0026-895X/02/6203-539–544$7.00
MOLECULAR PHARMACOLOGY
Vol. 62, No. 3
1746/1008153
Mol Pharmacol 62:539–544, 2002
Printed in U.S.A.

Blockade of Nitric-Oxide Synthase Reduces Choroidal Neovascularization

AKIRA ANDO,[1] AMY YANG, HIROYUKI NAMBU, and PETER A. CAMPOCHIARO

Departments of Ophthalmology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland

ABSTRACT

Nitric oxide (NO) promotes retinal and choroidal neovascularization, although different isoforms of nitric-oxide synthetase (NOS) are critical in each. Deficiency of endothelial NOS (eNOS) suppresses retinal but not choroidal neovascularization, whereas deficiency of neuronal NOS (nNOS) or inducible NOS (iNOS) suppresses choroidal, but not retinal neovascularization. In this study, we investigated the effect of N^G-monomethyl-L-arginine (L-NMMA), a nonspecific NOS inhibitor, in three models of ocular neovascularization. Oral administration of L-NMMA caused significant inhibition of choroidal neovascularization in mice with laser-induced rupture of Bruch's membrane and significantly inhibited subretinal neovascularization in transgenic mice with expression of vascular endothelial growth factor (VEGF) in photoreceptors (rho/VEGF mice) but did not inhibit retinal neovascularization in mice with ischemic retinopathy. By extensive mating among mice deficient in NOS isoforms, triple homozygous mutant mice deficient in all three NOS isoforms were produced. These mice had marked suppression of choroidal neovascularization at sites of rupture of Bruch's membrane and near-complete suppression of subretinal neovascularization in rho/VEGF mice but showed no difference in ischemia-induced retinal neovascularization compared with wild-type mice. These data indicate that NO is an important stimulator of choroidal neovascularization and that reduction of NO by pharmacologic or genetic means is a good treatment strategy. However, the situation is more complex for ischemia-induced retinal neovascularization for which NO produced in endothelial cells by eNOS is stimulatory, but NO produced in other retinal cells by iNOS and/or nNOS is inhibitory. Selective inhibitors of eNOS may be needed for treatment of retinal neovascularization.

Nitric oxide (NO) is a signaling molecule with pleiotropic effects. One of its well-characterized functions is as a mediator of vascular dilation and permeability (Furchgott and Zawadzki, 1980; Moncada et al., 1991). Vascular endothelial growth factor (VEGF) also increases dilation and permeability of blood vessels, and there is evidence to suggest that NO may be involved in the VEGF signaling pathway (Ku et al., 1993; Fischer et al., 1999). In some settings, levels of NO are elevated during VEGF-induced angiogenesis and tumor growth (Leibovich et al., 1994; Jenkins et al., 1995; Ziche et al., 1997). Furthermore, some types of neovascularization are suppressed by blockade of NO production (Ziche et al., 1994; Montrucchio et al., 1997; Gallo et al., 1998; Jadeski and Lala, 1999; Lee et al., 2000). However, NO inhibits other types of angiogenesis (Pipili-Synetos et al., 1994, 1995; Hatjikondi et al., 1996; Norrby, 1998; Sennlaub et al., 1999; Jia et al., 2000; Powell et al., 2000). Therefore, depending upon the setting, NO may have either proangiogenic or antiangiogenic effects.

There are three isoforms of nitric-oxide synthase (NOS), the enzyme that synthesizes NO. Each NOS isoform is coded by a separate gene and the isoforms have distinct but overlapping tissue distribution [for review, see Kone (2001)]. Neuronal NOS (nNOS or NOS1) is the predominant isoform expressed in neurons, but it is also expressed in other tissues, including skeletal muscle and airway epithelium. It is constitutively expressed, but its level of expression may also be modulated. Inducible NOS (iNOS or NOS2) is somewhat difficult to localize, because it generally has low or undetectable basal expression levels and is identifiable only in the presence of certain cytokines or other stimuli. It has been demonstrated in alveolar macrophages, bronchial airway epithelium, uterus, ileum, and platelets. Endothelial NOS (eNOS or NOS3) is the predominant isoform in vascular endothelial cells, but it is also expressed in some other tissues. It is not known why three isoforms of NOS are needed.

Several studies have investigated the localization of NOS isoforms in ocular tissues (Goureau et al., 1993, 1994; Yamamoto et al., 1993; Park et al., 1994; Fischer and Stell, 1999). These studies suggest that nNOS is present in several

This work was supported by National Eye Institute grants EY05951, EY12609, and P30-EY1765, a Lew R. Wasserman Merit Award (to P.A.C.) and unrestricted funds from Research to Prevent Blindness, a grant from Dr. and Mrs. William Lake, a grant from the Eccles Foundation, and a grant from the Ruth and Milton Steinbach Foundation. P.A.C. is the George S. and Dolores Dore Eccles Professor of Ophthalmology.

[1] Present address: Kansai Medical University, Moriguchi, Osaka, Japan

ABBREVIATIONS: NO, nitric oxide; VEGF, vascular endothelial growth factor; NOS, nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; L-NMMA, N^G-monomethyl-L-arginine; GSA, *Griffonia simplicifolia* lectin; TBS, Tris-buffered saline; CCD, charge-coupled device.

types of retinal neurons, particularly amacrine cells. It is also present in choroidal and extraocular blood vessels, and choroidal nerve fibers. Inducible NOS has been localized to retinal pigmented epithelial cells, Muller cells, and invading inflammatory cells.

