EFFECTS OF NITRIC OXIDE DONORS AND CYCLIC GMP ON INTRAOCULAR PRESSURE AND AQUEOUS HUMOR DYNAMICS

Hanna Kotikoski

Institute of Biomedicine
Pharmacology
University of Helsinki

University of Tampere
Medical School

Tampere University Hospital
Department of Ophthalmology

Academic Dissertation

To be presented, with the permission of the Medical Faculty of the University of Helsinki, for public examination in Lecture Hall 2, Biomedicum Helsinki, University of Helsinki, Haartmaninkatu 8, on 20 th September 2003, at 12 o'clock noon.

Helsinki 2003

EFFECTS OF NITRIC OXIDE DONORS AND CYCLIC GMP ON INTRAOCULAR PRESSURE AND AQUEOUS HUMOR DYNAMICS

Hanna Kotikoski

Institute of Biomedicine
Pharmacology
University of Helsinki

University of Tampere
Medical School

Tampere University Hospital
Department of Ophthalmology

Academic Dissertation

Supervisors: Professor Heikki Vapaatalo, MD
Institute of Biomedicine
Pharmacology
University of Helsinki
Helsinki, Finland

Professor Eeva Moilanen, MD
Medical School
Immunopharmacological Research Group
University of Tampere and Tampere University Hospital
Tampere, Finland

Reviewers: Professor Leila Laatikainen, MD
Department of Ophthalmology
Helsinki University Hospital
Helsinki, Finland

Docent Pauli Vuorinen, PhD
Chief Chemist
Centre for Laboratory Medicine
Tampere University Hospital
Tampere, Finland

Opponent: Professor Johan Stjernschantz, MD
Pharmacology and Drug Development
Unit of Pharmacology
Department of Neuroscience
University of Uppsala
Uppsala, Sweden

ISBN 952-91-6230-8 (paperback)
ISBN 952-10-1311-7 (PDF)
Yliopistopaino
Helsinki 2003

To my son

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-V):

I **Kotikoski H**, Alajuuma P, Moilanen E, Salmenperä P, Oksala O, Laippala P and Vapaatalo H. Comparison of nitric oxide donors in lowering intraocular pressure in rabbits: role of cyclic GMP. J Ocul Pharmacol Ther 2002;18:11-23.

II **Kotikoski H**, Vapaatalo H and Oksala O. Nitric oxide and cyclic GMP enhance aqueous humor outflow facility in rabbits. Curr Eye Res 2003;26:119-123.*

III **Kotikoski H**, Oksala O, Vapaatalo H and Aine E. Aqueous humor flow after single oral dose of isosorbide-5-mononitrate in healthy volunteers. Acta Ophthalmol Scand 2003;81:355-360.

IV **Kotikoski H**, Kankuri E and Vapaatalo H. Incubation of porcine iris-ciliary bodies to study the mechanisms by which nitric oxide donors lower intraocular pressure. Med Sci Monit 2003;9:BR1-7.

V **Kotikoski H**, Moilanen E, Vapaatalo H and Aine E. Biochemical markers of the L-arginine-nitric oxide pathway in the aqueous humor in glaucoma patients. Acta Ophthalmol Scand 2002;80:191-195.

ABBREVIATIONS

ACE	Angiotensin-converting enzyme
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
AUC	Area under curve, time/response curve
CO	Carbon monoxide
Cyclic GMP	Cyclic guanosine 3',5'-monophosphate
DMSO	Dimethylsulfoxide
GC	Guanylate cyclase
GSNO	S-nitrosoglutathione
GTP	Guanosine 5'-triphosphate
IOP	Intraocular pressure
ISMN	Isosorbide-5-mononitrate
L-NAME	N^G-nitro-L-arginine methyl ester
L-NIO	N-iminoethyl-L-ornithine
L-NMMA	N^G-monomethyl-L-arginine
L-NNA	N^G-nitro-L-arginine
NADPH	Nicotinamide adenine dinucleotide phosphate
Na^+/K^+ ATPase	Sodium-potassium adenosine triphosphatase
NANC	Nonadrenergic-noncholinergic
NO	Nitric oxide
NO_x	Nitrate + nitrite
NOR-3	(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-2-hexenamide
NOS	Nitric oxide synthase
cNOS	Constitutive nitric oxide synthase
eNOS	Endothelial nitric oxide synthase
iNOS	Inducible nitric oxide synthase
nNOS	Neuronal nitric oxide synthase
NZW	New Zealand White rabbit
NMDA	N-methyl-D-aspartate
ODQ	1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one
$ONOO^-$	Peroxynitrite

PAGE	Polyacrylamide gel electrophoresis
PKG	Protein kinase G
POAG	Primary open-angle glaucoma
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SIN-1	3-morpholino-sydnonimine
SNAP	S-nitroso-N-acetylpenicillamine
SNOG	S-nitrosothiol
SNP	Sodium nitroprusside
Spermine NONOate	N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine
YC-1	3-(5'-hydroxymethyl-2'furyl)-1-benzylindazole

ABSTRACT

The effects of various nitric oxide (NO) donors and cyclic GMP on intraocular pressure (IOP) were investigated in rabbits. Further, the mechanisms underlying these effects on aqueous humor dynamics were clarified by measuring aqueous humor outflow facility in rabbits and aqueous humor flow in healthy human volunteers. A novel tissue incubation method for screening potential NO donors and guanylate cyclase (GC) activators was evaluated using porcine iris-ciliary body. The possible clinical relevance of NO in aqueous humor dynamics in glaucoma patients was studied.

Topically or intravitreally administered compounds affecting the NO-cyclic GMP pathway lowered IOP in ocular normotensive rabbits. Zaprinast, a cyclic nucleotide phosphodiesterase (PDE 5/6) inhibitor, in combination with sodium nitroprusside (SNP), a NO-releasing reference compound, prolonged the response, suggesting the central role of cyclic GMP in IOP reduction. All NO donors and GC-activating compounds elevated the nitrite + nitrate (NO_x) concentration in aqueous humor, but the nitrite level was increased only after SNP administration. Cyclic GMP concentrations in GC activator (atriopeptin III)- and cyclic GMP analog (8-Br-cGMP)-treated eyes were higher than in the control eyes.

Since NO donors and a cyclic GMP (8-Br-cGMP) analog lowered IOP, it was of importance to clarify whether they influence aqueous humor outflow facility or aqueous humor production. Intracamerally administered SNP, nitrosocaptopril and 8-Br-cGMP enhanced aqueous humor outflow facility in anesthetized rabbits. ACE inhibition was not the mechanism of nitrosocaptopril since plain captopril had no effect on outflow facility. Aqueous humor flow was not significantly changed after a single oral dose of the NO donor isosorbide-5-mononitrate, as compared to placebo in healthy human subjects. Since IOP after placebo and isosorbide-5-mononitrate intake were at the same level, the rate of aqueous humor flow can be regarded as an indicator of the formation of aqueous humor. Since isosorbide-5-mononitrate, as a model of systemic NO donors, did not influence the rate of aqueous humor flow, enhanced aqueous humor outflow facility mainly explains the IOP-lowering effect of NO-releasing compounds.

In a tissue incubation model, various NO donors and GC activators increased cyclic GMP production in the porcine iris-ciliary body. ODQ, an inhibitor of GC, totally abolished the production of cyclic GMP after the administration of NO donors SNP and nitrosocaptopril. Captopril had no effect on cyclic GMP production, while the GC activators atriopeptin III and YC-1 increased the production dose-dependently.

Glaucoma patients had slightly higher concentrations of NO_x, nitrite and cyclic GMP in aqueous humor than the matched control patients, but the difference was not statistically significant. However, glaucoma medication may have masked real changes in the variables, which are possibly unbalanced in untreated patients.

In conclusion, various compounds affecting the NO-cyclic GMP pathway lowered IOP and enhanced aqueous humor outflow facility in ocular normotensive rabbits. A single oral dose of the NO donor isosorbide-5-mononitrate had no effect on aqueous humor flow and IOP in healthy volunteers, suggesting that the NO-cyclic GMP pathway has no significant effect on aqueous humor production. A contribution of cyclic GMP in the physiological regulation of IOP was supported by the findings in the porcine iris-ciliary body incubation method. A non-toxic NO-donating or GC-activating compound would represent a potential new mode of antiglaucomatous treatment.

1 INTRODUCTION

Glaucoma is among the leading causes of irreversible blindness in the world. It is a chronic progressive optic neuropathy which if not treated leads to visual impairment and even blindness. The pathomechanism of the disease is taken to be multifactorial. Elevated intraocular pressure (IOP) plays a significant role as a risk factor but is not a necessary component of glaucoma. Lowering of IOP is thought to be beneficial in slowing down glaucomatous damage to the optic nerve and visual field (Leske et al. 2003). Accordingly, all current pharmacological treatments of glaucoma are designed to reduce IOP and maintain it at levels presumed to prevent deterioration of the visual field and alterations in the optic nerve. Glaucoma drugs lower IOP by reducing the production of aqueous humor and/or by increasing the outflow of aqueous humor through trabecular or uveoscleral routes.

Nitroglycerin has been used for over a century in the treatment of cardiac diseases, but it was not until 1987 that the vasodilating endothelium-derived relaxing factor was identified as nitric oxide (NO) and nitroglycerin was shown to release NO (Ignarro et al. 1987, Palmer et al. 1987). NO is a gaseous messenger molecule which plays an important role in diverse physiological and pathophysiological processes in the body (for review, see Moncada and Higgs 1995, Moncada 1997, Ignarro et al. 1999). In the eye, NO is involved in a wide range of physiological events such as regulation of aqueous humor dynamics, neuronal visual processing and ocular hemodynamics, but it has also been related to the pathogenesis of eye diseases, including glaucoma, retinopathy, myopia and cataract (for review, see Becquet et al. 1997, Chiou 2001). There is good evidence to warrant the hypothesis that NO-releasing compounds and cyclic GMP, the second messenger of NO, lower IOP in animals. However, there are at present no antiglaucomatous drugs on the market whose effects are based on the nitric oxide-cyclic GMP pathway.

