Effects of Systemic NO Synthase Inhibition on Choroidal and Optic Nerve Head Blood Flow in Healthy Subjects

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PURPOSE. There is evidence from animal studies that nitric oxide (NO) is a major determinant of ocular blood flow. In humans NO synthase inhibition reduces pulsatile choroidal blood flow, but no data on optic nerve head (ONH) vasculature are available yet. The goal of this study was to investigate the effects of NO synthase inhibition on human choroidal and ONH blood flow using laser Doppler flowmetry.

METHODS. The study design was a randomized, placebo-controlled, double-masked, balanced three-way crossover. On separate study days 12 healthy male subjects received infusions of N\textsuperscript{G}-nitro-L-arginine (L-NMMA; either 3 mg/kg over 5 minutes followed by 30 μg/kg per minute over 55 minutes or 6 mg/kg over 5 minutes followed by 60 μg/kg per minute over 55 minutes) or placebo. The effects of L-NMMA or placebo on choroidal and ONH blood flow were measured with laser Doppler flowmetry. In addition, laser interferometric measurement of fundus pulsation was performed in the macula to assess pulsatile choroidal blood flow.

RESULTS. L-NMMA reduced all outcome parameters in the choroid and the ONH. The higher dose of L-NMMA caused a significant decrease in blood flow in the choroid (−26% ± 9%; P < 0.001) and the ONH (−20% ± 16%; P < 0.001) as evidenced from laser Doppler flowmetry and a significant decrease in fundus pulsation amplitude (−26% ± 5%; P < 0.001).

CONCLUSIONS. These results indicate that NO is continuously released in human choroidal and ONH vessels. (Invest Ophthalmol Vis Sci. 2000;41:3080–3084)

There is evidence from a variety of studies that nitric oxide (NO) plays an important role in the regulation of ocular blood flow. The initial step in the endogenous production of NO is the hydroxylation of the nitrogen in the guanidine group of L-arginine, a process that is catalyzed by the enzyme NO synthase (NOS). To date three isoforms of the NO synthase have been identified: inducible NOS, neuronal NOS, and endothelial NOS. Inducible NOS is only present under pathologic conditions in response to inflammatory or allergic reactions, the latter isoforms are expressed constitutively.

Different L-arginine analogues can be used as competitive inhibitors of NOS, which facilitates the investigation of the NO system in vivo. A variety of studies in different species using different methods have been performed, which indicate that choroidal blood flow is strongly reduced after the inhibition of NOS. With respect to optic nerve head (ONH) blood flow, few studies are available. In the cat, NOS inhibition significantly reduces ONH blood flow and blunts the response to flicker-induced vasodilation as evidenced from a laser Doppler flowmetry study. In the ONH of cats and monkeys a decrease in blood flow after the administration of an NOS inhibitor was seen in experiments using the radioactive microsphere technique. By contrast, an effect of NOS inhibition on blood flow in the human eye is only evident for the choroid from laser interferometric measurement of fundus pulsation, which estimates the pulsatile blood flow component in this vascular bed. These experiments have not yet been confirmed using a different method for the assessment of choroidal hemodynamics, and no data on the effect of NOS inhibition on ONH blood flow exist in humans. We, therefore, investigated the effect of NOS inhibition on choroidal and ONH blood flow in healthy subjects using laser Doppler flowmetry. This was done in an effort to elucidate whether constitutively formed NO contributes to basal vascular tone in the ONH, and whether the effects of NOS inhibition in the choroidal and the ONH vasculature are comparable.

METHODS

Subjects

The present study was performed in adherence to the Declaration of Helsinki and the Good Clinical Practice (GCP) guidelines of the European Union. After approval of the study protocol by the Ethics Committee of the Vienna University School of Medicine and after written informed consent was obtained, 12 healthy male subjects were studied (mean ± SD age, 25.6 ± 2.4 years). All subjects passed a prestudy screening during the

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4 weeks before the first study day, which included medical history and physical examination; 12-lead electrocardiogram; complete blood count; activated partial thromboplastin time; thrombin time; clinical chemistry (sodium, potassium, creatinine, uric acid, glucose, cholesterol, triglycerides, alanine aminotransferase, aspartate transcarbamylase, γ-glutamyltransferase, alkaline phosphatase, total bilirubin, and total protein); hepatitis A, B, C, and HIV serology; urine analysis; and an ophthalmic examination. Subjects were excluded if any abnormality was found as part of the pretreatment screening unless the investigators considered an abnormality to be clinically irrelevant. In addition, subjects with normal findings from the screening examinations and with ametropia of less than 3 diopters were included in the trial. Mean baseline intraocular pressure (IOP) of the subjects was 14.5 ± 2.1 mm Hg. During the last week after completion of the study a follow-up safety investigation was scheduled. This follow-up investigation included complete blood count, activated partial thromboplastin time, thrombin time, clinical chemistry (sodium, potassium, creatinine, uric acid, glucose, cholesterol, triglycerides, alanine aminotransferase, aspartate transcarbamylase, γ-glutamyltransferase, alkaline phosphatase, total bilirubin, and total protein), and urine analysis.