We recently used a genetic approach to investigate the effect of selective deficiency of each of the isoforms of NOS in three models of ocular neovascularization (Ando et al., 2002). Deficiency of any of the three isoforms resulted in reduced neovascularization in rho/VEGF transgenic mice, whereas nNOS or iNOS deficiency suppressed choroidal neovascularization, and eNOS deficiency selectively suppressed ischemia-induced retinal neovascularization. These data indicate differences regarding the relative importance of particular NOS isoforms in retinal and choroidal neovascularization but suggest that a net decrease in NO-producing capacity has a negative impact on ocular neovascularization. This implies that pharmacologic or genetic inhibition of all of the NOS isoforms should inhibit ocular neovascularization. In this study, we sought to test that hypothesis by investigating in three models of ocular neovascularization the effect of N^G-monomethyl-L-arginine (L-NMMA), which inhibits all three NOS isoforms. As a complementary approach, we generated triple knockout mice deficient in all three NOS isoforms and tested them in the three models of ocular neovascularization.

Materials and Methods

Genetically Engineered Mouse Models. The protocol for this study was approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. All mice were treated in accordance with the recommendations of the Association for Research in Vision and Ophthalmology and the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice with targeted disruption of the *eNOS* gene were obtained from Dr. Paul Huang (Boston, MA) (Huang et al., 1995). Mice with targeted disruption of the *iNOS* gene (Laubach et al., 1995) and mice with targeted disruption of the *nNOS* gene (Huang et al., 1993) were obtained from The Jackson Laboratory (Bar Harbor, ME). Each of the three types of NOS mutant mice was bred into a C57BL/6 background and then crossed with the other types. Heterozygous offspring were crossed multiple times to obtain triple knockouts deficient in all three NOS isoforms. The method of genotyping to identify the mutant *NOS* alleles has been described previously (Ando et al., 2001). Transgenic mice with increased expression of VEGF in photoreceptors were generated and characterized as described previously (Okamoto et al., 1997; Tobe et al., 1998a).

Model of Oxygen-Induced Ischemic Retinopathy. Ischemic retinopathy was produced in mice deficient in all 3 isoforms of NOS or control mice with the same genetic background (C57BL/6) by a method described by Smith et al. (1994). Seven-day-old (P7) mice and their mothers were placed in an airtight incubator and exposed to an atmosphere of 75 ± 3% oxygen for 5 days. Incubator temperature was maintained at 23 ± 2°C, and oxygen was measured every 8 h with an oxygen analyzer. After 5 days, the mice were removed from the incubator and placed in room air. Some of the wild-type C57BL/6 mice were treated once a day by gavage with 80 mg/kg of L-NMMA (Sigma-Aldrich, St. Louis, MO) between P12 and P17. At P17, after 5 days in room air, mice were sacrificed, and eyes were rapidly removed and frozen in optimum cutting temperature embedding compound (OCT; Miles Diagnostics, Elkhart, IN).

Quantitation of Retinal Neovascularization. Frozen sections (10 μm) of eyes were histochemically stained with biotinylated *Griffonia simplicifolia* lectin B4 (GSA; Vector Laboratories, Burlingame, CA) which selectively binds to vascular cells. Slides were incubated in methanol/H_2O_2 for 10 min at 4°C, washed with 0.05 M Tris-buffered saline, pH 7.6 (TBS), and incubated for 30 min in 10% normal porcine serum. Slides were incubated 2 h at room temperature with biotinylated GSA and after rinsing with 0.05M TBS, they were incubated with avidin coupled to peroxidase (Vector Laboratories) for 45 min at room temperature. After being washed for 10 min with 0.05 M TBS, slides were incubated with diaminobenzidine to give a brown reaction product, counterstained with eosin, and mounted with Cytoseal.

To perform quantitative assessments, 10-μm serial sections were cut through each eye and sections from one side of the eye, in which the iris was first visualized, to the last section on the other side of the eye, which contained peripheral iris, were collected. Sections roughly 50 to 60 μm apart were stained with GSA providing 13 sections per eye for analysis. GSA-stained sections were examined with an Axioskop microscope (Zeiss, Thornwood, NY) and images were digitized using a three-CCD color video camera (IK-TU40A; Toshiba, Tokyo, Japan) and a frame grabber. Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to delineate GSA-stained cells on the surface of the retina and their area was measured. The mean of the 13 measurements from each eye was used as a single experimental value.

Model of Choroidal Neovascularization. Choroidal neovascularization was generated by a technique described previously (Tobe et al., 1998b). Briefly, adult mice deficient in all three isoforms of NOS or NOS-sufficient mice with the same genetic background were anesthetized with ketamine hydrochloride (100 mg/kg body weight) and the pupils were dilated with 1% tropicamide. Three burns of 532-nm diode laser photocoagulation (75-μm spot size, 0.1-s duration, 120 mW) were delivered to each retina using the slit-lamp delivery system of an OcuLight GL Photocoagulator (Iridex, Mountain View, CA) and a hand-held cover slide as a contact lens. Burns were performed in the 9-, 12-, and 3-o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining choroidal neovascularization (Tobe et al., 1998b), so only burns in which a bubble was produced were included in the study. Some of the wild-type C57BL/6 mice were given drinking water containing 0.5 g/l L-NMMA for 2 weeks starting the day of laser treatment. After 14 days, the mice were killed with an overdose of pentobarbital sodium, and their eyes were rapidly removed and frozen in OCT.

