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Role of nitric oxide synthase isoforms for ophthalmic artery reactivity in mice

Panagiotis Laspas [a], Evgeny Goloborodko [a], Jan J. Sniatecki [a], Marcin L. Kordasz [a], Caroline Manicam [a], Leszek Wojnowski [b], Huige Li [b], Andreas Patzak [c], Norbert Pfeiffer [a], Adrian Gericke [a, *]

[a] Department of Ophthalmology, University Medical Center, Johannes Gutenberg University Mainz, Langenbeckstr. 1, 55131 Mainz, Germany
[b] Department of Pharmacology, University Medical Center, Johannes Gutenberg University Mainz, Obere Zahlbacher Str. 67, 55131, Mainz, Germany
[c] Institute of Vegetative Physiology, Charité-Universitätsmedizin Berlin, Charitéplatz 1, 10117 Berlin, Germany

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ABSTRACT

Nitric oxide synthases (NOS) are involved in regulation of ocular vascular tone and blood flow. While endothelial NOS (eNOS) has recently been shown to mediate endothelium-dependent vasodilation in mouse retinal arterioles, the contribution of individual NOS isoforms to vascular responses is unknown in the retrobulbar vasculature. Moreover, it is unknown whether the lack of a single NOS isoform affects neuron survival in the retina. Thus, the goal of the present study was to examine the hypothesis that the lack of individual nitric oxide synthase (NOS) isoforms affects the reactivity of mouse ophthalmic arteries and neuron density in the retinal ganglion cell (RGC) layer. Mice deficient in one of the three NOS isoforms (nNOS−/−, iNOS−/− and eNOS−/−) were compared to respective wild type controls. Intraocular pressure (IOP) was measured in conscious mice using rebound tonometry. To examine the role of each NOS isoform for mediating vascular responses, ophthalmic arteries were studied *in vitro* using video microscopy. Neuron density in the RGC layer was calculated from retinal wholemounts stained with cresyl blue. IOP was similar in all NOS-deficient genotypes and respective wild type controls. In ophthalmic arteries, phenylephrine, nitroprusside and acetylcholine evoked concentration-dependent responses that did not differ between individual NOS-deficient genotypes and their respective controls. In all genotypes except eNOS−/− mice, vasodilation to acetylcholine was markedly reduced after incubation with L-NAME, a non-isoform-selective inhibitor of NOS. In contrast, pharmacological inhibition of nNOS and iNOS had no effect on acetylcholine-induced vasodilation in any of the mouse genotypes. Neuron density in the RGC layer was similar in all NOS-deficient genotypes and respective controls. Our findings suggest that eNOS contributes to endothelium-dependent dilation of murine ophthalmic arteries. However, the chronic lack of eNOS is functionally compensated by NOS-independent vasodilator mechanisms. The lack of a single NOS isoform does not appear to affect IOP or neuron density in the RGC layer.

1. Introduction

Nitric oxide (NO) is a key messenger molecule regulating various functions in the eye, including neurotransmission, immune activity, and vasodilation (Linden et al., 2005; Toda and Nakanishi-Toda, 2007). Three isoforms of nitric oxide synthase (NOS) have been characterized; neuronal (nNOS), endothelial (eNOS), and inducible NOS (iNOS) (Forstermann and Sessa, 2012).

All three NOS isoforms were reported to be involved in regulation of vascular tone. For example, nNOS was shown to be expressed in blood vessels and to modulate vascular reactivity by both endothelium-dependent and independent mechanisms (Capettini et al., 2008; Forstermann et al., 1993; Seddon et al., 2009). While expression of iNOS can be induced in a wide range of cells and tissues by cytokines and other agents (Schoonover et al., 2000), its existence has also been demonstrated in the retina and

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; IOP, intraocular pressure; iNOS, inducible nitric oxide synthase; nNOS, neuronal nitric oxide synthase; L-NAME, N_{ω}-nitro-L-arginine methyl ester; RGC, retinal ganglion cell layer.

