

Altered Nitric Oxide System in Patients With Open-Angle Glaucoma

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Objective: To investigate the ocular blood flow response to systemic nitric oxide synthase inhibition in patients with primary open-angle glaucoma.

Methods: In 12 patients with glaucoma and 12 age-matched control subjects, subfoveal choroidal blood flow, optic nerve head blood flow, ocular fundus pulsation amplitude, intraocular pressure, and systemic hemodynamic parameters were measured at baseline and after inhibition of nitric oxide synthase by intravenous administration of NG-monomethyl-L-arginine.

Results: The increase in blood pressure in response to NG-monomethyl-L-arginine was comparable between the 2 study cohorts. In patients with glaucoma, the de-

crease of optic nerve head blood flow ($P = .03$) and fundus pulsation amplitude ($P < .001$) during nitric oxide synthase inhibition was significantly less pronounced than in healthy control subjects. A tendency toward a reduced response in choroidal blood flow was seen ($P = .051$ between groups) in patients with glaucoma.

Conclusions: This is the first in vivo study providing evidence for an altered ocular L-arginine/nitric oxide system in patients with glaucoma. Normalization of the ocular nitric oxide production may be beneficial in terms of normalization of ocular blood flow and neuroprotection of retinal ganglion cells.

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GLAUCOMA IS CONSIDERED one of the most common causes of blindness in industrialized nations.¹ It is characterized by damage of nerve fibers in the optic nerve head and selective loss of retinal ganglion cells, resulting in progressive reduction of the visual field. Elevated intraocular pressure (IOP) is the most important risk factor for glaucoma. However, some eyes with high IOP do not develop glaucoma; approximately 20% of patients with open-angle glaucoma have normal IOP levels.²⁻⁴ In recent years, additional concepts, like dysregulation of optic nerve head perfusion^{5,6} and ischemia-induced retinal ganglion cell death,⁷ have been established.

According to the vascular concept of glaucoma, glaucomatous ganglion cell damage is at least in part caused by chronic impairment of blood supply to the optic nerve head.⁵ It is suggested that decreased optic nerve head blood flow, as observed in patients with glaucoma,⁸⁻¹¹ is related to an altered endothelial function in the supplying vessels.^{12,13} There is evidence that constant formation of nitric oxide (NO) by the endothelial and neuronal isoforms of the enzyme NO synthase

(NOS) provides the maintenance of a basal vasodilator tone in the optic nerve head,^{14,15} which is a precondition of sufficient blood supply in this tissue. Changes in NO synthesis might therefore shift the delicate balance between vasoconstrictor and vasodilator endothelial agents resulting in altered blood supply.

Some in vitro studies indicate that the endothelial and neuronal isoforms of NOS are overexpressed in endothelial cells of glaucomatous optic nerve heads.^{16,17} Direct confirmation of altered NO production in patients with glaucoma is, however, lacking. Because direct measurement of NO production in ocular tissues is not feasible in vivo, we investigated the response of ocular blood flow to inhibition of NOS in patients with glaucoma and compared it with the response in healthy control subjects. Blood flow in the optic nerve head and in the subfoveal choroid, as well as pulsatile choroidal blood flow, were assessed.

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METHODS

SUBJECTS

Our study was performed in adherence with the Declaration of Helsinki and the Good Clini-