**Study Design**

Subjects were asked to refrain from alcohol and caffeine for at least 12 hours before trial days and were studied after an overnight fast. The study design was a randomized, placebo-controlled, double-masked, three-way crossover with a washout period of at least 5 days. On different study days all subjects received intravenous infusions of L-arginine (L-NMMA; Clinalfa, Läufelfingen, Switzerland; either 3 mg/kg per minute for 5 minutes followed by 30 μg/kg per minute over 55 minutes or 6 mg/kg over 5 minutes followed by 60 μg/kg per minute over 55 minutes) or placebo.

**Description of Study Days**

A resting period of at least 20 minutes in a sitting position was scheduled for all subjects. After stable hemodynamic conditions were achieved, which was ensured by repeated blood pressure measurements, baseline values of choroidal blood flow (CHBF), ONH blood flow (ONHBF) with laser Doppler flowmetry, fundus pulsation amplitude (FPA) with laser interferometry, NO concentration in exhaled air, blood pressure, and pulse rate were assessed. Thereafter the infusion of L-NMMA or placebo was started, and hemodynamic parameters and NO concentration in exhaled air were measured every 15 minutes.

**Rationale for L-NMMA as NOS Inhibitor and Dose Selection**

L-NMMA has been used in a variety of human studies and is well tolerated in healthy subjects. We have previously shown that L-NMMA induces a pronounced effect on ocular FPA and that this effect can be reversed by high-dose L-arginine.14 The doses of L-NMMA were selected on the basis of previous clinical trials. The continuous dose was chosen according to the pharmacokinetic–pharmacodynamic profile of this drug.17 In addition, we have previously shown that the selected dose regimen exerts a constant effect on ocular and cerebral hemodynamics.16

**Systemic Hemodynamics**

Systolic, diastolic, and mean arterial pressures (SBP, DBP, MAP) were measured on the upper arm by an automated oscillometric device. Pulse pressure amplitude (PPA) was calculated as SBP – DBP. Pulse rate (PR) was automatically recorded from a finger pulse oximetry device (HP–CMS patient monitor; Hewlett-Packard, Palo Alto, CA).

**Laser Doppler Flowmetry**

CHBF and ONHBF were assessed with laser Doppler flowmetry according to the method of Riva et al.18,19 The principles of laser Doppler flowmetry have been described in detail by Bonner and Nossal.20 Briefly, the vascularized tissue is illuminated by coherent laser light. Scattering on moving red blood cells (RBCs) leads to a frequency shift in the scattered light. In contrast, static scattering in tissue does not change light frequency but leads to randomization of light directions impinging on RBCs. This light diffusing in vascularized tissue leads to a broadening of the spectrum of scattered light (Doppler shift power spectrum, DSPS). From this DSPS the mean RBC velocity (VEL), the blood volume (VOL), and the blood flow (FLOW) can be calculated in relative units. In the present study the laser beam was directed to the fovea to assess blood flow in the submacular chorioid.19 Blood flow in the ONH was measured at the temporal neuroretinal rim.18 Care was taken to be sure that the measurement location did not include any visible vessels.

**Fundus Pulsation Technique**

Ocular fundus pulsation was assessed by laser interferometry as described by Schmetterer et al.21 Briefly, the eye is illuminated by the beam of a single mode laser diode (λ = 785 nm) along the optical axis. The light is reflected at both the front surface of the cornea and the retina. The two 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 FPA. The method has been shown to estimate the pulsatile blood flow in the choroidal vasculature.22

**Measurement of Exhaled NO**

Exhaled NO was measured with a chemoluminescence detector (NO analyzer, model 8840; Monitor Labs, Englewood, CO) connected to a strip-chart recorder. Calibration of the instrument was done with certified gases (300 ppb NO in N2; AGA, Vienna, Austria), using precision flowmeters. A baseline signal was obtained with pure nitrogen. Exhaled air (1000 ml/min) was allowed to enter the inlet port. Subjects were instructed to fully inflate their lungs, hold their breath for 10 seconds, and exhale for 10 seconds into a polytetrafluoroethylene tube. Three consecutive readings were taken at each measurement point under nasal occlusion. The end-expiratory values from the recorder readings were used for analysis. This assures that inspired NO from the ambient air does not distort the results.23