The present study was designed to clarify the roles of NO and cyclic GMP in the regulation of IOP. The IOP-lowering effect of NO donors and cyclic GMP analog found at the beginning of the project raised further questions regarding their mechanisms *in vivo* and *in vitro* and whether there are alterations in NO levels in glaucoma patients.

2 REVIEW OF THE LITERATURE

2.1 MODULATION OF INTRAOCULAR PRESSURE

2.1.1 Aqueous humor dynamics and intraocular pressure

IOP is maintained by a homeostatic balance of production and outflow of aqueous humor. When the eye is in a steady state, i.e. IOP remains stable, aqueous humor formation and drainage are equal.

2.1.1.1 Formation of aqueous humor

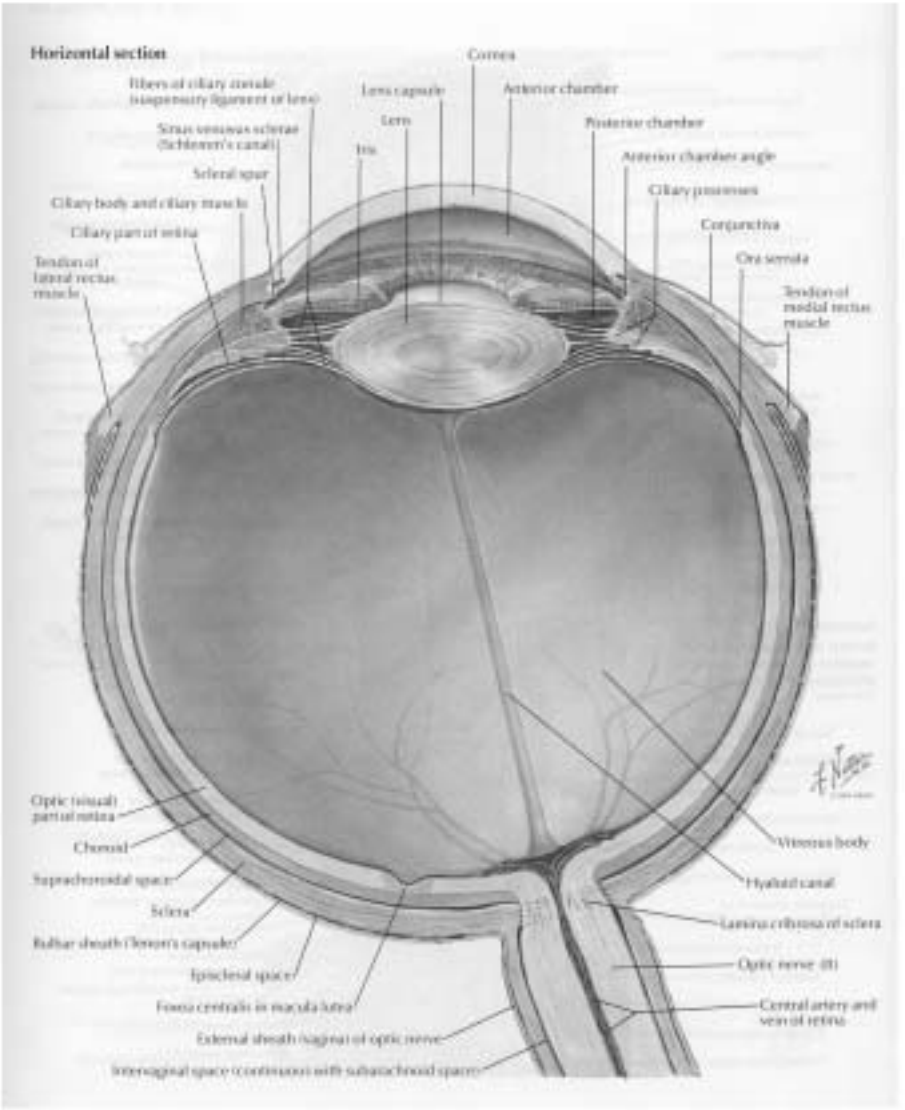
Aqueous humor is produced by the ciliary processes at approximately 2 - 3 µl/min and the entire volume of aqueous humor is replaced every 90-100 minutes (turnover) (see Brubaker 1994; for review, see Freddo 2001). There are three essential steps in the formation of aqueous humor. First, the blood circulation must be sufficient in the ciliary processes. Second, a portion of the plasma perfusing processes must be filtered into tissue spaces. Third, a portion of the filtrate must pass through the double-layered epithelium to enter the posterior chamber (see Brubaker 1994). The production of aqueous humor is the result of two primary driving forces: hydrostatic (pressure in liquid due to outside pressure) and oncotic pressures (pressure due to high-molecular substances such as proteins) between the posterior chamber and the ciliary process vasculature and stroma. These determine the net movement of fluid, electrolytes and small molecules across the ciliary body. Vascular tone, IOP and ion transport in the ciliary body epithelium combined with the blood-aqueous barrier further regulate the production of aqueous humor (see Kardon and Weingeist 1994, Kaufman 1994).

The ciliary processes produce aqueous humor by active secretion of solutes into the posterior chamber. The membrane-bound enzyme complex sodium-potassium adenosine triphosphatase (Na^+/K^+ ATPase) constitutes an energy-dependent active transport system which transfers Na^+ into the posterior chamber, resulting in water movement from the stromal pool into the posterior chamber (see Caprioli 1992, Kaufman 1994). Under normal conditions this active transport covers 80 – 90% of total aqueous formation and it is essentially pressure-insensitive near the physiologic IOP and operates at a constant rate

(see Kaufman 1994). Active transport of Cl^- and HCO^-_3 (formed in a reaction sequence catalyzed by carbonic anhydrase) may also occur to a lesser extent (see Caprioli 1992). In addition to active secretion, aqueous humor is produced by pressure-sensitive ultrafiltration of fluid from plasma into the posterior chamber. This ultrafiltration does not, however, contribute significantly to the formation of aqueous humor (see Caprioli 1992, Kaufman 1994). Aqueous humor, besides generating IOP, provides nutrition for the avascular ocular tissues which it bathes. It contains electrolytes, glucose, lactate, oxygen, ascorbate, amino acids, proteins, lipids and other substances of minor significance (see Caprioli 1992).

2.1.1.2 Outflow of aqueous humor

Aqueous humor passes from the posterior chamber through the pupil into the anterior chamber driven by a convective flow resulting from the temperature difference between the iris and the cornea (for review, see Freddo 2001) (Figure 1). Five routes have been suggested through which aqueous humor may exit the eye: 1) the trabecular pathway, 2) the uveoscleral pathway, 3) the corneal endothelial pathway, 4) the iris vessels, 5) the anterior vitreous. The trabecular and uveoscleral pathways are the two measurable ways by which aqueous humor escapes from the eye. The trabecular (or conventional) pathway is the principal route, draining over 90% of the aqueous humor in the normal eye (see Kardon and Weingeist 1994). The rate of uveoscleral drainage differs between species; in man this route accounts for about 10% of total outflow (Weinreb 2000). Direct measurements in human eyes have suggested that the uveoscleral pathway drains less than 15% of aqueous humor. However, uveoscleral outflow may alter in different age groups (for review, see Nilsson 1997). In the intact eye, the balance between the contractility of the ciliary muscle and the trabecular meshwork determines the total aqueous humor outflow. Contraction of the ciliary muscle alters the geometry of the trabecular meshwork, which in turn increases the trabecular outflow and finally reduces IOP. On the other hand, relaxation of the ciliary muscle leads to increased uveoscleral outflow (for review, see Nilsson 1997; see Wiederholt 2000).



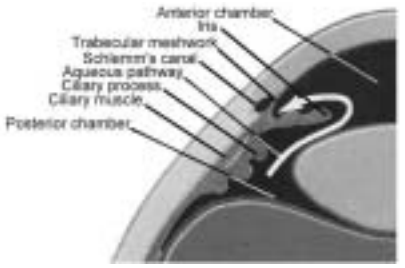


Figure 1. Aqueous humor pathway (Netter FH: Atlas of Human Anatomy, 1989; p. 82, Basle, Ciba-Geigy Limited).

Flow through the trabecular system is dependent on pressure, and the rate of flow is determined by the hydrostatic pressure and the resistance to flow (see Hart 1992). The trabecular pathway consists of the trabecular meshwork, pericanalicular connective tissue, the canal of Schlemm and collector channels leading into the scleral vessels and venous circulation. The trabecular meshwork is a multilayered net-like structure located in the periphery of the anterior chamber angle. Wastes in the aqueous humor are filtered by this meshwork as they pass through it. The endothelium of the meshwork possesses phagocytic capability which is important in maintaining the capacity of the entire filtration area, especially in conditions involving an abnormal accumulation of materials which may obstruct the outflow of aqueous humor (e.g. pigmentary dispersion syndrome, pseudoexfoliation syndrome) (see Kardon and Weingeist 1994). The trabecular meshwork comprises the principal resistance to aqueous outflow. Resistance to flow rises gradually through progressively smaller pores in the trabecular meshwork. It is thought that the juxtacanalicular tissue, the connective tissue separating corneoscleral portions of the meshwork from Schlemm's canal, and the inner wall of Schlemm's canal are the sites of highest resistance, thus having a role in the pathogenesis of the ocular hypertension characteristic of primary open-angle glaucoma (Ethier 2002, see Hart 1992; for review, see Bill 1993). Once aqueous humor has passed through the trabecular meshwork, the pericanalicular zone and Schlemm's canal, it has free access to the collector channels and venous plexuses (the deep intrascleral, mid-intrascleral and episcleral plexuses) (see Kardon and Weingeist 1994).

