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Nitric oxide/guanylate cyclase pathways and flow in anterior segment perfusion

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Abstract *Background:* The nitric oxide/guanylate cyclase pathway has been suggested to participate in the regulation of intraocular pressure. In the present study, the involvement of nitric oxide pathways on the outflow through the trabecular meshwork was assessed using pharmacological manipulation of the nitric oxide pathway. *Methods:* Anterior segments of human donor eyes were maintained in an organ culture perfusion system, and the effects of L-NAME, an inhibitor of nitric oxide synthase, on the flow rate was determined. In a second series, the effects of consecutive application of L-arginine as substrate for nitric oxide synthase, L-NAME, and sodium nitroprusside, a nitric oxide-donor, were studied. The cyclic GMP levels in the perfusate were assessed with an ELISA immunoassay kit. *Results:* In the first series of experiments, L-NAME caused a statistically significant decrease in flow rate of 10%, accompanied by a decrease in cGMP levels. In the second series, L-arginine did not alter flow, and the effect of L-NAME seen in the first series was prevented by the high preload of L-arginine. Nitroprusside caused a significant 10% increase of flow rate. In the perfusate, cGMP levels were not altered by L-arginine and L-NAME, but were increased after nitroprusside. *Conclusion:* Under organ culture perfusion conditions, modulation of the nitric oxide/guanylate cyclase system alters the flow rate through the trabecular meshwork within a total range of 20%; i.e. the difference between inhibition of NO synthesis and the presence of a NO-donor. These results indicate that the nitric oxide/guanylate cyclase system plays a role in aqueous humour dynamics and, therefore, in the regulation of intraocular pressure.

Introduction

Although increased ocular pressure (IOP) is debated as part of the definition of glaucoma, it is a major risk factor of the progression of visual field loss [7]. An increased outflow resistance in the trabecular meshwork is suggested to be the cause of an elevated IOP [3]. Therefore, it is of interest to investigate the endogenous regulatory pathways that control the flow through the trabecular meshwork. The involvement of the nitric oxide (NO)–guanylate cyclase system on the regulation of flow through the trabecular meshwork in human-donor eyes is subject of this study.

NO causes smooth muscle relaxation and is an essential intercellular regulator of resistance in blood vessels. NO is formed from L-arginine in a reaction catalysed by the enzyme nitric oxide synthase (NOS). It activates cytosolic guanylate cyclase leading to enhanced cyclic guanosine 3', 5'-mono phosphate (cGMP) levels, which triggers a cascade resulting in the relaxation of smooth muscle cells [9, 15]. Intraocular or topical application of NO-donors lowered IOP in rabbits and monkeys [2, 11, 12, 16, 19, 22]. In the trabecular meshwork and scleral spur, cells have been described as having smooth, muscle-like properties, which might indicate that NO may

alter the shape of these cells and, as a consequence, influence flow rate [14, 21, 24]. Furthermore, cells in the trabecular meshwork were reported to contain endothelial cell NOS (eNOS) [18] and various substances that increase intracellular cGMP levels and cause relaxation of isolated bovine trabecular meshwork strips [23]. Remarkably, in the trabecular meshwork of patients with primary open-angle glaucoma (POAG) fewer eNOS-containing cells were detected than in controls, indicating that an attenuation of the NO system might be involved in this disease [17].

These observations suggest a role for NO in the regulation of outflow resistance through the trabecular meshwork, and may be therefore a potential target of a new pharmacological approach to regulate IOP. The anterior segment perfusion model was used to test the hypothesis that NO is involved in the regulation of outflow through the human trabecular meshwork.

Materials and methods

Preparation of the anterior segments

Human donor eyes unsuitable for corneal transplantation were obtained from the Cornea Bank Amsterdam, located at the Netherlands Ophthalmic Research Institute. The age of the donor, time of death and time of enucleation are known. After enucleation, the eyes were kept on ice, preventing more excessive cell-loss and debris formation in the trabecular meshwork. Under aseptic conditions, the eyes were divided into an anterior- and a posterior segment by hemisection at the equator. Conjunctiva, vitreous, lens, and the uvea were removed carefully. The anterior segments were connected to a perfusion apparatus and perfused with Eagle's minimum essential medium (EMEM, supplemented with 2% foetal bovine serum, 100 U/ml penicillin, and 50 µg/ml streptomycin) at 37°C and 5% CO_2 in atmosphere. In the first series, 13 out of 16 anterior segments were cultured in EMEM at 37°C and 5% CO_2 in atmosphere, before they were transferred to the perfusion apparatus. In the second series, 10 out of 16 segments were cultured. Results were not influenced by culturing.