* Corresponding author. Tel.: +49 6131 17 5741; fax: +49 6131 17 7680.
E-mail addresses: adrian.gericke@gmx.net, adrian.gericke@unimedizin-mainz.de (A. Gericke).

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choroid under physiological conditions (Berra et al., 2005; Bhutto et al., 2010; Mishra and Newman, 2010; Steinle et al., 2009). Some studies suggested that iNOS is physiologically involved in regulation of vascular tone (Berra et al., 2005; Fagan et al., 1999). However, the majority of reports indicates that induction of iNOS expression is associated with impairment of vascular responses (Gunnett et al., 1998, 2001; Ichihara et al., 2000; Mishra and Newman, 2010). The third isoform, eNOS, is critically involved in endothelium-dependent regulation of vascular tone and blood flow (Faraci and Sigmund, 1999; Pollock et al., 1993). Its activity can be triggered by mechanical forces (Busse and Fleming, 1998) or by vasoactive substances (Forstermann et al., 1994), e.g., acetylcholine. Moreover, endothelium-derived NO is involved in numerous vasoprotective mechanisms, such as regulation of endothelial growth and permeability, inhibition of thrombocyte aggregation and of vascular smooth muscle cell proliferation, and limitation of LDL cholesterol oxidation (Forstermann and Munzel, 2006).

Disturbances of vascular NO production in the retina and optic nerve have been implicated in the pathophysiology of various ocular diseases, including glaucoma and diabetic retinopathy (Polak et al., 2007; Schmetterer et al., 1997; Toda and Nakanishi-Toda, 2007). However, it is still unknown whether the chronic blockade or lack of a specific NOS isoform leads to impaired reactivity of ocular blood vessels and to a decrease of neuron number in the retina, as typically seen in ocular hypoxia-ischemia (Hein et al., 2012; Kaur et al., 2008).

Thus, the major goal of the present study was to test the hypothesis that the chronic lack of an individual NOS isoform affects vascular responses in murine ophthalmic arteries. To examine the role of NOS in endothelium-dependent vasodilation, we examined responses to acetylcholine, an endothelium-dependent vasodilator in mouse ocular blood vessels (Gericke et al., 2011, 2014). To this end, we used mice with targeted disruption of individual NOS genes and various NOS inhibitors. Another purpose of this study was to examine whether mice lacking an individual NOS gene displayed alterations in neuron density in the retinal ganglion cell (RGC) layer.

2. Materials and methods

2.1. Animals

All mice were treated in accordance with the EU Directive 2010/63/EU for animal experiments, and all experiments were approved by the Animal Care Committee of Rhineland-Palatinate, Germany. The following mouse strains were used in this study: B6.129P2-NOS3^{tm1Unc}/J (eNOS−/−) (Shesely et al., 1996), B6.129P2-Nos2^{tm1Lau}/J (iNOS−/−) (Laubach et al., 1995), C57BL/6J (B6) wild type mice, 129S-Nos1^{tm1P1h} (nNOS−/−) (Huang et al., 1993), and B6129F2/J wild type mice, all from The Jackson Laboratory, Bar Harbour, ME, USA. The eNOS−/− and iNOS−/− mice were backcrossed with C57BL/6J B6 wild type mice and kept as inbreeding of homozygous animals (eNOS−/− and iNOS−/−, respectively). C57BL/6J B6 mice served as wild type controls for eNOS−/− and iNOS−/− mice. The nNOS−/− mice were obtained by breeding of heterozygous (±) mice, obtained from crossing 129S-Nos1^{tm1P1h} and B6129F2/J wild type mice. Homozygous (+/+) mice of this breeding served as controls. The breeding was continuously monitored by assessing the genetic status of each animal by PCR using DNA isolated from tail biopsies according to the protocols of The Jackson Laboratory. The age of mice was 314 ± 8 days and 313 ± 5 days in the nNOS−/− ($n = 8$) and the respective wild type group ($n = 8$), 232 ± 8 days and 236 ± 6 days in the iNOS−/− ($n = 8$) and the respective wild type group ($n = 8$), and 262 ± 7 days and 260 ± 8 days in the eNOS−/− ($n = 8$) and the respective wild type group ($n = 8$). The mean age of each NOS knockout genotype and its respective wild type control did not differ significantly. Mice were housed under standardized conditions with a 12 h light/dark cycle, temperature of 22 ± 2 °C, humidity of 55 ± 10%, and with free access to food and tap water.