cal Practice guidelines of the European Union. After approval of the study protocol by the ethics committee of the Medical University of Vienna and after written informed consent was obtained, 12 patients with primary open-angle glaucoma (POAG; mean age \pm SD, 60 \pm 12 years) and 12 age-matched control subjects (mean age \pm SD, 62 \pm 11 years) were studied. During the 4 weeks before the first study day, all subjects passed a prestudy screening, which included a physical examination and medical history, a 12-lead electrocardiography, complete blood cell count, clinical chemistry, and ophthalmic examination. Patients with glaucoma were defined based on the criteria of the Ocular Hypertension Treatment Study.¹⁸ An abnormal visual field (Humphrey 30-2 program) was accordingly defined as having a glaucoma hemifield test outside the normal limits and/or a corrected-pattern SD with $P < .05$. This criterion had to be fulfilled during at least 3 independent visual field-testing visits within 1 year before the study. Patients with exfoliation glaucoma, pigmentary glaucoma, a history of acute angle closure, a mean deviation of visual-field testing greater than 10 or visual-field defect within the central 10 degrees, a history of trabeculectomy or other ocular surgery, or with a visual acuity less than 12/20 or an uncontrolled IOP (>23 mm Hg within the last 3 months) were excluded. Cup-disc ratios and optic disc areas were determined using a slitlamp and a 78-diopter Volk lens. The optic disc margin was defined as the inner border of the scleral ring. Reading of the slitlamp scale was only done after the measurement was completed. Estimation of cup-disc ratios was calculated to the nearest 0.05. The mean highest-recorded IOP \pm SD in our patients was 28.4 \pm 2.4 mm Hg.

All of the patients were being treated with antiglaucoma therapy, including timolol alone ($n=4$), timolol in combination with latanoprost ($n=2$), timolol in combination with dorzolamide hydrochloride ($n=1$), dorzolamide hydrochloride alone ($n=3$), and latanoprost alone ($n=2$). Patients did not take their regular antiglaucoma therapy on the morning of the trial day. Some subjects participating in this clinical study took nontrial medication, including metoprolol ($n=1$), tamsulosin hydrochloride and spironolactone ($n=1$), pramipexole ($n=1$), atorvastatin calcium ($n=1$), formoterol and hesperidin ($n=1$), and lansoprazole ($n=1$) in the glaucoma group; and atorvastatin, tamsulosin, and diclofenac sodium ($n=1$), betahistine hydrochloride, ibuprofen, Ca^{2+} dobesilate, and vitamins ($n=1$), and finasteride ($n=1$) in the control group. Patients with glaucoma and control subjects were asked to discontinue their regular medication on the trial day.

DESIGN

The study was performed in an open design with 1 study day. The investigators who collected the measurements on the study day and evaluated the data were masked to the patients' study group. Subjects were asked to refrain from ingesting alcohol and/or caffeine for at least 12 hours before trial days. Patients and control subjects received NG-monomethyl-L-arginine (L-NMMA; Clinalfa, Läufelfingen, Switzerland), a competitive inhibitor of NOS.

STUDY DAYS

After stable hemodynamic baseline conditions were achieved (which was ensured by repeated blood pressure measurements), baseline values of choroidal and optic nerve head blood flow, fundus pulsation amplitude, blood pressure, and pulse rate were measured. Then, the intravenous infusion of the following dose of L-NMMA was started: 6 mg/kg of body weight per minute during a 5-minute period, followed by a continuous infusion of 60 μ g of L-NMMA per kilogram of body weight

per minute during a 25-minute period. Ocular and systemic hemodynamic parameters were measured at 10, 20, and 30 minutes after the start of infusion. Intraocular pressure was measured at baseline conditions and after 30 minutes of NOS inhibition.

L-NMMA AS NOS INHIBITOR AND SELECTED DOSES

NG-monomethyl-L-arginine is the most frequently used inhibitor of NOS in human trials. In addition, the hemodynamic effects of intravenous L-NMMA can be reversed by administration of a high dose of L-arginine, indicating specificity for NOS inhibition.¹⁹ The dose of L-NMMA was chosen on the basis of a previous clinical trial in healthy young subjects,¹⁵ in which choroidal and optic nerve head blood flows were significantly reduced after administration of 6 mg of L-NMMA per kilogram of body weight. The continuous dose was chosen according to the pharmacokinetic-pharmacodynamic profile of the drug.²⁰

DATA COLLECTION

Systemic Hemodynamics

Systolic, diastolic, and mean arterial blood pressures were measured on the upper arm by an automated oscillometric device. Pulse rate was automatically recorded from a finger pulse oxymetric device (HP-CMS patient monitor; Hewlett Packard, Palo Alto, Calif).