Perfusion

The anterior segments were mounted on a perfusion apparatus connected to a real-time flow measurement system, enabling continuous recording of pressure and flow. For a detailed description, see Dijkstra et al. [5, 6]. The anterior segments were perfused with EMEM under standard conditions: temperature was 37°C, the perfusion pressure kept constant at 10 mmHg, and 5% CO_2 in atmosphere. After a stabilisation period of 4 h during which EMEM was renewed every hour, drugs or placebo were administered. Anterior segments with a flow rate higher than 5 µl/min, lower than 1 µl/min, or with an irregular behaviour of the flow rate were excluded from this study. The anterior segments with a flow higher than 5 µl/min and irregular behaviour were discarded because of probable leakage, while a flow below 1 µl/min is indicative for a low vitality of the tissue [10]. Excluding the eyes with a flow rate below 1 µl/min also means that, although we have no access to information on the patient history of the donor, eyes with an extreme outflow obstruction were not included in this study.

In the first series of experiments with 16 human anterior segments, the following drugs were successively administered: 10^{-6} M nitro-L-arginine-methyl-ester hydrochloride (L-NAME) for 1 h followed by 10^{-4} M L-NAME for 1 h. At baseline and after each hour of perfusion with L-NAME, the perfusate was collected and frozen at –20°C for cGMP level assessment.

In the second series of experiments with 16 human anterior segments, the following drugs were successively administered: 2×10^{-4} M L-arginine hydrochloride (L-arginine) for 1 h, followed by 10^{-4} M L-NAME 1 h, and followed by 10^{-4} M sodium nitroprusside 1 h. At the end of each hour, the perfusate was collected and frozen at –20°C for cGMP detection. The frozen samples were examined with a cyclic GMP Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, Mich., USA) to determine cGMP concentrations ("duplo measurements").

In several cases, the lactate dehydrogenase (LDH) levels in the perfusate were measured. All drugs were obtained from Sigma (St. Louis, USA).

Data analysis

The average flow rate over the last 15 min at the end of each hour of perfusion was used to evaluate the effect of drugs. The effect of drug application on the flow rate was expressed as the relative change in percentage of the flow rate after addition of the drug, compared to the flow rate preceding the application. In this way, each anterior segment served as its own control, allowing for paired statistics. Since flow-rate data were not distributed normally, the non-parametric Wilcoxon matched pairs signed rank test was used for statistical evaluation of the effect of the various drugs on flow rate. However, analysis with Student's *t*-test gave essentially the same outcome. To test and validate our method, we analysed in the same way, the effect on flow rate induced by the change of the perfusion medium (EMEM), 1 h before addition of the first drug. No statistically significant effect was induced by this action. Additionally, a control experiment was carried out on 11 human donor eyes by following the flow rate while replacing the perfusion medium once every hour for the duration of the typical experiment in the first and second series.

The effects of the drug on cGMP levels were quantified by calculating the mean relative change in percentage of flow rate in comparison to baseline ± SEM. The cGMP levels were given as a ratio relative to baseline values and their 95% confidence interval. Baseline values were set at 1.00. The cGMP values measured in the perfusate are caused by diffusion through cell membranes and are assumed to be a reflection of the intracellular cGMP levels. The cGMP measurements proved to be problematic. There was a great variability between the different eyes. Another matter was, that due to the small amounts of perfusate, it was not possible to repeat the cGMP measurement at different dilutions. Finally, a few samples had to be excluded because their cGMP levels were too low. We decided to analyse all raw data to avoid bias created by excluding low values [1]. The null hypothesis for the statistical analysis of the cGMP data was that the change in cGMP levels of the various drugs is equal in all eyes. In the first experiment, the cGMP levels in the perfusate were fit to: cGMP $level_{j,\ i}$=cGMP $level_{j,\ i}=1\times A_i$ with j=eye 1 to 16, i=treatment 1 to 3 (baseline, 10^{-6} ML-NAME, 10^{-4} M L-NAME) and $A_1=1$, using the raw absorbance values. In the second experiment, the cGMP levels in the perfusate were fit to: cGMP $level_{m,\ n}$=cGMPm, $n=1\times A_n$ with m=eye 1-16, *n*=treatment 1–4 (baseline, L-arginine, L-NAME, nitroprusside) and $A_1=1$. This approach results in a best fit for all data after treatment with a drug and provides an index for the deviation of the data from 1.00 (baseline value) together with a 95% confidence interval.