**Data Analysis**

The effect of L-NMMA on ocular hemodynamics was calculated as percent change from baseline values. The maximum change from baseline during the observation period is presented as the...
percent change induced by L-NMMA. Statistically significant
effects of L-NMMA were assessed with three-way ANOVA for
repeated measures using the absolute values of all outcome
parameters. If significant changes were observed, the dose
dependency of the effects was investigated using two-way
ANOVA for repeated measures. To investigate whether
L-NMMA differentially affected ONHBF and CHBF the relative
data were used. This was done separately for the two doses by
using two-way ANOVA for repeated measures. Post-hoc analy-
sis for individual time points was performed with paired t-tests
using the Bonferroni correction for multiple comparisons. Data
are presented as mean ± SD. P < 0.05 was considered to be
significant.

RESULTS
No adverse events were observed during the study. In all
subjects blood pressure and PR returned to baseline levels
within 2 hours after the stop of L-NMMA infusion. As compared
with the prestudy screening, none of the subjects had any
relevant changes in laboratory parameters at the follow-up
investigation.

There were no significant differences between the baseline
values on the three trial days (Table 1). Placebo had no
consistent effect on systemic or ocular hemodynamics and did
not affect exhaled NO.

The effect of L-NMMA or placebo on systemic hemody-
namic parameters and concentrations of NO in the exhalant is
shown in Figure 1. L-NMMA induced a significant increase in
MAP (P < 0.0001), which was dose-dependent (P = 0.046).
This increase was 10% ± 6% for the lower dose of L-NMMA (3
mg/kg over 5 minutes followed by 30 µg/kg per minute over
55 minutes, P = 0.006) and 14% ± 6% for the higher dose of
L-NMMA (6 mg/kg over 5 minutes followed by 60 µg/kg per
minute over 55 minutes, P = 0.001). The effect of L-NMMA on
PPA was not significant (P = 0.11). However, the higher dose
tended to decrease PPA (−15% ± 17%). The systemic hyper-
tensive response to L-NMMA was paralleled by a decrease in PR
(P = 0.0001), which was not dose-dependent. The decrease in
PR was −12% ± 11% for the lower dose of L-NMMA (P = 0.015) and −18% ± 6% (P < 0.001) for the higher dose of
L-NMMA. The efficacy of the selected doses of L-NMMA was
also in evidence from the significant reduction of exhaled NO
concentrations (P = 0.0007). The maximum effect of the
lower L-NMMA dose was −55% ± 11% (P = 0.003), whereas
the effect of the higher L-NMMA dose −56% ± 25% (P = 0.014) was only slightly more pronounced.

TABLE 1. Hemodynamic Outcome Variables at Baseline on Different
Study Days

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>L-NMMA at Low Dose</th>
<th>L-NMMA at High Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>80 ± 8</td>
<td>78 ± 5</td>
<td>79 ± 8</td>
</tr>
<tr>
<td>PR, beats · min⁻¹</td>
<td>67 ± 7</td>
<td>69 ± 8</td>
<td>71 ± 9</td>
</tr>
<tr>
<td>Exhaled NO, ppb</td>
<td>100 ± 43</td>
<td>93 ± 44</td>
<td>92 ± 56</td>
</tr>
<tr>
<td>CHBF, arbitrary units</td>
<td>5.5 ± 1.9</td>
<td>5.4 ± 1.9</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td>ONHBF, arbitrary units</td>
<td>6.2 ± 2.3</td>
<td>6.3 ± 1.8</td>
<td>6.1 ± 2.2</td>
</tr>
<tr>
<td>FPA, µm</td>
<td>3.95 ± 1.13</td>
<td>4.04 ± 1.15</td>
<td>4.03 ± 1.26</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD (n = 12).

The effect of L-NMMA on the ocular hemodynamic outcome
variables is depicted in Figure 2. L-NMMA significantly
reduced FPA (P < 0.0001), FLOW in the choroid (P = 0.0001),
and FLOW in the ONH (P = 0.0007). The effect of L-NMMA on
FPA was −22% ± 9% for the lower dose (P < 0.001) and −26% ±
5% for the higher dose (P < 0.001). The effect of the higher
dose of L-NMMA on FLOW in the choroid was comparable
(−26% ± 9%; P < 0.001) to that on FPA, whereas the effect of
the lower dose of L-NMMA was less pronounced (−14% ±
11%; P = 0.037). The effect of L-NMMA on FLOW in the
choroid was dose-dependent (P = 0.008), whereas the effect
on FPA was not dose-dependent. The effects of L-NMMA on
FLOW in the ONH were smaller: The lower dose of L-NMMA
exerted an effect of −9% ± 11%, which was only significant 15
minutes after drug administration (P = 0.045). The higher dose
of L-NMMA induced a significant effect on FLOW in the ONH
−20% ± 16% (P = 0.002). The effect of the higher dose of