Laser Doppler Flowmetry

Choroidal and optic nerve head blood flows were measured by laser Doppler flowmetry (LDV-5000 System; Oculix Inc, Arbaz, Switzerland).^{21,22} Briefly, the vascularized tissue is illuminated by coherent laser light, while the laser beam is directed away from visible vessels. Scattering by moving red blood cells leads to a frequency shift in the scattered light. In contrast, static scatterers in tissue do not change light frequency but lead to randomization of light directions impinging on red blood cells, offering a reference signal. This light diffusion in vascularized tissue leads to a broadening of the spectrum of scattered light. From this spectrum, the mean red blood cell velocity and red blood cell volume within the scattering volume of the illuminated tissue can be calculated in relative units. The product of velocity and volume is the flow and is proportional to red blood cell flux. In our study, laser Doppler flowmetry was performed in the neuroretinal rim to assess optic nerve head blood flow and, in the fovea, to assess blood flow in the subfoveal choroid. The average period of the measurements in the optic disc and choroid was approximately 2 minutes, depending on the subject's skill in fixation.

To reduce the variability of blood flow data as assessed with this technique, laser Doppler flowmetry parameters were corrected to yield.²³ This correction takes into account that the signal obtained with laser Doppler flowmetry may depend on the absolute amount of re-emitted light, which affects the direct current level at the detector. The parameter yield is defined by the intensity of the returning light (direct current) divided by the signal amplification (gain). The influence of yield on the measurements was investigated by using a regression model (third order polynomial equation) applied on the logarithmic values of yield and laser Doppler flowmetry flow data in the choroid and the optic nerve head.

Table. Baseline Parameters of Ocular and Systemic Hemodynamic Variables in Patients With POAG and in Healthy Controls

Measurement	Patients With POAG, Mean \pm SD	Controls, Mean \pm SD	P Value*
SBP, mm Hg	129.5 \pm 7.0	131.9 \pm 17.4	.66
DBP, mm Hg	71.8 \pm 9.4	71.2 \pm 9.5	.86
MAP, mm Hg	91.1 \pm 7.2	91.4 \pm 11.5	.93
PR, beats/min	67.8 \pm 12.4	71.5 \pm 8.7	.41
IOP, mm Hg	16.6 \pm 3.1	13.7 \pm 2.3	<.02
FPA, μ m	3.5 \pm 0.8	4.4 \pm 0.9	<.02
ONHBF, arbitrary units	5.7 \pm 1.4	6.5 \pm 1.6	.2
ChBF, arbitrary units	5.8 \pm 1.8	7.4 \pm 3.2	.15

Abbreviations: ChBF, choroidal blood flow; DBP, diastolic blood pressure; FPA, fundus pulsation amplitude; IOP, intraocular pressure; MAP, mean arterial pressure; ONHBF, optic nerve head blood flow; POAG, primary open-angle glaucoma; PR, pulse rate; SBP, systolic blood pressure.

*Two-tailed *t* test.

Fundus Pulsation Technique

Ocular fundus pulsation amplitude, which estimates the pulsatile part of blood flow in the choroidal vasculature,²⁴ was assessed by laser interferometry.²⁵ Briefly, the eye is illuminated by the beam of a single-mode laser diode (wavelength, 783 nm) along the optical axis. The light is reflected at both the front surface of the cornea and the retina. The 2 re-emitted waves produce interference fringes, from which the distance changes between the cornea and retina during a cardiac cycle can be calculated. These distance changes are caused by the pulsatile inflow of blood through the arteries and by the nonpulsatile outflow through the veins. The maximum change in corneoretinal distance is called fundus pulsation amplitude.

Measurement of IOP

Intraocular pressure was measured with a handheld applanation tonometer (Perkins MK2; Clement Clarke, Edinburgh, Scotland). Before each measurement, 1 drop of 0.4% benoxinate hydrochloride combined with 0.25% fluorescein sodium was used for local anesthesia.

