEDF ratio compared with the ratio in BN rats.

**DISCUSSION**

Ischemia-induced retinal neovascularization is a commonly used model for studies of ROP and proliferative diabetic retinopathy. The present study demonstrated that BN rats have a higher susceptibility to ischemia-induced retinal neovascularization than SD rats. Retinal neovascularization is more severe and has a longer duration in BN rats than in SD rats after the hyperoxia treatment, suggesting that these differences might be strain-specific. The different susceptibilities were correlated with the differences in ischemic regulation of angiogenic stimulators and angiogenic inhibitors in the retina.

Both rats and mice have been used as an oxygen-induced retinopathy model. However, previous evidence showed that it was more difficult to induce retinal neovascularization in SD rats compared with C57 mice, using constant hyperoxia followed by exposure to normoxia (13,20). Although retinal neovascularization can be induced in mice by exposure to constant 75% oxygen for 5 days followed by exposure to normoxia, albino SD rats require cycling between hyperoxia and normoxia to induce typical retinal neovascularization (18,20). Therefore, most previous studies using constant hyperoxia were limited to mice. The present study showed that pigmented BN rats develop typical retinal neovascularization after ischemia, indicating that the strain differences in ischemia regulation are not strain-specific but are rather related to the strain-specific differences in ischemia-induced neovascularization.
exposure to constant hyperoxia, to an extent comparable
to that in mice. Therefore, differences between SD rats and
mice in susceptibility to retinal neovascularization may
not be ascribed to different species. Our results demon-
strate that pigmented BN rats are as efficient a neovas-
cularization model as mice. Because of the large size of rat
eyes compared with mouse eyes, using rats for ischemia-
induced retinal neovascularization model offers some ad-
vantages, e.g., easy intravitreal injection and retinal
examination. Therefore, the findings that BN rats are
highly sensitive to neovascularization will broaden the
application of this animal model for studies of retinal
neovascularization, especially in searching for therapeutic
antiangiogenic reagents.

The mechanism underlying retinal neovascularization is
not completely understood. However, there is evidence
that a delicate balance between angiogenic stimulators
and angiogenic inhibitors plays a key role in regulating
angiogenesis (2,25). Under certain hypoxic conditions, as
found in diabetic retinopathy, the angiogenic stimulators
are overproduced, whereas the angiogenic inhibitors are
decreased (2,9,23). The subsequent disruption of the bal-
ance between antiangiogenic and angiogenic factors re-
sults in neovascularization in the eye. VEGF is a primary
angiogenic stimulator (6,26–28), and PEDF has been iden-
tified as a major angiogenic inhibitor in the eye (8,9). Thus,
we used the VEGF-to-PEDF ratio as representative of the
balance between angiogenic stimulators and inhibitors to
better understand the molecular basis responsible for the
different susceptibilities to ischemia-induced retinal neo-
vascularization between different rat strains. Our results
showed that VEGF increased gradually from postnatal
days 12 to 16 in hyperoxia-treated BN rats, reaching a peak
with a fivefold increase over the control (Fig. 5). In the
same animals, PEDF gradually decreased by 2-fold, when
compared with their age-matched controls (Fig. 4), result-
ing in a substantially increased VEGF-to-PEDF ratio (10-
fold) (Fig. 6). In SD rats undergoing the same treatment,
the VEGF-to-PEDF ratio was only slightly higher (2.6-fold)
than the normal controls at these early stages. After
postnatal day 14, VEGF levels quickly dropped, whereas
PEDF recovered to control levels, resulting in a normal
VEGF-to-PEDF ratio. The gradual increase of the VEGF-
to-PEDF ratio in BN rats from postnatal days 12 to 16
correlated with the progression of retinal neovasculariza-

FIG. 4. Retinal PEDF level changes in hyperoxia-treated BN and SD
rats. A: Western blot analysis of PEDF. Retinas from three hyperoxia-
treated (H) BN rats, three hyperoxia-treated SD rats, three age-
matched normal control (C) BN rats, and three age-matched normal
control SD rats were excised at each of the indicated time points
(postnatal days 12–16). The retinas of each group were pooled and
homogenized. The same amount (100 μg) of total protein from each
group was blotted with a specific anti-PEDF antibody. B: The same blot
from A was stripped and rebotted with an antibody specific to β-actin.
The bands were semiquantified by densitometry and normalized with
β-actin levels. C: Relative PEDF levels in hyperoxia-treated animals
were expressed as percentages of levels in the age-matched controls
(mean ± SE, n = 2). Mr., relative molecular mass; P, postnatal day.