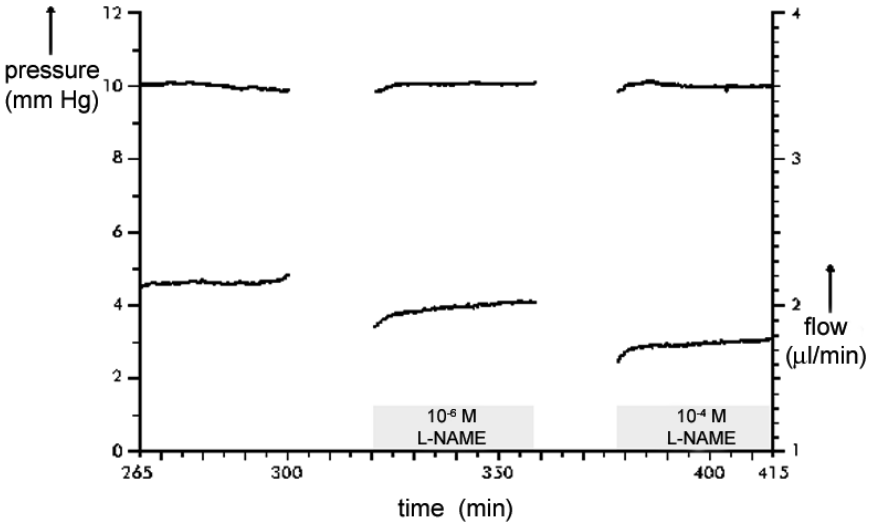

Fig. 1 Example of the recording of pressure (10 mmHg, constant) and flow rate in response to perfusion with 10^{-6} M L-NAME and 10^{-4} M L-NAME

Results

Effects of L-NAME

The mean age of the donors of the first series of experiments was 67 years, with a range from 50 to 79 years. The mean enucleation time, the time between death of the donor and placing the eyes on ice, was 9±4 h (± SD). The mean post-mortem time, the time between the death of the donor and preparation of the anterior segment, was 22±11 h. Some anterior segments were kept in culture medium before being perfused; mean duration in culture was 23.3±14.4 h.

For these experiments, two different concentrations of L-NAME, an inhibitor of the NO-producing enzyme NOS, were applied in order to prevent the presupposed endogenous synthesis of NO. It was expected that the subsequent decrease of intracellular cGMP levels and contraction of the smooth muscle-like cells of the trabecular meshwork lead to an increased resistance in the outflow pathway. The mean baseline flow was 2.5±.2 µl/min (± SEM). After perfusion with 10^{-6} M L-NAME for 1 h, the flow decreased in 11 of 16 studied anterior segments, with a mean change of -3.8±1.8% (± SEM). Statistical analysis showed that this effect, although being modest, was significant (P<0.04). After perfusion with 10^{-4} M L-NAME for 1 h, flow decreased in 14 of 16 experiments, compared to baseline flow. The mean decrease of flow rate, compared to baseline, was -10.4%±2.2% (P<0.001). In Fig. 1, an example of a recording of both flow and pressure in the course of an experiment of the first series is shown.

When baseline cGMP levels were set at a value of 1.00, there was no effect of 10^{-6} M L-NAME on cGMP levels (0.98; with a 95% confidence interval 0.32-2.00), but 10^{-4} M L-NAME decreased cGMP levels significantly (0.44; with a 95% confidence interval 0.19–0.79).

Effects of L-arginine and nitroprusside

The mean age of the donors for the second series was 65 years, with a range from 23 to 78 years. The mean enucleation time was 9±3 h (± SD), the mean post-mortem time was 25±6 h, and the mean time in culture was 26.4±16.7 h

In the second group of experiments, 16 anterior segments were perfused. First, L-arginine, the substrate to NOS, was applied to compensate for a possible washout of endogenous L-arginine thus limiting NOS activity. An increase in the production of NO was expected leading to an enhanced flow through the trabecular meshwork. L-Arginine is also competitive with L-NAME, which is a L-arginine analogue, in its reaction with NOS. After preloading the tissue with L-arginine, it was anticipated that the effect of L-NAME was reduced. Finally, sodium nitroprusside was applied, a NO-donor, thus by-passing the activity of the endogenous enzyme NOS.

The anterior segments were perfused with 2×10^{-4} M L-arginine, 10^{-4} M L-NAME, and 10^{-4} M sodium nitroprusside, consecutively. The mean baseline flow was 2.4±0.2 µl/min. L-Arginine did not alter flow (–1.9±1.6%), and a subsequent perfusion with L-NAME did not change the flow (–1.0±1.9%). After sodium nitroprusside, flow increased in all of the 16 experiments. The average increase was 10.2±2.2% (P<0.001). In Fig. 2, an example of a recording is shown.