Uveoscleral outflow of aqueous humor is normally independent of IOP and the rate of flow appears to be fairly constant. If the IOP is stabilized at levels above the normal, the outflow through the uveoscleral routes tends to increase, but much less than that through Schlemm's canal. If the IOP is reduced from the normal level to that of the episcleral venous pressure, the flow through the uveoscleral pathway is very little affected, while drainage via the trabecular pathway ceases (Bill 1967). Aqueous humor slowly seeps through the base of the iris and extracellular spaces in the ciliary muscle into the suprachoroidal space and anterior choroid, where it leaks through the scleral wall into the surrounding periocular orbital tissues (Weinreb 2000, see Hart 1992, Brubaker 1994). The driving force for the uveoscleral outflow is the difference in pressure between the anterior chamber and the suprachoroidal space (for review, see Nilsson 1997). The uveoscleral pathway is amenable to direct pharmacological influence. Contraction of the ciliary muscle

fibers, as after the administration of pilocarpine, leads to compression of the extracellular spaces among the muscle fibers, and the uveoscleral outflow decreases. On the other hand, if there is relaxation of the muscle fibers, as after atropine administration, the spaces are expanded and the uveoscleral outflow is increased (Weinreb 2000; for review, see Nilsson 1997).

2.1.2 Autonomic nerve system and intraocular pressure

The ocular structures which regulate IOP have cholinergic as well as adrenergic receptors. The ciliary body contains nerve terminals throughout its epithelium, muscle and vasculature. The ciliary epithelium has α_2- and β_2-adrenergic receptors. Stimulation of the α-receptors or inhibition of the β-adrenergic receptors leads to reduced aqueous humor formation (Prünte and Markstein 2000). The ciliary muscle has a high density of cholinergic nerve terminals, primarily deriving from the ciliary ganglion (Ruskell and Griffith 1979). Stimulation of these muscarinic receptors (M_3-subtype) results in contraction of the ciliary muscle and further alteration in the trabecular meshwork configuration, leading to reduced resistance to aqueous humor outflow. The trabecular meshwork contains both adrenergic and cholinergic nerve endings, about a third of them being adrenergic (Nomura and Smelser 1974). Adrenergic agonists such as adrenaline increase outflow facility through direct action on the trabecular meshwork and via the uveoscleral pathway. In the human trabecular meshwork, β-adrenergic receptors are mainly of the β_2-subtype. However, it is an open question whether the effects of adrenaline on outflow are mediated via α- or β-adrenergic receptors (Wiederholt 2000). It is possible that cholinergic stimulation acts directly on the endothelium of the trabecular meshwork or on the canal of Schlemm and this effect might consist in endothelium-dependent nitric oxide-mediated smooth muscle relaxation (for review, see Vapaatalo 1995). Nonadrenergic-noncholinergic (NANC) nerves are responsible for the relaxation of smooth-muscle cells and thus vasodilation in ocular circulation (Haefliger and Dettmann 1998).

2.1.3 Blood pressure and intraocular pressure

There is evidence of a link between blood pressure level and IOP. Several studies suggest an increased risk of open-angle glaucoma in persons with systemic hypertension (Klein and Klein 1981, Leske and Podgor 1983, Wilson et al. 1987; for review, see Hayreh 1999). On the other hand, patients with normal tension glaucoma evince a high incidence of low

systemic blood pressure (for review, see Hayreh 1999). It has been found that subjects with a systolic blood pressure of 160 mmHg or over are 2.2 times more likely to have IOP over 20 mmHg. Changes in IOP are positively correlated with changes in systolic blood pressure (McLeod et al. 1990). However, low blood pressure levels related to an individual's circadian rhythm can occur simultaneous with high IOPs at night, reducing blood flow to the optic nerve head below critical levels and thus resulting in optic nerve damage (Wax et al. 2002). Even though glaucoma is related to altered blood pressure, it has been found that a sustained decrease in systemic blood pressure of approximately 15 mmHg after a bolus intravenous injection of either hydralazine or prizidilol does not result in ocular hypotension in rabbits (Woodward et al. 1989).

2.2 GLAUCOMA

2.2.1 Definition and pathogenesis

Glaucoma is a multifactorial disease involving progressive optic neuropathy and altered intraocular hemodynamics. The term glaucoma thus refers to a syndrome of many causes rather than to a single disease. There is variation in the way glaucoma is defined in current clinical research (Bathija et al. 1998). The essential pathological process in the condition is progressive loss of axons of ganglion cells, leading to a decreased amount of neural tissue in the optic nerve head. The configuration of the nerve head changes, resulting in enlargement of the disc cup, loss of disc rim, increased pallor, changes in vessels, splinter hemorrhage, peripapillary atrophy and retinal nerve fiber layer defects (for review, see Infeld and O'Shea 1998).

Mechanical and vascular theories for the pathogenesis of glaucomatous optic neuropathy have been presented. According to the mechanical theory, increased IOP damages the *lamina cribrosa* and the neural axons of retinal ganglion cells. The vascular theory assumes that glaucomatous optic neuropathy is a consequence of insufficient blood flow due to either increased IOP or other contributing factors which reduce ocular blood flow (for review, see Flammer et al. 2002). Thus glaucoma can be divided into **IOP-dependent** and **IOP-independent types** (Schulzer et al. 1990). The predisposition to glaucomatous optic neuropathology varies individually. In the IOP-dependent type, an IOP exceeding the tolerance of the healthy eye (usually over 21 mmHg) causes optic disc disorders. First, the

drainage of aqueous humor from the eye becomes impaired, leading to raised IOP. This, together with other less well identified pathogenic factors, damages the optic nerve, resulting in loss of visual field. Aqueous humor drainage may be obstructed due to developmental or degenerative abnormalities in the trabecular outflow pathways or to outflow abnormalities secondary to some other ocular or systemic disease (see Phelps 1994).

Patients with **ocular hypertension** have abnormally high IOP, usually higher than 21 mmHg, but normal optic discs and visual fields. Some of these patients may eventually develop optic nerve damage over the years, but most will only have increased IOP.

Normal-tension glaucoma (previously low-tension glaucoma) is regarded as a clinical entity, defined as a chronic progressive optic neuropathy resulting in typical optic nerve head changes, retinal nerve fiber layer defects, and characteristic visual field defects. In addition, the chamber angle is open and IOP values within statistical normal limits (lower than 22 mmHg) (Lee et al. 1998; for review, see Hoyng and Kitazawa 2002). It has been shown that between one third and one half of patients with glaucoma do not have IOP higher than 21 mmHg (Tielsch et al. 1991). There is evidence that treatment of normal-tension glaucoma by lowering IOP can slow the glaucomatous process. A reduction of at least 30% in IOP is needed to induce a favorable alteration in this disease (for review, see Hoyng and Kitazawa 2002).

2.2.2 Glaucoma subtypes

Classically, glaucoma can be classified into **primary** or **secondary** types according to the etiology. **Primary glaucomas** result from developmental or degenerative abnormalities which are often hereditary and affect the channels of aqueous humor outflow. Reduced aqueous humor outflow facility in primary open-angle glaucoma might be due to change in trabecular endothelial cell density and functional capacity (see Migdal 1994, Phelps 1994). **Secondary glaucomas** involve a variety of ocular disorders, systemic disorders, injuries or toxic medications which primarily damage other ocular tissues and secondarily affect the outflow channels.

Glaucoma can also be classified according to the pathogenic mechanism involved. In **open-angle glaucoma** the chamber angle has its normal configuration and aqueous humor flows through the trabecular meshwork and has access to the outflow channels. In **closed-angle glaucoma** the root of the iris lies against the trabecular meshwork and prevents aqueous humor from entering the meshwork. This state may be partial or complete, intermittent or constant and reversible or permanent (see Phelps 1994). A more detailed classification of glaucoma is shown in Table 1.

Table 1. The classification of glaucoma according to Phelps (1994).

I ADULT GLAUCOMAS

A. Primary open-angle glaucoma (including ocular hypertension and low-tension glaucoma)

B. Primary closed-angle glaucoma
   1. Relative pupillary block
   2. Plateau iris
   3. Malignant glaucoma

C. Secondary glaucomas
   1. Exfoliative glaucoma
   2. Pigmentary glaucoma
   3. Corticosteroid-induced glaucoma
   4. Glaucoma associated with iritis
   5. Glaucoma after trauma
   6. Lens-induced glaucoma
   7. Glaucoma in aphakic eye
   8. Glaucoma secondary to high episcleral venous pressure
   9. Glaucoma associated with intraocular tumor
   10. Neovascular glaucoma
   11. Ghost cell glaucoma
   12. Iridocorneal endothelial syndrome
   13. Posterior polymorphous corneal dystrophy
   14. Angle closure secondary to ciliary swelling

II CHILDHOOD GLAUCOMAS

A. Primary congenital or infantile glaucoma

B. Secondary glaucomas in children
   1. Secondary to or associated with other ocular abnormalities
   2. Secondary to systemic diseases

2.2.3 Prevalence

Glaucoma is the third most prevalent cause of blindness in the world, accounting for over 5 million blind people or 13.5% of the total burden of world blindness (for review, see Infeld and O'Shea 1998, Roodhooft 2002). It is common in Western countries; the prevalence of primary open-angle glaucoma has been estimated in various surveys as 1.1 – 3.0% of Western populations (for review, see Infeld and O'Shea 1998). However, in industrial countries there is a high proportion of undetected cases, possibly 50% in some nations (Grehn 2001). The prevalence increases with age after the age of 40 years, being well below 1% in persons under 65 years, approaching 1% around 70 years and about 3% in persons older than 75 years (for review, see Leske 1983). The prevalence of primary open-angle glaucoma is 4 to 5 times higher in blacks than in whites, whereas primary angle closure glaucoma is diagnosed most often in Asians (Quigley 1996). In Finland about 63 000 patients obtained glaucoma medicine reimbursement in 2001 according to the statistics of the Social Insurance Institute.