Quantitation of Choroidal Neovascularization. Frozen serial sections (10 μm) were cut through the entire extent of each burn and histochemically stained with biotinylated GSA as described above. Histomark Red (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used to give a red reaction product that is distinguishable from melanin. Some slides were counterstained with Contrast Blue (Kirkegaard and Perry).

To perform quantitative assessments, GSA-stained sections were examined with an Axioskop microscope and images were digitized using a three-CCD color video camera and a frame grabber. Image-Pro Plus software was used to delineate and measure the area of GSA-stained blood vessels in the subretinal space. For each lesion, area measurements were made for all sections on which some of the lesion appeared and added together to give the integrated area measurement. Using the Image-Pro Plus software, the GSA-stained area of choroidal neovascularization was delineated and the area was calculated. Only lesions in which good sections were obtained through the entire lesion so that a valid area measurement could be made on each were included in the analysis. There seemed to be little variability among lesions in individual mice and all excluded lesions were qualitatively similar in size to included lesions and were excluded solely due to inability to obtain an accurate measurement because of poor quality of some sections.

Mice with Increased Expression of VEGF in Photoreceptors. Extensive mating was done to obtain mice that were homozygous for mutant alleles for all *NOS* loci and carried a rho/VEGF

transgene (Okamoto et al., 1997). Mice with the same genetic background that were wild-type at all three *NOS* loci and carried a rho/VEGF transgene were used as controls. At P21, mice were anesthetized with ether, the descending aorta was clamped, the right atrium was cut, and 1 ml of phosphate-buffered saline containing 50 mg/ml of fluorescein-labeled dextran (2×10^6 average mw; Sigma-Aldrich) was infused through the left ventricle as described previously (Tobe et al., 1998a). The eyes were removed and fixed for 1 h in 10% phosphate-buffered formalin. DNA was isolated and used for genotyping, but the investigator who evaluated the retinal neovascularization remained masked with respect to genotype. The cornea and lens were removed and the entire retina was carefully dissected from the eyecup, radially cut from the edge of the retina to the equator in all four quadrants, and flat-mounted in Aquamount with photoreceptors facing upward. Flat-mounts were examined by fluorescence microscopy and images were photographed, scanned, labeled, and printed.

Quantitation of VEGF-Induced Neovascularization. Retinal flat-mounts were examined by fluorescence microscopy at 400× magnification, which provides a narrow depth of field so that when focusing on NV on the outer edge of the retina, the remainder of the retinal vessels are out of focus, allowing easy delineation of the NV (Tobe et al., 1998a). The outer edge of the retina, which corresponds to the subretinal space in vivo, is easily identified; therefore, there is standardization of focal plane from slide to slide. Images were digitized using a three-CCD color video camera and a frame grabber. Image-Pro Plus software was set to recognize fluorescently stained neovascularization and used to delineate each of the lesions throughout the entire retina and calculate the number of lesions per retina, the area of each lesion, and the total area of neovascularization per retina.

Statistical Analyses. For each of the mouse models, the effect of L-NMMA or complete NOS deficiency on amount of neovascularization was analyzed using a linear mixed model (Verbeke and Molenberghs, 2000). Including the mouse in the model adjusted the estimated effect of experimental variable for correlation in measurements from the two eyes within a mouse. Measurements from within the same mouse were assumed to be exchangeable when modeling correlation structure. Variance components models were also run and adjusted for correlation of measurements from animals in the same litter; however, in most analyses, these effects were negligible once adjusting for correlation of measurements within each mouse did not affect estimated effect of genotype, and hence were dropped from the models. When necessary, a transformation (log or square root) of measurements before analysis was used so that the distribution of measurements better met the normally distributed outcome assumption of the linear mixed model. All analyses were performed using SAS software (SAS Institute, Inc. Cary, NC).

Results

Mice Treated with L-NMMA and Mice Deficient in All Three NOS Isoforms Have Markedly Reduced Choroidal Neovascularization after Rupture of Bruch's Membrane. In adult C57BL/6 mice, 14 days after laser-induced rupture of Bruch's membrane, there was extensive choroidal neovascularization at rupture sites (Fig. 1A), as has been noted in several previous studies (Tobe et al., 1998b; Seo et al., 1999; Kwak et al., 2000; Ando et al., 2001; Mori et al., 2001a,b). In contrast, C57BL/6 mice treated with 0.5 g/l L-NMMA in their drinking water starting the day of laser showed very little choroidal neovascularization at rupture sites 14 days after laser (Fig. 1B). To assess the effect of eliminating NOS activity in another way, triple homozygous mutant mice deficient in all three NOS isoforms were produced by extensive mating among mice deficient in NOS