L-Arginine and L-NAME did not alter baseline cGMP levels in the perfusate (L-arginine 0.90, 95% confidence interval 0.24–3.40; L-NAME 0.84, 95% confidence in-

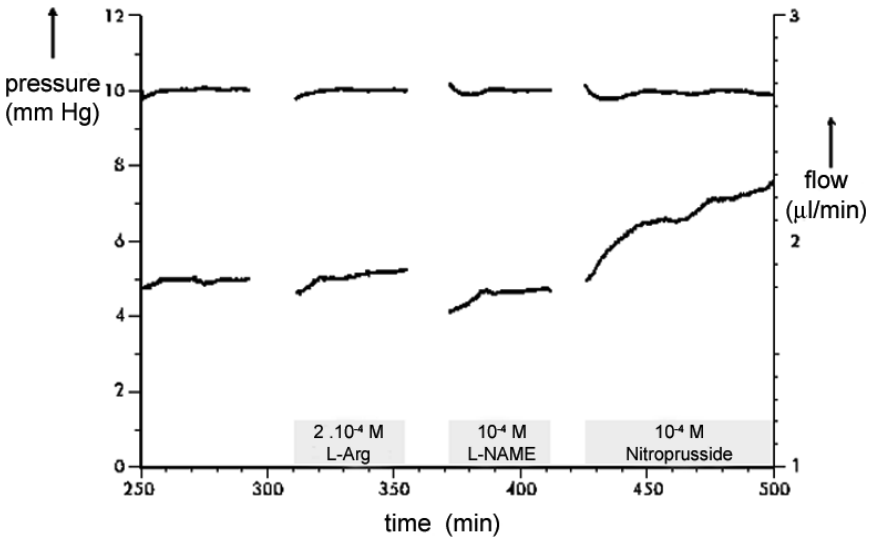

Fig. 2 Example of the recording of pressure and flow rate in response to perfusion with 2×10^{-4} M L-arginine, 10^{-4} M L-NAME and 10^{-4} M sodium nitroprusside

terval 0.25–2.86). Nitroprusside increased cGMP levels (147, 95% confidence interval 28–751).

Control series

The mean age of the 11 donors in the third, placebo, group was 69 years, with a range from 48 to 85 years. The mean enucleation time was 7±3 h (± SD) and the mean post-mortem time until culture was 29±12 h. The baseline flow was 1.6±0.2 µl/min (mean ± SEM) and was not altered significantly by the first, second and third administration of placebo. Mean changes were 0.4±3.5%, –0.1±3.2% and 3.8±4.3%, respectively. In several anterior segments, the LDH levels were measured as a control for cell death during perfusion. The LDH levels did not increase after administration of high concentrations of L-arginine, L-NAME or sodium nitroprusside (data not shown). Since LDH levels did not differ much among those eyes, we may assume that all eyes had the same vitality and that the drugs did not alter the vitality of the anterior segments.

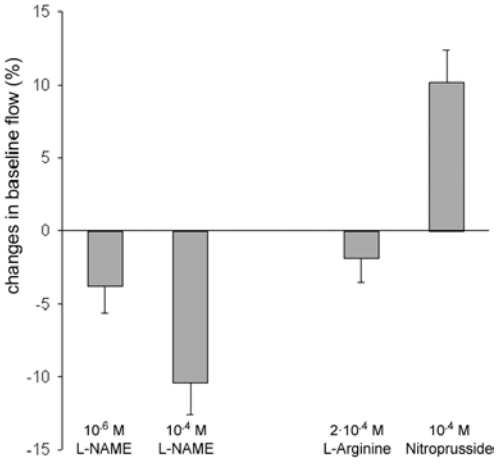

Fig. 3 Mean change from baseline flow values after application of the different drugs in the two experiments. Change is expressed as a percentage ± SEM

Discussion

The purpose of this study was to investigate the possible role of NO in modulating aqueous humour outflow through the human trabecular meshwork. The results of the experiments using the anterior segment perfusion system show that the total modulation range of the flow rate in response to decreasing and increasing the NO levels is 20%. This result indicates that the NO/guanylate cyclase pathway plays a role in the modulation of flow through the trabecular meshwork. Our findings are summarised in Fig. 3.