DATA ANALYSIS

Statistical analysis was performed using the CSS Statistical Software for Windows (Statsoft Inc, Tulsa, Okla). For data description, the effect of L-NMMA on ocular hemodynamic parameters was expressed as the percentage of change from baseline values. Statistically significant effects of L-NMMA were assessed with an analysis of variance (ANOVA) model for repeated measurements using the absolute values of all outcome parameters. Baseline data were compared between groups using unpaired *t* tests. A 2-tailed *P* value <.05 was considered significant.

RESULTS

Patients with POAG had a mean deviation in visual field testing of -3.43 (range, -9.23 to -0.88), a mean \pm SD cup-disc ratio of 0.71 ± 0.11 , and a mean optic disc area of 2.43 mm². Baseline values of ocular and systemic variables are presented in the **Table**. Patients with POAG had significantly higher IOP values ($P < .02$) and significantly lower

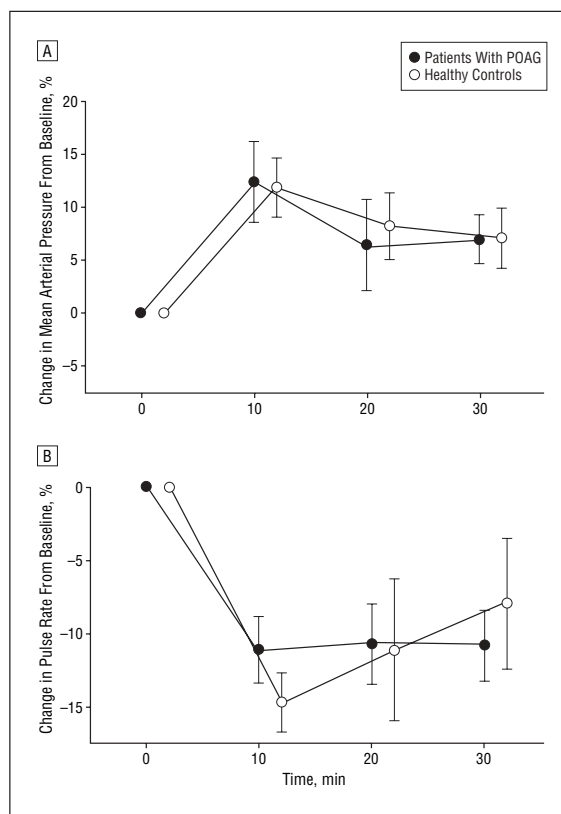


Figure 1. Effect of nitric oxide synthase inhibition with NG-monomethyl-L-arginine on mean arterial pressure (A) and pulse rate (B) in patients with primary open-angle glaucoma (POAG; n=12) and in healthy controls (n=12).

fundus pulsation amplitude values ($P < .02$) as compared with control subjects. No significant difference between patients with POAG and healthy controls was observed in systemic hemodynamic parameters or in subfoveal choroidal and optic nerve head blood flows at baseline.

Figure 1 shows the effects of NOS inhibition on mean arterial pressure and pulse rate in patients with POAG and in healthy control subjects. Patients with POAG and healthy control subjects showed comparable reactivity of blood pressure and pulse rate in response to L-NMMA. Systolic, diastolic, and mean arterial blood pressures increased in patients with POAG, as well as in healthy controls (systolic blood pressure, $P = .88$; diastolic blood pressure, $P = .98$; mean arterial pressure, $P = .97$; ANOVA between groups). As expected, L-NMMA reduced subjects' pulse rate in both groups ($P = .54$; ANOVA between groups). NG-monomethyl-L-arginine did not affect IOP in patients with POAG or healthy control subjects ($P = .60$; ANOVA between groups; data not shown).