2.2.4 Risk factors

Elevation of IOP from the individual normal level is one of the most important risk factors in glaucoma. IOP in the normal population ranges from 10 to 21 mmHg with a mean of about 16 mmHg. The risk of ocular damage and visual loss rises with increasing levels of pressure. The risk of visual field defects in persons with IOP over 21 mmHg is approximately five to six times higher than in persons with lower levels (for review, see Leske 1983). The progression of glaucoma is closely linked to the lowering of IOP after treatment, the risk decreasing by about 10% with each mmHg of IOP reduction (Leske et al. 2003). It should be borne in mind, however, that IOP is influenced by many factors, e.g. age of patient, sex, race, family history, blood pressure, menstrual cycle, season of the year, mental stress, use of alcohol and nonalcoholic liquids and physical exercise, and IOP measurements are influenced by the type of tonometer used, ocular rigidity, squeezing of the lids, position of patient and time of day (for review, see Leske 1983; see Leopold 1984).

Age is one of the well-known risk factors in glaucoma and it plays an important role in the development and progression of glaucomatous optic neuropathy (Leske et al. 1996; for review, see Hayreh 1999). It is known that in later life there are reduced numbers of nerve

fibers and age-related changes in the supporting structures of the optic disc or blood supply. Older persons are thus more susceptible to glaucomatous injury (see Migdal 1994, Greve et al. 1998).

Positive family history and genetic disposition are known to increase the risk of glaucoma (Wilson et al. 1987, Tielsch et al. 1994, Leske et al. 1996, Nemesure et al. 1996, Wolfs et al. 1998; for review, see Leske 1983). In a population-based familial aggregation study in Rotterdam, the lifetime risk of glaucoma was almost 10 times higher in first-degree relatives of glaucoma patients than in siblings and offspring of controls (Wolfs et al. 1998). Maternal history of glaucoma was reported twice as often as paternal history in the Barbados Eye Study (Nemesure et al. 1996). In 1996 and 1997, the first major gene loci associated with an increased risk of primary open-angle glaucoma were identified (Stoilova et al. 1996, Stone et al. 1997) and many “glaucoma genes” have since been mapped (Lichter 2001).

Among black populations, primary open-angle glaucoma appears at an earlier age and with greater severity (Greve et al. 1998), and a more rapid progression of the disease has been observed (for review, see Leske 1983). It has been reported that blacks have larger optic nerve cups than whites (Beck et al. 1985), but it is not known whether these cups are preglaucomatous changes or whether they are simply more susceptible to damage by high IOP. Furthermore, the prevalence of glaucoma-related blindness in blacks is 6.8 to 8 times greater than in whites (Wilson et al. 1987). The highest figures for angle closure glaucoma come from Asia; it is most common among the Chinese, while open-angle glaucoma is more evenly distributed in the world (Quigley 1996). In Japan, at least one in two patients have normal-tension glaucoma (Araie et al. 1994).

The risk of glaucoma is about 2-4-fold in patients with myopia (Mitchell et al. 1997, Grodum et al. 2001). The association between myopia and glaucoma is strong at lower IOP levels, implying that myopia is an important risk factor for normal-tension glaucoma (Grodum et al. 2001). Myopia is also a serious risk factor underlying progression of primary open-angle glaucoma. Patients with a combination of myopia and glaucoma have a higher progression rate and more vision-threatening visual field defects (Wilson et al. 1987, Greve et al. 1998).

Vascular factors play a role in the pathogenesis of primary open-angle glaucoma, particularly normal-tension glaucoma, and they can be divided into systemic and local risk factors. Both high and low systemic blood pressures have been regarded as risk factors for glaucoma (Wilson et al. 1987, Kaiser et al. 1993, Tielsch et al. 1995, Leske et al. 1996, Bonomi et al. 2000; for review, see Hayreh 1999). It has been shown that the effect of blood pressure on glaucoma is modified by age, the association being stronger among older patients. It has been hypothesized that increased blood pressure in the early course of systemic hypertension might protect the ganglion cells and their axons from damage resulting in increased blood flow or greater hydrostatic resistance to closure of small vessels. Subsequently, when damage to the small vessels has occurred and resistance to flow increased, a positive association between hypertension and optic nerve damage can be detected (Tielsch et al. 1995). Low perfusion pressure (blood pressure – IOP) is strongly associated with an increased prevalence of primary open-angle glaucoma (Tielsch et al. 1995, Bonomi et al. 2000). Low systemic blood pressure may reduce local perfusion, particularly in the presence of IOP elevation or poor autoregulation (Graham and Drance 1999). Analysis of blood pressure indicates that systemic hypotension is a far more important risk factor for glaucomatous damage than systemic hypertension (Tielsch et al. 1995). Nocturnal arterial hypotension is an important risk factor for glaucoma, especially among hypertensive patients taking oral hypotensive medication (for review, see Hayreh 1999), and patients with greater blood pressure dips are more likely to evince progressive visual field defects (Graham and Drance 1999). The major cause of reduced ocular blood flow is vascular dysregulation, and blood flow may also be reduced in glaucoma patients in other parts of the body. Vascular dysregulation leads to low perfusion pressure and insufficient autoregulation, and further unstable ocular perfusion, ischemia and reperfusion damage (for review, see Flammer et al. 2002). Other cardiovascular diseases such as coronary artery disease, cardiac arrhythmias, conduction abnormalities and congestive heart failure are associated with glaucoma (Peräsalo et al. 1992; for review, see Hayreh 1999). The prevalence of peripheral vasospasm is increased in glaucomatous optic neuropathy, especially normal-tension glaucoma (for review, see Gasser and Flammer 1991, Flammer et al. 1999, Gasser 1999). Vascular diseases such as migraine (Wang et al. 1997) and diabetes (Wilson et al. 1987, Klein et al. 1994, Mitchell et al. 1997) have been suggested to be associated with glaucoma. Local vascular risk factors, including hemorrhages of the disc, peripapillary atrophy and choroidal sclerosis, lead to progression of glaucomatous disease (Araie et al. 1994, Hendrickx et al. 1994).

Pseudoexfoliation syndrome, i.e. the accumulation of fibrillar extracellular material in ocular tissues, has been found to be associated with increased IOP and glaucoma (for review, see Damji et al. 1998). In eyes with pseudoexfoliation IOP usually rises to a high level over a short time and fluctuations in IOP are sometimes marked (Skuta 1994, see Flammer 2001). Subjects with pseudoexfoliation have a 5- to 10-fold risk of glaucoma and this is independent of other known glaucoma risk factors (Ekström 1993, Ringvold et al. 1991, Hirvelä et al. 1995, Mitchell et al. 1997, Ritch 2001). It has been proposed that pseudoexfoliation is genetically inherited (Allingham et al. 2001; for review, see Damji et al. 1998). The risk of developing glaucoma is cumulative over time and in eyes with pseudoexfoliation it may develop earlier, more frequently and more severely in men (Ritch 2001). Exfoliation accelerates the progression of glaucoma (Ritch 2001, Leske et al. 2003). A combination of pseudoexfoliation and elevated IOP increases the risk of chronic open-angle glaucoma 67-fold as compared with a no-exposure group (Ekström 1993).

2.2.5 Pharmacotherapy in glaucoma

The ideal antiglaucomatous drug would be a substance which lowers IOP, facilitates blood flow to the retina and prevents ischemic neuronal cell death. However, the lowering of IOP is currently the only proven approach in reducing the risk of glaucomatous damage (Leske et al. 2003) and thus remains the primary goal of therapy (Soltau and Zimmermann 2002). The level of IOP is a function of the rate of aqueous humor production (inflow) and resistance in the outflow channels (outflow). Aqueous humor is produced by the epithelium of the ciliary processes. IOP can be reduced either by inhibiting the production of aqueous humor or by increasing aqueous outflow via interaction with receptors within the ciliary body or the outflow pathways. The glaucoma medications currently used are presented in Table 2.

Table 2. Glaucoma medications (Coleman and Brigatti 2001, Soltau and Zimmermann 2002).