The results of the first series show that L-NAME statistically significantly inhibits the outflow. L-NAME is an inhibitor of NOS [8], and it may be concluded that inhibition of endogenous NOS activity causes a significant reduction of flow. The reduction of the flow was accompanied by a reduction of cGMP levels measured in the perfusate. These observations indicate that there is a basal NOS activity under the conditions of perfusion culture, resulting in a basal NO production leading to a positive modulation of flow rate of 10%. The activity of the two constitutive forms of NOS (eNOS and bNOS) present in trabecular meshwork cells, as described by Nathanson and McKee [18], may underlie the basal NO production. That the effect of L-NAME on the flow is

relatively modest may possibly be caused by a washout of L-arginine from the tissue, thus limiting the NO production. This hypothesis was tested in the second series, but no significant increase in flow occurred after adding L-arginine to the medium. From this, it is concluded that in the anterior segment perfusion model, a lack of L-arginine is not responsible for the small effect of L-NAME on flow rate. This conclusion is consistent with the high affinity of the enzyme NOS for L-arginine (K_m ~ 2 μM) [4], indicating that a rate-limiting effect of L-arginine levels on the reaction was not likely. After preloading the tissue with L-arginine, subsequent perfusion with L-NAME did not result in a decreased flow. Since L-NAME is a N^{ω}-monosubstituted L-arginine analogue, it competitively binds NOS with L-arginine. The high preload with L-arginine may, therefore, prevent the inhibitory effect by L-NAME on NOS. Application of sodium nitroprusside, a NO donor, bypassing the activity of endogenous NOS, boosted the NO levels to maximum values, and resulted in a statistically significant *increase* in flow rate of 10%. The measured cGMP levels in the perfusate in the second series of experiments also support the flow-data with no change after perfusion with L-arginine or L-NAME, but significantly increased in response to nitroprusside application.

It has been established that intraocular or topical application of NO donors lowered IOP in rabbits and monkeys [2, 11, 16, 19, 22], but whether the underlying mechanism is an enhancement of outflow has not been shown. In the living primate eye, the nitrovasodilators nitroglycerine and hydralazine increased outflow with 92% and 28%, respectively. These values are much higher than those we have encountered, however, the results with nitro-glycerine were obtained only after intracameral bolus injection with 10^{-3} M, and lower doses of nitro-glycerine had no significant effect on outflow in that study [19].

The detection of only relatively small effects in the flow rate demanded a critical evaluation of the sensitivity of our perfusion system. The analysis of the changes in flow rate as a result of the change of perfusion medium 1 h before drug was added, and the results of the control series, showed that the changes in flow rate were under these conditions on average below 4%, and not significantly different. Therefore, changes in the range of 10% are well within the detection range of our system. It also implies that in our hands a 4 h period was enough to stabilise the outflow, although in constant flow studies a stabilisation period of 24 h seems necessary.

Although the aim of our experiments was not to determine the effector mechanism of the NO-mediated regulation of outflow, we may speculate that a relaxation of myo-fibroblast-like cells in the trabecular meshwork is involved [13]. The finding that isolated trabecular meshwork strips relax after application of NO-donors, including 10^{-4} M sodium nitroprusside, is in line with this view [23]. The relaxant effect was more pronounced when the strips were precontracted with carbachol [23] In our study, no such precontraction of the meshwork was applied, which may explain why the changes we observed were relatively moderate. Wiederholt et al. showed that nitroprusside has a relaxant effect on ciliary muscle strips as well [23]. In the intact eye, the ciliary muscle exerts a force on the meshwork enlarging the intratrabecular spaces thereby facilitating outflow. Therefore, the NO-mediated relaxation of ciliary muscle and meshwork will have an opposite effect on outflow [24]. However, NO-donors applied to the intact eye, achieve a significant decrease of IOP and it seems, therefore, that the effect of NO on the trabecular meshwork prevails [2, 11, 12, 16, 19, 22, 23].

Finally, the influence of a neural component on outflow has been speculated on. The presence of bNOS [17] is an indication in that direction and, moreover, Selbach et al. demonstrated in a recent study that afferent nerve fibres contact elastic fibres in the trabecular meshwork in the cynomolgus monkey eye [20]. Furthermore, they found abundant efferent nerve fibres that stained with nicotinamide adenine dinucleotide phosphate-diaphorase (NADPHd) that colocalises with NOS reaction. These nitrergic nerve terminals were most numerous in the cribriform layer and scleral spur. These results suggest a nervous regulation of outflow facility guided by the tonus in the elastic fibres. Unfortunately, an effect of nervous regulation of flow via afferent fibres is not assessable in our model.

Further studies should be done to investigate the role of NO in the trabecular meshwork and the CM in more detail, and to assess the contribution of the neuronal regulation of the outflow.

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