Effects of NOS inhibition on ocular hemodynamic parameters are shown in **Figure 2**. In the eyes of patients with POAG, the percent decrease of optic nerve head blood flow during NOS inhibition was significantly less pronounced than in healthy control subjects at 30 minutes (mean \pm SD, patients with POAG, $5.4\% \pm 7.0\%$; healthy controls, $12.0\% \pm 8.4\%$; $P = .030$; ANOVA between groups). The decrease in subfoveal cho-

roidal blood flow during administration of L-NMMA was comparable between groups at 30 minutes, but a clear tendency toward a reduced response in patients with POAG was seen (mean \pm SD, patients with POAG, 9.9% \pm 9.0%; healthy controls, 15.3% \pm 10.8%; $P = .051$; ANOVA between groups). The decrease of fundus pulsation amplitude during NOS inhibition was significantly less in POAG eyes as compared with healthy control eyes at 30 minutes (mean \pm SD, patients with POAG, 7.2% \pm 5.6%; controls, 15.2% \pm 5.3%; $P < .001$; ANOVA between groups).

COMMENT

This is the first study performed in vivo that investigated the local L-arginine/NO system in human POAG. The main finding of our trial is that patients with POAG show an abnormal blood flow response to systemic inhibition of NOS with L-NMMA in the optic nerve head and the choroid as compared with healthy controls, despite a comparable increase in systemic blood pressure. This indicates local alterations of the L-arginine/NO system in this disease.

Endothelial cell dysfunction in glaucoma has already been proposed to be found in glaucoma, based on previous in vitro and in vivo studies. Patients with normal-pressure glaucoma exhibit a reduced response in forearm blood flow to acetylcholine, indicating generalized vascular endothelial dysfunction in this subgroup of patients with POAG.²⁶ This is also supported by the observation that isolated dissected arteries from gluteal fat biopsy specimens from patients with normal-tension glaucoma show enhanced responses to 5-hydroxytryptamine and endothelin-1.²⁷

Based on our results, several explanations are possible. Most likely, the reduced blood flow response to L-NMMA is indicative of increased basal NOS activity in glaucomatous ocular tissues. The blunted reactivity of blood flow to NOS inhibition may therefore reflect impaired inhibition of NOS. Therefore, higher doses of L-NMMA would be required to achieve comparable reductions in ocular blood flow. This is in keeping with an in vitro study^{16,17} that showed that the levels of the 3 isoforms of NOS are increased in the optic nerve head of patients with glaucoma. Increased levels of endothelial NOS in optic nerve head vessels may be assumed to be neuroprotective by causing vasodilation and thereby increasing blood flow. This increase may be considered a compensatory mechanism counteracting reduced blood flow. By contrast, the inducible and, in part, the neuronal isoforms are supposed to be involved in triggering the apoptotic cascade owing to the association of NO to proapoptotic factors, and to induce neurotoxic effects owing to the free radical properties of NO.¹⁶

Alternatively, it may be speculated that basal NO production by the endothelial NOS is reduced in ocular tissues. This argument is supported by the observation of reduced optic nerve head perfusion⁸⁻¹¹ and by lower plasma and aqueous humor concentrations of cyclic guanosine 3c,5c-monophosphate and NO₂ in patients with POAG, as compared with healthy controls.²⁸ Therefore,