Class of drug	Generic names	Mechanisms	Decrease in IOP	Duration of action	Main adverse effects
Topical cholinergic agonists	Pilocarpine, carbachol, ecothiophate iodide	aqueous outflow ↑	20-30%	6 h to 1 week	Bronchial secretion ↑, nausea, vomiting, diarrhea, myopia ↑, eye or brow pain, vision ↓
Topical β adrenergic antagonists	Timolol, levobunolol, carteolol, metipranolol, betaxolol	aqueous production ↓	15-35%	12-36 h	Congestive heart failure, bronchospasm, bradycardia, depression, confusion, impotence, worsening of myastenia gravis, dry eye syndrome
Topical α_1, β_2 adrenergic agonists	Adrenalin, dipivefrin, apraclonidine, brimonidine	aqueous outflow ↑ and aqueous production ↓	20-30%	8-12 h	Blood pressure ↑, tachyarrhythmias, bronchospasm, tremor, headache, anxiety, fatigue, conjunctival hyperemia, macular edema
Topical (T) or oral (O) inhibitors of carbonic anhydrase	T: dorzolamide, brinzolamide O: acetazolamide, methazolamide	aqueous production ↓	20-30%	6-12 h	Malaise, anorexia, depression, paresthesias, metabolic acidosis, renal stones, blood dyscrasias, allergic reactions, bitter or sour taste
Topical prostaglandin analogs	Latanoprost, travoprost, bimatoprost, unoprostone	uveoscleral aqueous outflow ↑	up to 50%	24-40 h	Iris pigmentation ↑, hypertrichosis, pigmentation of lashes ↑, conjunctival hyperemia, iritis, cystoid macular edema

2.3 NITRIC OXIDE AND CYCLIC GMP

Results of a study conducted over 20 years ago showed that an intact endothelium was required for the acetylcholine-induced relaxation of vascular smooth muscle, and the authors described the endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki 1980). Nitric oxide was discovered in 1987 to be a relaxant responsible for endothelium-dependent relaxation of blood vessels following treatment with acetylcholine (Ignarro et al. 1987, Palmer et al. 1987). In 1988, the amino acid L-arginine was found to be the precursor of NO synthesis by vascular endothelial cells (Palmer et al. 1988). NO is a gaseous, colorless, highly reactive short-lived signaling molecule which regulates various physiological and pathophysiological processes in the body. It is formed in various cell types in the body, including vascular endothelium, macrophages, central nervous system, NANC nerves, cerebellum and other tissues. NO is a small lipophilic molecule which diffuses freely through biological membranes and rapidly reaches the intracellular compartments of nearby cells, leading to the regulation of various cellular processes (for review, see Ignarro 1990, Moilanen and Vapaatalo 1995, Ignarro 2002).

2.3.1 Biosynthesis of nitric oxide

NO is synthesized from L-arginine by three NO synthase (NOS) isoforms: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) NOS (Figure 2). In addition to L-arginine, this reaction catalyzed by NOS requires molecular oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), and other cofactors such as tetrahydrobiopterin (BH_4), flavin adenine dinucleotide, flavin mononucleotide and heme (iron protoporphyrin IX) to produce NO and citrulline (for review, see Bredt and Snyder 1994, Farrell and Blake 1996, Stuehr 1997, Marletta et al. 1998, Alderton et al. 2001). Constitutively expressed e NOS and nNOS are Ca^{2+}/calmodulin-dependent, and they were first identified in vascular endothelial cells (eNOS) and certain central and peripheral nonadrenergic-noncholinergic neurons (NANC nerves) (for review, see Änggård 1994, Marletta et al. 1998). These enzymes release NO for short periods in response to receptor mediated Ca^{2+} increase and they are also regulated by shear-induced stress in the vasculature. NO released by eNOS and nNOS acts as a transduction mechanism in several physiological responses subserving e.g. vasodilation. The third enzyme, iNOS, is induced after activation of macrophages, endothelial cells and a number of other cells by bacterial products or

proinflammatory cytokines, and once expressed produces large amounts of NO for long periods. High levels of NO have a cytotoxic role in invading micro-organisms and tumor cells, and might participate in other pathological processes such as tissue damage and pathological vasodilation. Inducible NOS is Ca^{2+}-independent and it requires cofactors such as BH_4. The induction of iNOS can be inhibited by e.g. glucocorticoids (Korhonen et al. 2002; for review, see Moncada et al. 1991, Moncada 1992, Änggård 1994, Bredt and Snyder 1994, Farrell and Blake 1996, Marletta et al. 1998). NO may also be formed to some extent in a NOS-independent pathway involving chemical reduction of inorganic nitrite/nitrate to NO in acidic conditions (for review, see Weitzberg and Lundberg 1998).

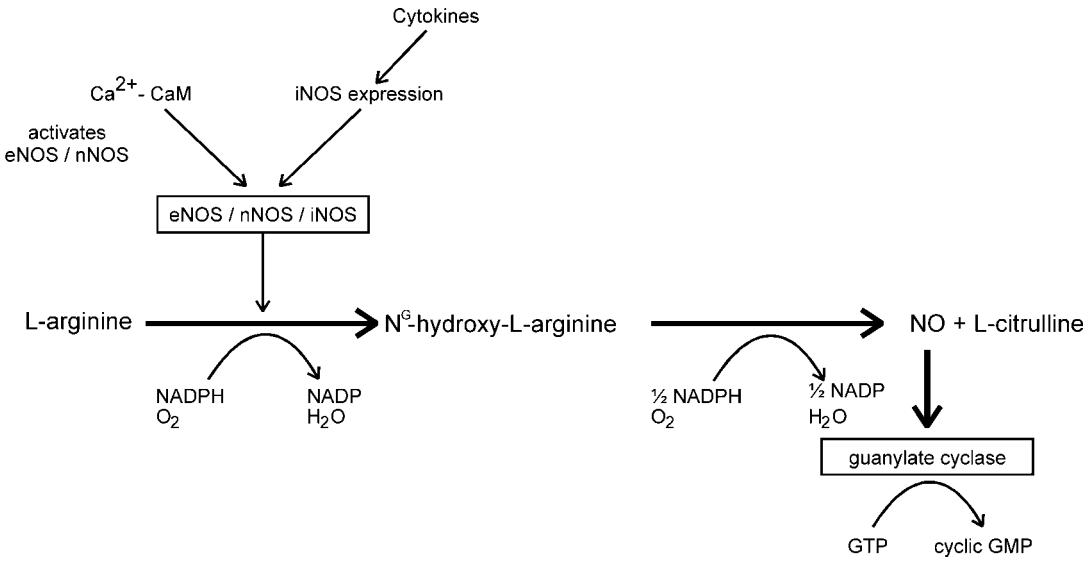


Figure 2. Biosynthesis of NO.
CaM, calmodulin; cyclic GMP, cyclic guanosine 3',5'-monophosphate; GTP, guanosine triphosphate; iNOS inducible nitric oxide synthase; NADP, nicotinamine adenine dinucleotide; NADPH, nicotinamine adenine dinucleotide phosphate; NO, nitric oxide

The synthesis of NO from L-arginine can be inhibited by analogs of L-arginine, i.e. NOS inhibitors, which act by competing with L-arginine at the active NOS sites such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), N^G-nitro-L-arginine methyl esther (L-NAME) and N-iminoethyl-L-ornithine (L-NIO) (for review, see Änggård 1994, Alderton et al. 2001, Vallance and Leiper 2002). Highly selective iNOS inhibitors such as 1400W compete with arginine and presumably bind to arginine-binding sites of NOS isoforms. Enzyme dimerization and cofactor blockers may also inhibit the synthesis of NO (for review, see Vallance and Leiper 2002). In experimental systems, NO may be inhibited by the addition of oxyhemoglobin, or the effects of NO on the guanylate cyclase (GC) can be blocked by methylene-blue (for review, see Änggård 1994). NO itself appears to exert feedback inhibition of NOS, perhaps by interacting with the enzyme's heme prosthetic group (for review, see Bredt and Snyder 1994).

Nitric oxide may exist as the nitroxyl anion (NO^-), nitric oxide ($NO^\bullet$) or the nitrosonium cation (NO^+) depending on its oxidation state. Interconversion of NO^-, $NO^\bullet$ and NO^+ can take place in cellular conditions and consequently all three species must be considered in order to account fully for the biological activity of NO (for review, see Hughes 1999, Gow and Ischiropoulos 2001). In air, NO reacts rapidly with oxygen to form brown fumes of nitrogen dioxide (NO_2) which is capable of inducing tissue damage. When NO_2 is applied to aqueous medium (water, ultrafiltrate or plasma), it hydrolyzes to equimolar amounts of nitrite (NO_2^-) and *in vivo* may be further oxidized by erythrocyte hemoglobin to nitrate (NO_3^-) (for review, see Feelisch 1991, Farrell and Blake 1996). In blood the basal concentrations of nitrite are thus low while those of nitrate are about 100 times higher (for review, see Moncada and Higgs 1993). There are four main targets for NO reactions in cells: metals, reduced thiols, molecular oxygen and other reactive oxygen species, e.g. superoxide (O_2^-). Superoxide ions form in a fast reaction peroxynitrite ($ONOO^-$), a powerful oxidant which can modify proteins and lipids by nitration (for review, see Vallance and Leiper 2002).

2.3.2 Functions of nitric oxide

Endogenous NO has a significant role in many bioregulatory systems and host defence mechanisms, including the control of vascular tone which is important in blood flow and pressure, inhibition of platelet aggregation and adhesion, neurotransmission and

macrophage cytotoxicity (for review, see Moncada and Higgs 1993, Ignarro et al. 1999, Moilanen et al. 1999). NO is probably the major endogenous vasodilator (for review, see Bredt and Snyder 1994, Ignarro et al. 1999). It constitutes a highly diffusible first messenger and it is synthetized on demand. There are both direct and indirect effects of NO on molecular level. The former are mediated by the NO molecule itself, while the latter are mediated by reactive nitrogen species produced by the interaction of NO with oxygen (O_2) or superoxide radicals ($O_2^{\cdot-}$). At the low concentrations (< 1 μM) of NO produced by eNOS and nNOS, the direct effects prevail while at higher concentrations (> 1 μM) of NO, produced by iNOS, the indirect effects predominate (for review, see Murad 1999, Davis et al. 2001).

The direct effects of NO often involve its interaction with metal complexes (for review, see Davis et al. 2001). The formation of cyclic guanosine 3',5'-monophosphate (cyclic GMP) accounts for many of the physiological effects of NO (for review, see Ignarro 1990, Bredt and Snyder 1994, Beckman and Koppenol 1996, Ignarro et al. 1999, Murad 1999). NO may also interact with nonheme iron-containing and zinc-containing proteins or form S-nitrosothiols by nitrosylation (for review, see Davis et al. 2001, Hogg 2002).