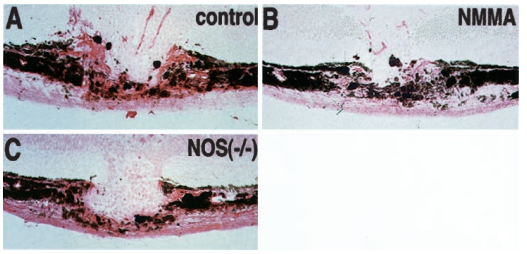

Fig. 1. Reduced choroidal neovascularization after rupture of Bruch's membrane in mice treated with L-NMMA and mice deficient in all 3 NOS isoforms. C57BL/6 mice (A, control), C57BL/6 mice treated with 0.5 g/l L-NMMA (B, NMMA), or mice with a C57BL/6 background deficient in all three isoforms of NOS (C, NOS−/−) had rupture of Bruch's membrane in three locations in each eye by laser photocoagulation. After 2 weeks, the mice were sacrificed and serial frozen sections were cut through each rupture site and stained with GSA, which selectively stains vascular cells. Mice from the control group showed large areas of red-stained choroidal neovascularization (A, arrows), whereas mice treated with L-NMMA (B) and NOS-deficient mice (C) had very little choroidal neovascularization at Bruch's membrane rupture sites.

isoforms. These mice appeared normal on gross examination and were able to reproduce. Fourteen days after laser-induced rupture of Bruch's membrane, mice deficient in all three isoforms of NOS had little or no choroidal neovascularization at rupture sites (Fig. 1C). Quantitation of the amount of choroidal neovascularization by image analysis confirmed that compared with control mice with the same genetic background, mice treated with L-NMMA or mice deficient in all three NOS isoforms showed a dramatic reduction in the amount of choroidal neovascularization at Bruch's membrane rupture sites (Table 1).

Mice Treated with L-NMMA and Mice Deficient in All Three NOS Isoforms Have Markedly Reduced VEGF-Induced Neovascularization in the Retina. As demonstrated in several previous studies (Tobe et al., 1998a; Ozaki et al., 2000; Ando et al., 2001), P21 rho/VEGF transgenic mice showed numerous areas of subretinal neovascularization. These were seen as numerous hyperfluorescent spots in retinal whole mounts of fluorescein-labeled dextran perfused mice (Fig. 2A), but were more easily resolved in high-magnification views, in which they were seen as hyperfluorescent tufts of vessels partially surrounded by retinal pigmented epithelial cells (Fig. 2C, arrows). The narrow depth of field at high magnification makes it easier to distinguish the neovascularization from retinal vessels, which appear as out-of-focus streaks and blurs in the background. Transgene-posi-

TABLE 1

Mice treated with L-NMMA and mice deficient in all three NOS isoforms have markedly reduced choroidal neovascularization after rupture of Bruch's membrane

Each value represents the mean ± S.E.M. of the sum of the areas of neovascularization on each section that included a part of the lesion. If one of the serial sections tore or was unusable, then an accurate measurement could not be obtained, and that rupture site was not included in the analysis. The investigator was masked during sectioning to eliminate any possibility of bias regarding elimination of rupture sites. *P* values were obtained using a linear mixed model, which adjusted for correlation in measurements from the same mouse, assuming that eyes were exchangeable. A log transformation was used for data analysis.

Mice	Rupture sites	Mice	Area of NV	*P*-value
	n	*n*	10^{-3} *mm*^{2}	
Control	18	3	0.045 ± 0.005	
L-NMMA–treated	27	5	0.015 ± 0.002	<0.0001
NOS-deficient	22	5	0.012 ± 0.002	<0.0001

NV, neovascularization.