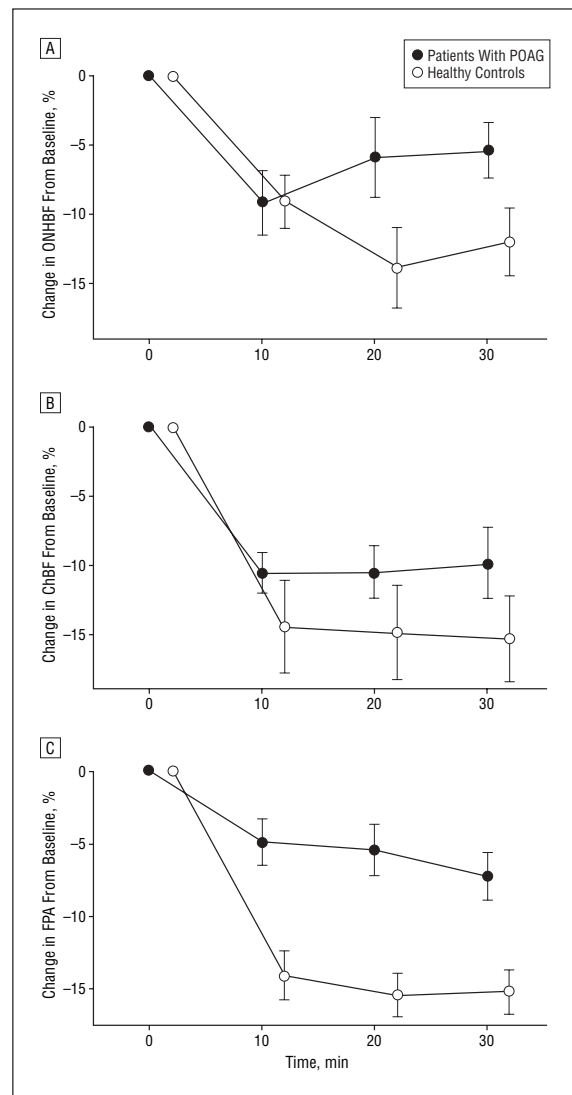


Figure 2. Effect of nitric oxide synthase inhibition on optic nerve head blood flow (ONHBF) (A), subfoveal choroidal blood flow (ChBF) (B), and ocular fundus pulsation amplitude (FPA) (C) in patients with primary open-angle glaucoma (POAG; n = 12) and in healthy controls (n = 12). Patient and control groups showed significant differences in ONHBF and FPA measurements.

the relative effect of NOS inhibition on blood flow might be smaller as compared with controls, because less NO is blocked. Finally, an altered reactivity of glaucomatous ocular vessels to L-NMMA may be postulated. The ocular dose-response curve to NOS inhibition may be shifted to the right. Multiple factors could provoke a shift in vascular reactivity, such as increased vasoconstrictory mediators, increased deactivation of NO, alterations in the cyclic guanosine 3c,5c-monophosphate and second messenger cascade in the vascular smooth muscle, or loss of endothelial cells. The latter theory is supported by the observation that microvascular changes and capillary dropout can be observed in the human glaucomatous optic nerve head.²⁹

To finally answer the question of whether the results in the present trial are a consequence of either increased

or decreased NO synthesis in ocular tissues or reflect altered reactivity, dose-response curves to L-NMMA would be required. Ethical considerations do, however, render such a study impossible.

Importantly, reduced reactivity to NOS inhibition in patients with POAG was observed with 2 independent methods. This appears particularly important, because both methods have some limitations in assessing ocular blood flow.³⁰ In addition, one needs to consider that patients with glaucoma were on regular topical antiglaucoma medication, which could (in principle) have modified the hemodynamic response to NOS inhibition in our study. This appears, however, to be unlikely, because the ocular hemodynamic effects of topical antiglaucoma drugs are generally considered to be small.³¹

Our results are critically important on the specificity of L-NMMA as an inhibitor of NOS. L-arginine analogues may exert pharmacological effects other than NOS inhibition, including muscarinic antagonism, inhibition of cytochrome *c* reduction by ferrous iron, and blockade of vasodilatation caused by amiloride and dibutyl cyclic adenosine monophosphate.³² On the other hand, we have previously shown that L-arginine reverses the effect of L-NMMA on choroidal blood flow and restores the response to systemic hypercapnia.¹⁹ In addition, it has been shown that L-NMMA inhibits the acetylcholine-induced, but not the sodium nitroprusside-induced, increase in femoral blood flow.³³

Our data indicate an abnormal NO system in the optic nerve head and in the choroid in patients with glaucoma as compared with healthy controls. The NO system may be an attractive target system for therapeutic interventions in glaucoma.

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