The indirect effects of NO include oxidation, nitrosation and nitration (for review, see Davis et al. 2001). Cytokine-induced NO production mediates cytotoxicity in the target cells of macrophages (for review, see Farrell and Blake 1996). In a reaction with O_2 (auto-oxidation) NO forms dinitrogen trioxide (N_2O_3), which can mediate DNA deamination and nitrosylation. By reacting with superoxide ($O_2^{\cdot-}$) NO produces peroxynitrite ($ONOO^-$), which is a toxic nitrating agent and a powerful oxidant, modifying proteins, lipids, tyrosine and nucleic acids (for review, see Beckman and Koppenol 1996, Davis et al. 2001).

2.3.3 Biosynthesis of cyclic GMP

The two pathways known to generate cyclic GMP by guanylate cyclases (GCs) are considerably different. Particulate guanylate cyclase is activated by peptide ligands which bind to cell membrane receptors possessing transmembrane domains contiguous with intracellular GC. Four membrane receptor guanylate cyclases have been cloned and characterized in humans and rats. Guanylate cyclase A, also called atrial natriuretic peptide receptor type A, binds atrial natriuretic peptide (ANP) and brain natriuretic peptide

(BNP). Guanylate cyclase B, also called atrial natriuretic peptide receptor type B, is selectively activated by natriuretic peptide type C (CNP). A third membrane receptor GC, also called guanylate cyclase C, is the intestinal receptor for *Escherichia coli* heat-stable enterotoxin, which is activated by this enterotoxin and endogenous intestinal peptide guanylin (Schmidt et al. 1993). A fourth membrane receptor-cyclase, i.e. human retinal guanylate cyclase, has been cloned and expressed (Shyjan et al. 1992).

Soluble GC is a heme-containing protein found in the cytosolic fraction of virtually all mammalian cells, with the highest concentrations in lung and brain. Several isoforms of soluble GC have been cloned and characterized (for review, see Hobbs 1997). Soluble GC is regulated by NO, carbon monoxide (CO) and a number of other endogenously formed molecules, but NO is the most potent and effective activator (for review, see Schmidt et al. 1993). The binding of NO to the heme group of soluble GC, a heterodimeric hemoprotein, by dislocating the heme-iron causes an immediate alteration in the enzyme's conformation and an increase in catalytic activity resulting in a 50- to 200-fold increase in the velocity of conversion of magnesium guanosine 5'-triphoshate (MgGTP) substrate to cyclic GMP and pyrophosphate (for review, see Ignarro 1990, Bredt and Snyder 1994). Soluble GC is activated by NO at a fairly low concentration (10-100 nM), reflecting the high affinity of NO for the soluble GC heme moiety (for review, see Hobbs 1997, Davis et al. 2001). Since NO easily permeates biological membranes, endothelium-derived NO can activate cytosolic GC in diverse cell types located in close proximity to its cell of origin (for review, see Ignarro 1990, Davis et al. 2001). The result is an increase in intracellular cyclic GMP leading to diverse physiological effects. One of the significant actions of cyclic GMP is the relaxation of smooth muscle cells (for review, see Farrell and Blake 1996).

2.3.4 Functions of cyclic GMP

Cyclic GMP has a central role in several physiological phenomena, e.g. cardiac and smooth muscle relaxation, cellular calcium movements important for platelet aggregation, the retinal rod response to light, olfactory reception, steroidogenesis and renal and intestinal ion transport. Signal transduction pathways can be composed of any types of soluble and particulate GCs, and any of an array of cyclic GMP mediators, including cyclic GMP-gated ion channels, cyclic GMP-stimulated or inhibited phosphodiesterases and cyclic GMP-dependent protein kinases. Disorder in some step of the signaling transduction

pathway leads to pathological conditions; overactivity is associated with endotoxic shock and secretory diarrhea, underactivity with hypertension (for review, see Schmidt et al. 1993, Biel et al. 1998, Smolenski 1998).

2.3.5 Nitric oxide releasing compounds

NO donors produce NO when applied to biological systems, where they either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency. These compounds include the organic nitrates, S-nitrosothiols, sydnonimines, NONOates, sodium nitroprusside and furoxans (Feelisch 1998).

2.3.5.1 Organic nitrates

Organic nitrates are nitric acid esters of mono- and polyhydric alcohols and most of them are only sparingly soluble in water. Clinically used compounds include glyceryl trinitrate, isosorbide dinitrate and isosorbide-5-mononitrate (for review, see Feelisch 1991, Feelisch 1998). Ferid Murad and co-workers analyzed the mechanisms of action of glyceryl trinitrate and other related vasodilators in 1977 and suggested that these compounds release NO, which enhances cyclic GMP production and relaxes smooth muscle (Arnold et al. 1977, Katsuki et al. 1977a, Katsuki et al. 1977b). Organic nitrates require either enzymatic or non-enzymatic bioactivation for NO release to occur (for review, see Feelisch 1998). The most important indications for organic nitrates are angina pectoris, acute myocardial infarction and congestive heart failure. Chronic administration of organic nitrates leads to the development of tolerance. The precise incidence of tolerance with these compounds is not known. The mechanism underlying tolerance is not completely understood and probably involves several independent factors. Proposed mechanisms for the development of nitrate tolerance include depletion of reduced sulphydryl groups, desensitization of GC, increased activity of cyclic GMP phosphodiesterase, reflex neurohormonal activation, shift in extravasal volume, increased endothelin-1 production and increased vascular superoxide (for review, see Glasser 1999). Experimental and clinical observations suggest that tolerance may be a consequence of intrinsic abnormalities in the vasculature, including enhanced endothelial production of oxygen-derived free radicals (for review, see Münzel and Harrison 1997).

2.3.5.2 S-nitrosothiols

S-nitrosothiols are sulphur analogs of organic nitrites. At least two S-nitrosothiols have been prepared as stable solids and characterized: S-nitroso-N-acetylpenicillamine (SNAP) and S-nitrosoglutathione (GSNO) (for review, see Butler and Rhodes 1997, Feelisch 1993, Feelisch 1998). S-nitrosothiols decompose to yield the corresponding disulfide and NO. Another important reaction of S-nitrosothiols is transnitrosation, i.e. the transfer of bound NO from one thiol group to another (for review, see Feelisch 1998).

2.3.5.3 Sydnonimines

The most thoroughly studied compound of sydnonimines is molsidomine (N-ethoxycarbonyl-3-morpholino-sydnonimine). Molsidomine is a prodrug which is converted by liver esterases to the active metabolite 3-morpholino-sydnonimine (SIN-1). SIN-1 is a vasorelaxant and anti-platelet agent and these activities are thought to be mediated mainly by the release of NO (for review, see Feelisch 1998). SIN-1 decomposes to produce NO in an oxygen-dependent process. It undergoes rapid nonenzymatic hydrolysis to the open-ring form SIN-1A. Oxygen promotes conversion to a cation radical intermediate from which NO is released and more stable SIN-1C is formed. In the course of this reaction superoxide (O_2^-) is formed, which together with NO can form peroxynitrite (Feelisch et al. 1989, for review, see Feelisch 1998). SIN-1 does not induce tolerance in *in vitro* experiments (Hinz and Schröder 1999).

2.3.5.4 NONOates

NONOates are adducts of NO with nucleophiles. They have the ability to generate NO spontaneously in a chemically predictable manner which correlates directly with their biologic effect (Morley and Keefer 1993). It is thought that NONOates generate NO by acid-catalyzed dissociation with regeneration of the free nucleophile and NO, although enzymatic metabolism *in vivo* cannot be excluded. The decomposition of NONOates is pH-dependent, proceeding at a very slow rate at values over pH 9, a moderate rate at physiological level and almost instantaneously at acidic pH (for review, see Feelisch 1998). Spermine NONOate does not induce tolerance in *in vitro* experiments (Hinz and Schröder 1998).

2.3.5.5 Sodium nitroprusside

The mechanism of NO release from sodium nitroprusside (SNP) is incompletely understood. In biological systems both non-enzymatic and enzymatic NO release from SNP may occur. SNP decomposition leads to the formation of NO, disulfide and cyanide. SNP is used clinically to reduce blood pressure, e.g. hypertensive emergencies (for review, see Feelisch 1998).

2.3.5.6 Furoxans

Furoxans are a group of heterocyclic compounds which have been shown to exert a variety of NO-related bioactivities. Furoxans have been demonstrated to increase potently the activity of soluble GC. They liberate NO after reacting with sulphydryl groups of low molecular weight thiols and proteins (Feelisch et al. 1992). Some furoxans have been reported to release NO spontaneously and independently of thiols (Hecker et al 1995).

2.4 NITRIC OXIDE AND THE EYE

Nitric oxide is a mediator of physiological and pathophysiological processes in the eye, for example regulation of aqueous humor dynamics, vascular tone, retinal neurotransmission, retinal ganglion cell death by apoptosis, phototransduction and ocular immunological responses (for review, see Haefliger et al. 1994, Becquet et al. 1997, Haefliger et al. 1999). Both underproduction and overproduction of NO may contribute to pathological processes in degenerative diseases (glaucoma, retinal degeneration, cataract) or inflammatory diseases (uveitis, retinitis) in the eye. These diseases might thus be treated by compensating for NO deficiency with NO donors or NO precursors or by reducing overproduction of NO by inhibiting iNOS activity, respectively (for review, see Becquet et al. 1997, Chiou 2001).