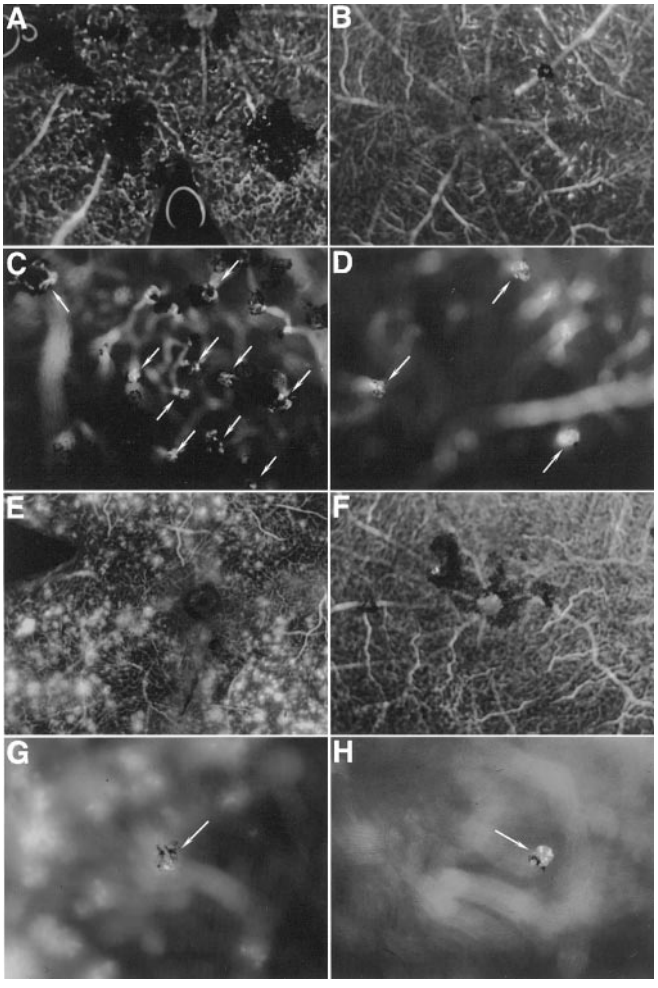

Fig. 2. Reduced VEGF-induced neovascularization in mice treated with L-NMMA and mice deficient in all three NOS isoforms. Hemizygous rhodopsin promoter/VEGF transgenic mice (rho/VEGF mice) were divided into two groups: one group was given vehicle alone (A and C) and the other was given 80 mg/kg/day L-NMMA by gavage between P7 and P21 (B and D). Multiple matings were done to obtain mice deficient in all three NOS isoforms that also carried a rho/VEGF transgene (E-H). At P21, mice were perfused with fluorescein-labeled dextran, and retinal whole mounts were examined by fluorescence microscopy. The retinas of vehicle-treated rho/VEGF mice showed numerous small foci of neovascularization (A) that are more easily distinguished in a high power view (C, arrows). The neovascularization is partially surrounded by black retinal pigmented epithelial cells. The retinas of rho/VEGF mice treated with L-NMMA showed very little neovascularization (B), which is confirmed in high power views (D, arrows). Mice deficient in all three NOS isoforms had fragile retinal capillaries that showed focal disruptions and leakage spots when perfused with normal pressure (E and G) but were well preserved when slowly perfused with low pressure (F and H). Regardless of how the perfusion was done, the retinas showed very little neovascularization (G and H, arrows).

tive rho/VEGF mice treated once a day by gavage with 80 mg/kg of L-NMMA showed many fewer areas of neovascularization (Fig. 2, B and D, arrows). Quantitation by image analysis confirmed that there were significantly fewer neovascular lesions and a much smaller total area of neovascularization per retina in transgenic mice treated with L-NMMA transgenics compared with control transgenics (Table 2).

It appeared that the retinal capillary bed was more fragile in NOS-deficient mice that carried a VEGF transgene, because perfusion of fluorescein-labeled dextran in the standard way resulted in many small disruptions in the capillary network shown by numerous spots of fluorescent leakage throughout the retina (Fig. 2E). High-magnification views showed that the leakage spots were at the level of retinal capillaries, not at the outer border of the retina (Fig. 2G). With slow, low-pressure perfusion of NOS-deficient mice that carried a VEGF transgene, the retinal vessels remained intact and there was no fluorescent leakage (Fig. 2F). Regardless of whether or not there was perfusion-induced leakage, NOS-deficient mice that carried a VEGF transgene had very little neovascularization (Fig. 2, G and H, arrows). Image analysis showed a profound decrease in the number of neovascular lesions and the total area of neovascularization per retina (Table 2).

Mice Treated with L-NMMA and Mice Deficient in All Three NOS Isoforms Have No Significant Reduction in Ischemia-Induced Neovascularization in the Retina. Mice with oxygen-induced ischemic retinopathy show extensive preretinal neovascularization, which is visualized as clumps of GSA-stained vascular cells on the surface of the retina (Fig. 3A). Mice with oxygen-induced ischemic retinopathy treated with 80 mg/kg of L-NMMA once a day by gavage during the ischemic period from P12 to P17 also showed prominent preretinal neovascularization (Fig. 3B). Extensive preretinal neovascularization was also seen in mice with ischemic retinopathy that were deficient in all three NOS isoforms (Fig. 3C). Measurement of the area of preretinal neovascularization per section by image analysis showed that neither L-NMMA-treated mice ($n = 7$; area = 0.0091 ± 0.007; $p = 0.8848$) nor NOS-deficient mice ($n = 5$; area = 0.0095 ± 0.0011; $p = 0.8590$) showed a significant decrease compared with control mice ($n = 4$; area = 0.0092 ± 0.0011).

Discussion

Although the presence of multiple NOS isoforms makes it somewhat difficult to clarify the physiology of NO, it makes it

TABLE 2

Mice treated with L-NMMA and mice deficient in all three NOS isoforms have markedly reduced VEGF-induced neovascularization in the retina

Each value represents the mean ± S.E.M. calculated from the experimental values generated from each mouse (n). P values were obtained using a linear mixed model, which adjusts for correlation in measurements from the same mouse, assuming that eyes were exchangeable. A log transformation was used when analyzing number and average size of NV lesions and a square root transformation was used for total area of NV lesions. Transformation reduced the variance of the data relative to the mean values and resulted in greater statistical power for detecting differences between groups.

Mice	n	Number of NV Lesions	Total Area of NV	Size of NV Lesions
			10^{-3} mm^2	
Control	14	64.2 ± 15.69	17.33 ± 3.52	0.319 ± 0.028
L-NMMA-treated	14	19.1 ± 3.97	6.60 ± 1.10	0.379 ± 0.024
P value		0.0244	0.0308	
NOS-deficient	14	1.6 ± 0.84	0.73 ± 0.42	0.419 ± 0.067
P value		<0.0001	<0.0001	NS

NV, neovascularization.