FIGURE 1. Time course of mean arterial pressure (MAP), PR, and
exhaled NO concentration after administration of L-NMMA (batched
bars: 3 mg/kg over 5 minutes followed by 30 µg/kg per minute over
55 minutes; solid bars: 6 mg/kg over 5 minutes followed by 60 µg/kg
per minute over 55 minutes) or placebo (hollow bars). Data are
presented as mean ± SD (n = 12). Asterisks indicate significant effects
of L-NMMA versus baseline.
The effect of NOS inhibition on NO concentration in exhaled air was also constant over the whole study period. However, this effect was not dose-dependent in the present trial. There is evidence that most of the NO detected in exhaled air arises from the upper airways. Although our data clearly indicate that the doses of L-NMMA administered were capable of inhibiting NOS in the airways, they cannot be extrapolated to the extent or time course of NOS inhibition in other organs. Hence, data on exhaled NO concentrations do not necessarily reflect quantitative NOS inhibition at the level of the ocular blood vessels.

Effects of L-NMMA on CHBF in the present trial were evidenced from both methods used, and in general a high degree of consistency was observed. Although the effect of the higher dose of the NOS inhibitor on FPA and FLOW in the choroid was almost identical, the lower dose exerted slightly higher effects on FPA than on FLOW. The reason for this observation may either be related variability of results with these techniques or related to slight changes in pulsatility of the CHBF. The latter could be subject to changes in the blood pressure profile or to changes in choroidal vascular resistance and may result in an over- or underestimation of CHBF effects when only the pulsatile component is assessed. In the present study we observed only small changes in PPA. A small decrease in PPA as observed during the high dose of L-NMMA would result instead in a reduction of the pulsatile flow component.

With respect to our data obtained in the ONH, the limitations of this method have to be considered. A recent study in monkeys indicates that after section of the posterior ciliary arteries supplying the posterior part of the ONH, blood flow as measured with the laser Doppler flowmetry does not change. The authors attribute this finding to limited sampling depth of this method and speculate that only the anterior parts of ONH vasculature, which are normally supplied by retinal arterioles, contribute to the signal. Whether this interpretation is true remains to be confirmed. However, a severe intervention such as dissection of supplying arteries may lead to redistribution of blood flow in adjacent vascular beds and hamper the interpretation of such data. Moreover, reflectance spectra from the ONH indicate that the penetration depth of laser light in the red or near-infrared is much higher as also mentioned by Petrig et al. These optical considerations indicating that deeper layers of ONH vasculature contribute to the laser Doppler flowmetry signal is further supported by A-scans as obtained with low coherence interferometry, where a significant portion of light arises from structures located behind the retinal surface.

In the present study we did not measure IOP during the administration of L-NMMA. However, we have previously shown that L-NMMA in the selected doses does not affect IOP. Moreover, laser interferometry is very sensitive to even slight disturbances in the tear film. Hence, applanation tonometry often makes it difficult to get technically adequate interferograms. Our results may also be relevant for future investigations of endothelial function of ONH blood vessels in glaucoma. A recent study reported that patients with normal tension glaucoma have an impaired endothelial responsiveness to agents altering the l-arginine/NO pathway. Evidence for abnormal NO production in glaucoma also comes from an in vitro study in which altered levels of NOS isoforms were observed in

**DISCUSSION**

In the present trial a dose-dependent effect of L-NMMA on CHBF was evidenced using laser Doppler flowmetry. Moreover, this is the first study to show that NO contributes to vascular tone in human ONH blood vessels. However, the effect of L-NMMA on blood flow in the ONH was less pronounced than that in the choroid. During the observation period the selected dose regimen of L-NMMA produced stable effects on ocular blood flow as evidenced from all ocular hemodynamic parameters assessed in the present study, which is in keeping with a previous study. Hence, the doses chosen for this clinical trial can be recommended for future clinical trials investigating the interaction of the NO system with other vasoactive agents in the eye.

![L-NMMA or placebo](image-url)
glaucomatous ONH tissue. The present study indicates that alterations in the L-arginine/NO system in the ONH may also be investigated by intravenous administration of L-NMMA in humans. We have previously shown that this method may be used to detect altered endothelial function in the choroid in patients with long-standing diabetes. In conclusion, our results indicate that NO is continuously released in human choroidal and ONH vessels.

References