2.2. Intraocular pressure measurement

Non-invasive measurements of intraocular pressure (IOP) were performed in conscious mice using the Icare® Tonolab rebound tonometer (Bon Optic, Lübeck, Germany) especially designed for rats and mice. Proparacaine 0.5% eye drops (URSAPHARM Arzneimittel GmbH, Saarbrücken, Germany) were administered to each eye before examination. Twelve IOP measurements have been taken per eye, and the overall mean of all 24 measurements was calculated for each mouse.

2.3. Measurements of vascular reactivity

After mice had been killed by CO_2 inhalation, the eyes were immediately removed together with the retrobulbar tissue and placed in ice-cold Krebs buffer with the following ionic composition (in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 11 glucose (all chemicals were from Carl Roth GmbH, Karlsruhe, Germany). Ophthalmic arteries were isolated under a dissecting microscope, placed in an organ chamber filled with cold Krebs buffer and cannulated and sutured onto micropipettes, as reported previously (Gericke et al., 2009). Vessels were pressurized via the micropipettes to 50 mm Hg under no-flow conditions using two reservoirs filled with Krebs buffer and imaged using a video camera mounted on an inverted microscope. Video sequences were captured to a personal computer for off-line analysis. The organ chamber was continuously perfused with oxygenated and carbonated Krebs buffer at 37 °C and pH 7.4. Arteries were allowed to equilibrate for 30 min before further intervention. Viability of vessels was assessed as satisfactory when at least 50% constriction from resting diameter in response to high KCl solution (100 mM) was achieved.

Next, cumulative concentration–response curves were obtained for the α_1-adrenoceptor agonist phenylephrine (10^{-8}–10^{-4} M, Sigma–Aldrich, Munich, Germany). In arteries preconstricted with phenylephrine to 50%–70% of the initial vessel diameter, concentration–response curves for the endothelium-dependent vasodilator acetylcholine (10^{-9}–10^{-4} M, Sigma–Aldrich) and for the endothelium-independent vasodilator nitroprusside (10^{-9}–10^{-4} M, Sigma–Aldrich) have been obtained.

Because the lack of a single NOS isoform has been reported to be functionally compensated in other vascular beds (Lamping et al., 2000; Meng et al., 1998), we used various inhibitors to examine whether such a compensation occured in endothelium-dependent vasodilation. After a concentration–response curve for acetylcholine (10^{-9}–10^{-4} M) had been obtained in arteries preconstricted with phenylephrine, vessels were washed for 10 min and then incubated with either the arginine analog N_ω-nitro-L-arginine methyl ester (L-NAME, 10^{-4} M, Sigma–Aldrich), a non-isoform-selective NOS inhibitor, 7-nitroindazole (10^{-5} M, Sigma–Aldrich), a selective blocker of neuronal NOS (nNOS), or aminoguanidine (3×10^{-4} M, Sigma–Aldrich), a selective inhibitor of inducible NOS (iNOS). Experiments with the three inhibitors were conducted in all mouse genotypes. Since no highly selective inhibitors for eNOS are yet known, the contribution of eNOS to vasodilation responses had to be derived from experiments using nNOS, iNOS and nonspecific NOS inhibition. The used concentrations of all three NOS inhibitors were proven effective in other vascular preparations (Boulanger et al., 1998; Gunnett et al., 1998, 2001; Patzak et al., 2008; Ren et al., 2001; Stapleton et al., 2007). The incubation time for each