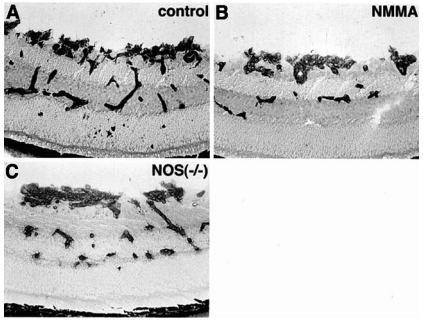

Fig. 3. Mice treated with L-NMMA and mice deficient in all three NOS isoforms show prominent ischemia-induced retinal neovascularization. C57BL/6 mice or mice in a C57BL/6 background deficient in all three NOS isoforms (C, NOS −/−) were placed in high oxygen at P7. At P12, they were removed to room air and wild type mice were divided into two groups and treated with 80 mg/kg/day of L-NMMA (B, NMMA) or vehicle (A, control) by gavage. On P17, the mice were sacrificed and ocular frozen sections were stained with GSA using diaminobenzidine, which gives a brown reaction product that localizes vascular cells. The retinas were counterstained with eosin, which allows identification of the retinal surface. The brown-stained tissue above the inner border of the retina represents retinal neovascularization and was prominent in all three groups of mice.

especially difficult to sort out the role of NO in pathophysiology. In some tissues, NO seems to promote neovascularization; in others, it seems to antagonize it. To address the role of NO and the various NOS isoforms in ocular neovascularization, we tested mice deficient in eNOS, nNOS, or iNOS in three models. We found that VEGF-induced retinal neovascularization was markedly reduced when any of the three isoforms was deficient; choroidal neovascularization was reduced by deficiency of iNOS and nNOS, but not eNOS; and ischemia-induced retinal neovascularization was reduced by deficiency of eNOS, but not iNOS or nNOS (Ando et al., 2002). We interpreted these data to mean that NO is proangiogenic in the retina and choroid and predicted that broad-spectrum NOS inhibitors would reduce retinal and choroidal neovascularization. In the present study, we tested that prediction and found that it was only partially correct. Pharmacologic inhibition of NOS reduced choroidal neovascularization and VEGF-induced retinal neovascularization but did not reduce ischemia-induced retinal neovascularization. To complement the pharmacologic studies, we also took a genetic approach and performed multiple crosses to obtain mice deficient in all three NOS isoforms. These mice, which have maximal blockade of NO-producing activity, had dramatic inhibition of choroidal neovascularization and VEGF-induced retinal neovascularization, but no significant reduction in ischemia-induced retinal neovascularization. This surprising finding indicates that the lack of effect of L-NMMA on ischemia-induced retinal neovascularization is not caused by some characteristic of the drug, such as lesser activity inhibiting eNOS compared with iNOS and nNOS. Instead, it indicates that although reduction in eNOS activity decreases ischemia-induced retinal neovascularization, that effect is abrogated by concomitant reduction in iNOS and nNOS. Therefore, our interpretation of the results in single NOS knockout mice, that NO is proangiogenic in the retina and choroid, seems to be an oversimplification, because the NO generated by iNOS and/or nNOS in cells adjacent to endothelial cells in the presence of retinal ischemia must have an antiangiogenic effect.

While working out the mechanism by which iNOS- and/or nNOS-derived NO reduces ischemia-induced neovascularization in the retina is a worthy pursuit that could help to further clarify the complex cascade of events that contribute to neovascularization in ischemic retina, our findings have important therapeutic implications that deserve immediate attention. Our data suggest that broad-spectrum NOS inhibitors dramatically inhibit choroidal neovascularization. We used systemic administration of a NOS inhibitor in our models, but it is likely that local administration would be the best approach for treatment of ocular neovascularization in patients. Previous studies have demonstrated that eNOS-deficient mice have hypertension (Huang et al., 1995) and nNOS-deficient mice have behavioral abnormalities (Nelson et al., 1995). We did not observe any gross abnormalities in triple NOS mutant mice and investigation for blood pressure or behavioral abnormalities are beyond the scope of our study. Systemic administration of a broad spectrum NOS inhibitor has been shown to cause hypertension but was otherwise well tolerated (Lloyd et al., 2001). Local administration of a NOS inhibitor to avoid hypertension and other possible side effects seems to be the most prudent approach. As soon as sufficient pharmacokinetic and safety data are available, local administration of NOS inhibitors should be tested in patients with choroidal neovascularization.

Choroidal neovascularization is a major public health problem that occurs in a number of diseases in which there are abnormalities in the Bruch's membrane/retinal pigmented epithelial complex, the most common of which is age-related macular degeneration (Campochiaro, 2000). Current treatments for choroidal neovascularization focus on ablation of neovascularization and do not address the underlying angiogenic stimuli; therefore, they are plagued by recurrent neovascularization, which usually results in severe loss of vision. Development of pharmacologic antiangiogenic treatment for choroidal neovascularization is desperately needed. Two pharmacologic agents are currently being tested in clinical trials, an anti-VEGF antibody and an aptamer that binds VEGF (Guyer et al., 2001; Schwartz et al., 2001). Both are VEGF antagonists that are administered by intravitreous injection. NOS inhibitors are small molecules that should be able to gain access to the retina and choroid after periocular injection and will not require intravitreous injection, which is advantageous. In addition, because they act at a different site in the neovascularization cascade, they will complement the VEGF antagonists that are under investigation.

Effective antiangiogenic agents are also needed for ischemic retinopathies, the most common of which is diabetic retinopathy (Klein et al., 1984). Our results suggest that selective eNOS inhibitors rather than broad-spectrum NOS inhibitors should be tested in patients with diabetic retinopathy.