2.4.1 Localization of nitric oxide synthases in the eye

In the eye, the capacity to form NO is found in various tissues, and both the constitutive and inducible isoforms of NOS have been identified. Endothelial NOS has been found to be present in the vascular endothelium and smooth muscle cells of the anterior segment, choroid and retina. In addition to the ciliary vascular endothelium, eNOS (Osborne et al. 1993, Haufschild et al. 1996, Geyer et al. 1997; for review, see Becquet et al. 1997; see

Ellis and Nathanson 1998) and nNOS (Meyer et al. 1999) are highly enriched in the nonpigmented ciliary epithelium, and isolated human and porcine ciliary processes have been shown to produce NO (Haufschild et al. 2000). Using NADPH-diaphorase, a technique which identifies all three isoforms of NOS, the ciliary muscle and outflow pathway, i.e. the trabecular meshwork, Schlemm's canal, collecting channels and draining veins, have been found to be markedly enriched in NOS (Nathanson and McKee 1995a, Geyer et al. 1997; for review, see Becquet et al. 1997; see Ellis and Nathanson 1998). NOS has demonstrated in NADPH-diaphorase staining in nerve fibers in the limbus, in the cornea (endothelium, epithelium and peripheral cornea) and in the lens epithelium. Neuronal and inducible NOS have been identified in different parts of the retina (Meyer et al. 1999; for review, see Becquet et al. 1997). After cytokines and endotoxin stimulation iNOS may be detected in the iris/ciliary body and vessels (for review, see Becquet et al. 1997). All three isoforms of NOS are present in the human optic nerve head; iNOS, however, is present only in glaucomatous eyes or in eyes with retinal ischemia in rats, not in normal eyes (Neufeld et al. 1997, Neufeld et al. 2002b).

2.4.2 Role of nitric oxide in different sites in the eye

In the anterior segment of the eye, NO regulates cellular responses in conjunctiva, trabecular meshwork and ciliary muscle. In a pig model, NO has been found to be produced in the acute phase of allergic conjunctivitis and it mediates vasodilation, leading to increased vascular permeability and edema (Meijer et al. 1996). NO might be related to the regulation of aqueous humor dynamics by acting at the ciliary muscle, the aqueous humor outflow pathway or both (for review, see Becquet et al. 1997). For details of the mechanism, see 2.5. It has been suggested that overproduction of NO may result in the pathogenesis of endotoxin-induced uveitis as a proinflammatory mediator leading to hyperemia and cellular infiltration (for review, see Becquet et al. 1997, Koss 1999, Chiou 2001). Prostaglandin $F_{2\alpha}$ has been found to cause hyperemia on the surface of the eye by activating NOS (Astin et al. 1994).

NO has a dual role in the pathogenesis of retinal diseases (e.g. retinitis) or degeneration (e.g. ischemic retinopathy, age-related macular degeneration and retinitis pigmentosa) (for review, see Becquet et al. 1997, Chiou 2001). NO mediates ischemic damage and promotes neuronal cell death by the production of free radicals. On the other hand, NO

has an important role in the regulation of the regional blood flow in the retina. It improves blood flow during or immediately after ischemia and thus reduces the amount of damaged tissue (for review, see Becquet et al. 1997, Koss 1999). The choroid appears to be under the influence of a basal release of NO, which maintains the vasodilatory tone of choroidal vessels, improving the delivery of nutrients to the retina. NO also has a vasodilatory function for blood flow in the optic nerve head (see Tamm and Lütjen-Drecoll 1998a).

2.5 NITRIC OXIDE, CYCLIC GMP AND INTRAOCULAR PRESSURE

In the anterior segment of the eye, NO donors or nitrovasodilators may regulate IOP at the level of ciliary muscle, trabecular meshwork and endothelial and vascular smooth muscle cells in the aqueous drainage system. Compounds affecting the NO-cyclic GMP pathway have been reported to lower IOP in some animal and human experiments (Table 3). NO donors and cyclic GMP analogs may be involved in the modulation of aqueous humor dynamics by inducing relaxation of ciliary muscle, leading to decreased trabecular meshwork resistance and thus alteration in the outflow facility of aqueous humor, which results in lowered IOP. There is evidence that the trabecular meshwork has intrinsic contractile elements which can be relaxed by NO, leading to increased aqueous humor outflow (for review, see Becquet et al. 1997; see Ellis and Nathanson 1998, Haefliger and Dettmann 1998, Tamm and Lütjen-Drecoll 1998b, Wiederholt 1998). In contrast to NO, atriopeptin acts on particulate GC by binding with a cell surface receptor (Shahidullah and Wilson 1999). The ciliary body, dissected free from the ciliary epithelium, has shown only slight stimulation of GC activity, while greater stimulation has been found in the ciliary processes and iris, proposing a role of atriopeptin in aqueous humor formation (Nathanson 1987). The mechanism of action of another GC activator, YC-1, may be related to its ability to stabilize soluble GC in its active configuration (for review, see Hobbs 1997).

Table 3. Effects of NO-cyclic GMP pathway-related compounds on intraocular pressure *in vivo*.

Compound	Mechanism	Dose	Route of Administration	Animal or human	Effect on IOP	Reference
L-Arginine	Precursor of NO	0.5%	Topical (50 µl)	Rabbit	Lower	Chiou et al. 1995
		10 g/100 ml	Intravenous	Rabbit and human	Lower	Chuman et al. 2000
Nitroglycerin	Nitrovasodilator	9.7-194 µg/min	Intravenous	Human (glauc.)	Lower	Wizemann and Wizemann 1980
		0.03%	Topical (2 x 25 µl)	Rabbit	Lower	Nathanson 1988
		0.003-0.1%	Topical (2 x 25 µl)	Rabbit	Lower	Nathanson 1992
		0.1%	Topical (10 x 5 µl)	Monkey	Lower	Schuman et al. 1994
		0.05 and 0.1%	Topical (50 µl)	Monkey (norm. and glauc.)	No effect	Wang and Podos 1995
Isosorbide-mononitrate	Nitrovasodilator	0.5%	Topical	Human (POAG/ocul.hypert.)	No effect	Diestelhorst et al. 1991
		20 mg	Oral	Human	No effect	Iannaccone et al. 2000
Isosorbide dinitrate	Nitrovasodilator	40 mg	Oral	Human	Lower	Wizemann and Wizemann 1980
		0.1-0.3%	Topical (2 x 25 µl)	Rabbit	Lower	Nathanson 1992
SNP	NO donor	1-2%	Topical	Rabbit	Increase	Krupin et al. 1977
		0.1%	Topical (2 x 25 µl)	Rabbit	Lower	Nathanson 1992
SIN-1	NO donor	0.1%	Topical (2 x 25 µl)	Rabbit	Lower	Nathanson 1992
		20 mM	Intravitr./intracam.	Rabbit	Lower	Behar-Cohen et al. 1996
SNAP	NO donor	20 mM	Intravitr./intracam.	Rabbit	Lower	Behar-Cohen et al. 1996
8-Br-cGMP	cGMP analog	4%	Topical (50 µl)	Rabbit	Lower	Becker 1990
		1%	Subconj. (100 µl)	Rabbit	Lower	Busch et al. 1992
BB-cGMP	cGMP analog	0.35 and 3.5 mM	Topical (50 µl)	Rabbit	Lower	Stein and Clack 1994
		35 mM	Topical (70 µl)	Rabbit (ocular hypertensive)	Lower	Stein and Clack 1994

Table 3. Continued. The effects of NO-cyclic GMP pathway-related compounds on intraocular pressure *in vivo*.

Compound	Mechanism	Dose	Route of Administration	Animal or human	Effect on IOP	Reference
Natriuretic peptides	Activator of GC	0.5%	Topical (50 µl)	Rabbit	Lower	Sugrue and Viader 1986
		10 µg	Intravitr./intracam.	Rabbit	Lower	Sugrue and Viader 1986
		0.3 nmole	Intravitreal (10 µl)	Rabbit	Lower	Nathanson 1987
		2-4 µg	Intravitreal (20 µl)	Rabbit	Lower	Mittag et al. 1987
		6.25-100 µg/kg	Intravenous	Rabbit	Lower	Tsukahara et al. 1988
		100 µg	Intravenous	Human (glauc.)	Lower	Diestelhorst and Krieglstein 1989
		10 µg	Intravitreal (10 µl)	Rabbit	Lower	Korenfeld and Becker 1989
		97-390 pmol/kg/min	Intravenous	Monkey	Lower	Samuelsson-Almén et al. 1991
		81-162 pmol/ml	Intracameral perf.	Monkey	No effect	Samuelsson-Almén et al. 1991
		0.2 and 2 nmol	Intravitreal (20 µl)	Rabbit	Lower	Takashima et al. 1996
		0.02, 0.2, 2 nmol	Intravitreal (20 µl)	Rabbit	Lower	Takashima et al. 1998
		10 µg	Intracameral	Rabbit	Lower	Fernandez-Durango et al. 1999

BB-cGMP, 2'-O-(4-benzoyl)benzoyl cyclic guanosine 3',5'-monophosphate; 8-Br-cGMP, 8-bromo-cyclic guanosine 3',5'-monophosphate; cGMP, cyclic guanosine 3',5'-monophosphate; GC, guanylate cyclase; IOP, intraocular pressure; NO, nitric oxide; POAG, primary open-angle glaucoma; SNAP, S-nitroso-N-acetylpenicillamine; SNP, sodium nitroprusside; SIN-1, 3-morpholino-sydnonimine

Nitrergic nerves might dilate episcleral vessels, thereby lowering episcleral venous pressure and further the resistance to aqueous humor outflow, leading to decreased IOP (see Tamm and Lütjen-Drecoll 1998b). Since constitutive NOS is present in the ciliary epithelium, a possible role of NOS in the regulation of aqueous humor formation may be proposed. Systemic NOS inhibition by intravenous NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) causes a significant decrease in IOP, suggesting that the ocular hypotensive effect may be due in part to a blood-flow dependent decrease in aqueous production (Kiel et al. 2001). Topical application of NOS inhibitors does not prevent an IOP increase induced by water intake in rabbits (Fleischhauer et al. 2001).