FIG. 5. Retinal VEGF level changes in hyperoxia-treated BN and SD
rats. A: Retina samples from the same hyperoxia-treated (H) and
control (C) rats as those in Fig. 4 were blotted with an antibody
specific to VEGF. B: The same membrane was rebotted with the
anti-β-actin antibody. The bands were semiquantified by densitometry
and normalized with β-actin. C: VEGF levels in hyperoxia-treated
animals were expressed as percentages of levels in their respective
controls (mean ± SE, n = 2). P, postnatal day. Mr., relative molecular
mass.

FIG. 6. The VEGF-to-PEDF ratios in hyperoxia-treated BN and SD rats
during the development of neovascularization. The VEGF and PEDF
levels at each time point were first normalized by β-actin (Figs. 4 and
5). The normalized VEGF level was divided by the respective PEDF
levels in the same group to calculate the VEGF-to-PEDF ratio. The
ratio in each hyperoxia-treated group was expressed as a percentage of
ratios in the respective controls.
tion during this period, whereas a quick decline in the VEGF-to-PEDF ratio in SD rats in the same period corresponded to an early regression of neovascularization. The roles of other angiogenic stimulators and inhibitors in the different susceptibilities to retinal neovascularization in these two rat strains remain to be revealed, as there are multiple angiogenic factors in the retina.

To determine whether different basal levels of VEGF and PEDF in normal BN and SD rats are responsible for their different sensitivities to ischemia-induced retinal neovascularization, we measured retinal VEGF and PEDF levels in age-matched normal SD and BN rats. The result showed that both VEGF and PEDF levels are higher in BN rats than in SD rats at postnatal day 16, resulting in no significant change in the VEGF-to-PEDF ratio in normal animals of these two strains. This finding suggests that the different susceptibilities to retinal neovascularization may not arise from the basal balance between VEGF and PEDF in these rats. Instead, the different regulation of VEGF and PEDF in these two rat strains in response to ischemia may be responsible for different susceptibilities to ischemia-induced retinal neovascularization.

Both VEGF and PEDF levels are known to be regulated by oxygen concentration. The different responses of the VEGF-to-PEDF ratio in hyperoxia-induced retinopathy in SD and BN rats suggest a quantitative difference in the regulatory pathways of VEGF and PEDF expression under hypoxia in these two strains of rats. Our results have demonstrated that both VEGF and PEDF mRNA levels are changed in hyperoxia-treated animals, suggesting that the regulation occurs at the RNA level. Under ischemia, VEGF induction is known to be mediated by the HIF-1 pathway (29). The strain difference in the induction of VEGF is thus likely to be ascribed to a different response of the HIF-1 pathway to hypoxia. In contrast, the regulation of PEDF by hypoxia is unclear. The factors accounting for the strain difference in PEDF suppression in these two rat strains remain to be identified.

The present study showed that retinal neovascularization is more severe and enduring in BN than in SD rats after the same hyperoxia treatment. These results demonstrate that pigmented BN rats are more susceptible to ischemia-induced retinal neovascularization than albino SD rats. Strain differences have been observed previously in other neovascularization animal models. Different strains of inbred mice have shown a 10-fold difference in response to VEGF- or basic fibroblast growth factor–stimulated angiogenesis in the corneal micropocket assay (30). In a VEGF-induced choroidal neovascularization model, the pigmented BN and Long-Evans rats demonstrated more severe neovascularization when compared with the albino Wistar-Kyoto and Lewis rats (31). It is interesting that this report and our results seem to suggest a linkage between pigmentation and the sensitivity to choroidal or retinal neovascularization, although it is unknown how pigmentation is associated with susceptibility to retinal neovascularization. In the ischemia-induced retinopathy model, animals from postnatal days 7 to 18 are used. At this stage, the eyes had not yet opened. Furthermore, previous evidence has shown that light intensity does not affect ischemia-induced retinopathy in rats (32). Therefore, different levels of light-related stress in the retina of pigmented and albino rats are unlikely to be responsible for different sensitivities to ischemia-induced retinal neovascularization in pigmented BN and albino SD rats. Instead, genetic factors are more likely to be responsible for the different regulation of VEGF and PEDF and, subsequently, the susceptibilities to retinal neovascularization. These two strains of rats will be useful
for the identification of genetic factors accounting for the resistance or susceptibility to retinal neovascularization.

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