References

Ando A, Yang A, Mori K, Yamada H, Yamada E, Takahashi K, Saikia J, Kim M, Melia M, Fishman M, et al. (2002) Nitric oxide is proangiogenic in the retina and choroid. *J Cell Physiol* **191:**116–124.

Campochiaro PA (2000) Retinal and choroidal neovascularization. *J Cell Physiol* **184:**301–310.

Fischer S, Clauss M, Wiesnet M, Dieter R, Schaper W, and Karliczek GF (1999) Hypoxia induces permeability in brain microvessel endothelial cells via VEGF and NO. *Am J Physiol* **276:**C812–C820.

Fischer AJ and Stell WK (1999) Nitric oxide syntase containing cells in the retina, pigmented epithelium, choroid and sclera of the chick eye. *J Comp Neurol* **405:**1–14.

Furchgott RF and Zawadzki JV (1980) The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature (Lond)* **288:**373–376.

Gallo O, Masini E, Morbidelli L, Franchi A, Fini-Storchi I, Vergari WA, and Ziche M (1998) Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. *J Natl Cancer Inst* **90:**587–596.

Goureau D, Hicks D, Courtois Y, and Kozak YD (1994) Induction and regulation of nitric oxide synthase in retinal Muller cells. *J Neurochem* **63:**310–317.

Goureau O, Lepoivre M, Becquet F, Hartmann MP, and Courtois Y (1993) Differential regulation of inducible nitric oxide synthase by fibroblast growth factors and transforming growth factor beta in bovine retinal pigmented epithelial cells: inverse correlation with cellular proliferation. *Proc Natl Acad Sci USA* **90:**4276–4280.

Guyer DR, Martin DM, Klein M, Haller J, The Eye-Tech Study Group (2001) Anti-VEGF therapy in patients with exudative age-related macular degeneration. *Invest Ophthalmol Vis Sci (Suppl)* **42:**S522.

Hatjikondi O, Ravazoula P, Kardamakis D, Dimopoulos J, and Papaioannou S (1996) In vivo experimental evidence that the nitric oxide pathway is involved in the X-ray-induced antiangiogenicity. *Br J Cancer* **74:**1916–1923.

Huang PL, Dawson TM, Bredt DS, Snyder SH, and Fishman MC (1993) Targeted disruption of the neuronal nitric oxide synthetase gene. *Cell* **75:**1273–1286.

Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, and Fishman MC (1995) Hypertension in mice lacking the gene for endothelial nitric oxide synthetase. *Nature (Lond)* **377:**239–242.

Jadeski LC and Lala PK (1999) Nitric oxide synthase inhibition by N^G-nitro-L-arginine methyl ester inhibits tumor-induced angiogenesis in mammary tumors. *Am J Pathol* **155:**1381–1390.

Jenkins DC, Charles IG, Thomsen LL, Moss DW, Holmes LS, Baylis SA, Rhodes P, Westmore K, Emson PC, and Moncada S (1995) Roles of nitric oxide in tumor growth. *Proc Natl Acad Sci USA* **92:**4392–4396.

Jia L, Wu CC, Guo W, and Young X (2000) Antiangiogenic effects of S-nitrosocaptopril crystal as a nitric oxide donor. *Eur J Pharmacol* **391:**137–144.

Klein R, Klein BEK, Moss SE, Davis MD, and DeMets DL (1984) The Wisconsin Epidemiologic Study of Diabetic Retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years. *Arch Ophthalmol* **102:**520–526.

Kone BC (2001) Molecular biology of natriuretic peptides and nitric oxide syntheses. *Cardiovasc Res* **51:**429–441.

Ku DD, Zaleski JD, Liu S, and Brock TA (1993) Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. *Am J Physiol* **265:**H586–H592.

Kwak N, Okamoto N, Wood JM, and Campochiaro PA (2000) VEGF is an important stimulator in a model of choroidal neovascularization. *Invest Ophthalmol Vis Sci* **41:**3158–3164.

Laubach VE, Shesely EG, Smithies O, and Sherman PA (1995) Mice lacking inducible nitric oxide synthetase are not resistant to lipopolysaccharide-induced cell death. *Proc Natl Acad Sci USA* **92:**10688–10692.

Lee PC, Kibbe MR, Schuchert MJ, Stolz DB, Watkins SC, Griggith BP, Billiar TP, and Shears LL (2000) Nitric oxide induces angiogenesis and upregulates $\alpha_v\beta_3$ integrin expression on endothelial cells. *Microvasc Res* **60:**269–280.

Leibovich SJ, Polverini PJ, Fong TW, Harlow LA, and Koch AE (1994) Production of angiogenic activity by human monocytes requires an L-arginine/nitric oxide-synthase-dependent effector mechanism. *Proc Natl Acad Sci USA* **91:**4190–4194.

Lloyd PG, Yang HT, and Terjung RL (2001) Arteriogenesis and angiogenesis in rat ischemic hindlimb: role of nitric oxide. *Am J Physiol* **281:**H2528–H2538.

Moncada S, Palmer RMJ, and Higgs EA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol Rev* **43:**109–142.

Montrucchio G, Lupia E, de Martino A, Battaglia E, Arese M, Tizzani A, Bussolino F, and Camussi G (1997) Nitric oxide mediates angiogenesis induced in vivo by platelet-activating factor and tumor necrosis factor-alpha. *Am J Pathol* **151:**557–563.

Mori K, Ando A, Gehlbach P, Nesbitt D, Takahashi K, Goldsteen D, Penn M, Chen T, Mori K, Melia M, et al. (2001a) Inhibition of choroidal neovascularization by intravenous injection of adenoviral vectors expressing secretable endostatin. *Am J Pathol* **159:**313–320.

Mori K, Duh E, Gehlbach P, Ando A, Takahashi K, Pearlman J, Mori K, Yang HS, Zack DJ, Ettyreddy D, et al. (2001b) Pigment epithelium-derived factor inhibits retinal and choroidal neovascularization. *J Cell Physiol* **188:**253–263.

Nelson RJ, Demas GE, Huang PL, Fishman MC, Dawson VL, Dawson TM, and Snyder SH (1995) Behavioral abnormalities in male mice lacking neuronal nitric oxide synthetase. *Nature (Lond)* **378:**383–386.

Norrby K (1998) Nitric oxide suppresses bFGF- and IL-1-alpha-mediated but not VEGF165-mediated angiogenesis in natively vascularized mammalian tissue. *APMIS* **106:**1142–1148.

Okamoto N, Tobe T, Hackett SF, Ozaki H, Vinores MA, LaRochelle W, Zack DJ, and Campochiaro PA (1997) Transgenic mice with increased expression of vascular endothelial growth factor in the retina: a new model of intraretinal and subretinal neovascularization. *Am J Pathol* **151:**281–291.

Ozaki H, Seo M-S, Ozaki K, Yamada H, Yamada E, Hofmann F, Wood J, and Campochiaro PA (2000) Blockade of vascular endothelial cell growth factor receptor signaling is sufficient to completely prevent retinal neovascularization. *Am J Pathol* **156:**679–707.

Park C-S, Pardhasaradhi K, Gianotti C, Villegas E, and Krishna G (1994) Human retina expresses both constitutive and inducible isoforms of nitric oxide synthase mRNA. *Biochem Biophys Res Commun* **205:**85–91.

Pipili-Synetos E, Haralabopoulos G, Andriopoulou P, Peristeris P, and Maragoudakis ME (1994) Evidence that nitric oxide is an endogenous antiangiogenic mediator. *Br J Pharmacol* **111:**894–902.

Pipili-Synetos E, Papageorgiou A, Sakkoula E, Sotiropoulou G, Fotsis T, Karakiulakis G, and Maragoudakis ME (1995) Inhibition of angiogenesis, tumour growth and metastasis by the NO-releasing vasodilators, isosorbide mononitrate and dinitrate. *Br. J Pharmacol* **116:**1829–1834.

Powell JA, Mohamed SN, Kerr JS, and Mousa SA (2000) Antiangiogenesis efficacy of nitric oxide donors. *J Cell Biochem* **80:**104–114.

Sennlaub F, Courtois Y, and Goureau O (1999) Nitric oxide synthase-II is expressed in severe corneal alkali burns and inhibits neovascularization. *Invest Ophthalmol Vis Sci* **40:**2773–2779.

Seo M-S, Kwak N, Ozaki H, Yamada H, Okamoto N, Fabbro D, Hofmann F, Wood JM, and Campochiaro PA (1999) Dramatic inhibition of retinal and choroidal neovascularization by oral administration of a kinase inhibitor. *Am J Pathol* **154:**1743–1753.

Smith LEH, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, and D'Amore PA (1994) Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci* **35:**101–111.

Schwartz SD, Blumenkranz M, Rosenfeld PJ, Miller JW, Haller J, Fish G, Lobes L, Singerman L, Green WL, Reimann J (2001) Safety of rhuFab V2, an anti-VEGF antibody fragment, as a single intravitreal injection in subjects with neovascular age-related macular degeneration. *Invest Ophthalmol Vis Sci (Suppl)* **42:**522S.

Tobe T, Okamoto N, Vinores MA, Derevjanik NL, Vinores SA, Zack DJ, and Campochiaro PA (1998a) Evolution of neovascularization in mice with overexpression of vascular endothelial growth factor in photoreceptors. *Invest Ophthalmol Vis Sci* **39:**180–188.

Tobe T, Ortega S, Luna L, Ozaki H, Okamoto N, Derevjanik NL, Vinores SA, Basilico C, and Campochiaro PA (1998b) Targeted disruption of the *FGF2* gene does not prevent choroidal neovascularization in a murine model. *Am J Pathol* **153:**1641–1646.

Verbeke G and Molenberghs G (2000) *Linear Mixed Models for Longitudinal Data,* Springer-Verlag, Inc., New York.

Yamamoto R, Bredt DS, Snyder SH, and Stone RA (1993) The localization of nitric oxide synthetase in the rat eye and related cranial ganglia. *Neuroscience* **54:**189–200.

Ziche M, Morbidelli L, Choudhuri R, Zhang H-T, Donnini S, Granger HJ, and Bicknell R (1997) Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. *J Clin Invest* **99:**2625–2634.

Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA, Geppetti P, and Ledda F (1994) Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration in vitro promoted by substance P. *J Clin Investig* **94:**2036–2044.

Address correspondence to: Peter A. Campochiaro, M.D., Maumenee 719, Johns Hopkins University School of Medicine, 600 N. Wolfe Street, Baltimore, MD 21287-9277. E-mail: pcampo@jhmi.edu