2.6 NITRIC OXIDE AND GLAUCOMA

Nitric oxide may have effects on the development or progression of glaucoma; these are summarized in Table 4. Altered NOS activity in ciliary muscle and outflow pathways has been found in patients with primary open-angle glaucoma. A frank structural loss of NOS activity has been shown in the longitudinal fibers of the ciliary muscle. These abnormalities can be causally related to glaucoma or they may be a manifestation of the disease or its treatment (Nathanson and McKee 1995b).

In addition, it has recently been hypothetized that NO or NO-derived radicals might result in neurotoxic glaucomatous effects at the optic nerve head and retina, leading to optic nerve head degeneration and visual field loss. All three isoforms of NOS are present in increased amounts in the optic nerve head of patients with primary open-angle glaucoma. The increased expression of iNOS and nNOS suggests that the glaucomatous optic nerve head is exposed to enhanced concentrations of NO, which plays a major neurodestructive role in the chronic degeneration of axons in the optic nerve head (Neufeld et al. 1997). On the other hand, overexpression of these enzymes may reflect a mechanism compensatory to the lowered NO concentrations found in glaucoma patients. Increased IOP has apparently been a major causative factor for the overproduction of NO in an experimental animal model of glaucoma (Siu et al. 2002) in consequence of iNOS activation (Shareef et al. 1999). It has been suggested that glaucomatous visual field loss as a manifestation of retinal ganglion cell death occurs possibly through apoptosis. Apoptosis can be induced by glutamate activation of the N-methyl-D-aspartate (NMDA) membrane receptor, which stimulates the production of large amounts of NO as well as free radical superoxide anion in the mitochondria in retinal ganglion cells. NO then reacts with superoxide to form highly toxic peroxynitrite, which, in turn, triggers cell death (Quigley et al. 1995, see Haefliger and Dettmann 1998). Increased concentrations of glutamate have been found in the vitreous body of glaucomatous humans and monkeys (Dreyer et al. 1996). Damage to the optic nerve head and retinal ganglion cells might be avoided by inhibiting induction or activity of iNOS (Neufeld et al. 1999, Neufeld et al. 2002a; for review, see Neufeld 1999). Treatment with an iNOS inhibitor may stop progression of the glaucomatous process in eyes with already established damage (Neufeld et al. 2002a). However, the increased presence of eNOS in vascular endothelia could be neuroprotective in causing vasodilation and increased blood flow in the optic nerve head (Neufeld et al. 1997).

Table 4. Nitric oxide in glaucoma: diverse physiological and pathological functions and a variety of therapeutic approaches. Adapted from Ellis and Nathanson (1998).

Effect of NO	Site of action	Mechanism
Effects on aqueous humor production	NOS in ciliary epithelium	Likely inhibition
Effects on aqueous humor outflow	NOS in ciliary muscle	Increasing resistance in normal eyes, but less effect in POAG
	NOS in trabecular meshwork spindle cells	Probably reducing resistance
	NOS in trabecular meshwork and Schlemm's canal endothelium	Regulation of outflow via effects on Na, K, Cl co-transporter
	NOS in collecting channels and distal veins	Reducing resistance
Effects on ocular blood flow	Vascular tissue of choroid, retina and optic nerve head	Use of NO agonists to alter blood flow
	Arterial endothelium	Interfere with hormone and flow-mediated NO mechanism
	Vascular smooth muscle	Interfere with NO mechanisms
Limiting NO-mediated glutamate excitotoxicity	Ganglion cells and optic nerve	Blocks NMDA-receptors
Blocking NOS-initiated apoptosis	Ganglion cells	Blocks downstream cell death
Preventing NOS-mediated damage	Optic nerve	
Optimizing energy metabolism	Ganglion cell	NO alters ATP levels via Na pump regulation
Limiting action of oxygen free radicals		Remove or reduce generation of free radicals by NO
		Reverse modulating effects of free radicals on cell energy metabolism by blocking cyclic GMP/PKG pathway

ATP, adenosine 5'-triphosphate; cyclic GMP, cyclic guanosine 3',5'-monophosphate; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, nitric oxide synthase; PKG, protein kinase G; POAG, primary open-angle glaucoma

In conclusion, IOP is the result of a homeostatic balance of production and outflow of aqueous humor. Aqueous humor, produced by the ciliary processes, passes from the posterior chamber through the pupil into the anterior chamber and exits mainly through trabecular and uveoscleral pathways. IOP is regulated by cholinergic as well as adrenergic receptors. Glaucoma is a progressive optic neuropathy involving altered intraocular hemodynamics. Increased IOP is one of the most important risk factors for glaucoma, but it might also be at normal level concomitant with the disorder. Other risk factors for glaucoma include age, genetic disposition, black race, myopia, vascular factors such as arterial hyper- and hypotension and pseudoexfoliation. In the eye, NO has an important role in certain physiological processes, e.g. regulation of aqueous humor dynamics. On the other hand, NO is involved in several diseases of the eye. Compounds affecting the NO-cyclic GMP pathway may modulate aqueous humor dynamics by reducing trabecular meshwork resistance, resulting in increased aqueous humor outflow facility and thus lowered IOP. Patients with primary open-angle glaucoma have been found to have abnormalities in NO-containing cells in the ciliary muscle and the outflow pathway. On the other hand, overproduction of NO in the optic nerve head may lead to glaucomatous damage to the optic nerve and visual field.

3 AIMS OF THE STUDY

The aim of the present study was to investigate the roles of NO and cyclic GMP in the regulation of IOP and the mechanisms of their IOP-lowering effects using experimental animals, and the possible clinical relevance of NO in aqueous humor dynamics in glaucoma patients.

The specific aims were:

1. To compare the ocular hypotensive effects of different NO donors and guanylate cyclase activators in rabbits (Study I) and to evaluate the role of cyclic GMP in this process by measuring cyclic GMP production in the porcine iris-ciliary body (Study IV).

2. To clarify the IOP-lowering mechanism of the NO/cyclic GMP pathway by measuring aqueous humor outflow facility in rabbits (Study II) and aqueous humor production in healthy human volunteers (Study III) treated with NO-releasing compounds.

3. To investigate the possible connection of NO to aqueous humor dynamics in glaucoma patients (Study V).

4 MATERIALS AND METHODS

4.1 EXPERIMENTAL ANIMALS

New Zealand White (NZW) rabbits of both sexes were used (2.5-3.5 kg, n = 36 in Study I and 2.9-3.5 kg, n = 28 in Study II). The animals were purchased from the National Laboratory Animal Center, University of Kuopio, Kuopio, Finland; Harlan of UK, Bicester, UK or Harlan of Netherlands, Horst, the Netherlands. They were housed individually in Scanbur plastic cages (Scanbur BK A/S, Lellinge, Denmark); all rabbits were maintained conventionally during the study with regulated air temperature (15–21°C), relative humidity (40–70%), an artificial light cycle (12 hours light/12 hours darkness) and ventilation (air volume change 15 times/hour). They received about 100 g/day of pellet standard rabbit fodder (Stanrab, Special Diet Services, Witham, UK) and had free access to drinking water.

Porcine eyes (n = 45, Study IV) were obtained from an abattoir and from each eye about five tissue samples of iris-ciliary body were detached.

4.2 PATIENTS AND STUDY DESIGNS

The flow of aqueous humor after a single oral dose of isosorbide-5-mononitrate (ISMN), 10 mg, was investigated in Study III. Ten healthy human volunteers (five males and five females, mean age 22 years, range 19-26) with no history of ocular or systemic diseases participated in the study. The subjects were scheduled for four ambulatory visits, which included a prestudy visit and three treatment days (in random order): day 1 (ISMN or placebo), day 2 (ISMN or placebo) and day 3 (topical timolol maleate used as a positive control). In the timolol experiment, the treatment was applied in one eye while the other eye served as control. Isosorbide-5-mononitrate (Ormox® 10 mg, Orion Oy, Espoo, Finland) and identical-looking placebo capsules were packed in the Tampere University Hospital Pharmacy. Timolol maleate (Oftan® Timolol 5 mg/ml) and 5 % sodium fluorescein solution were from Santen Oy, Tampere, Finland.

Biochemical markers of the L-arginine-nitric oxide pathway in the aqueous humor were investigated in glaucoma patients (Study V). The prospective study involved 38 consecutive glaucoma patients undergoing unilateral cataract surgery in the glaucomatous eye (8 males and 30 females, mean age 75 years, range 45-87). Glaucoma patients were compared to 38 matched cataract controls (8 males and 30 females, mean age 77 years, range 59-88).

4.3 PHYSIOLOGICAL MEASUREMENTS

4.3.1 Intraocular pressure

In Study I IOP was measured with a pneumatonometer in conscious rabbits (Modular One Tonometer, Mentor, Cambridge, MA, USA) after topical anesthesia with 0.4 % oxybuprocain (Oftan® Obucain, Santen Oy, Tampere, Finland). One hour before the test compound application, a control measurement was taken for both eyes. Thirty μl of the test compounds or vehicle were administered in the inferior conjunctival sac, or 50 μl of the test compounds or vehicle were injected in the vitreous humor. Thereafter, IOP was measured at 0.5, 1, 2, 3, 4 and 5 hours, if not otherwise indicated.

In Study II IOP in anesthetized rabbits was measured manometrically in cannulated eyes with 27 G needles after topical anesthesia with 0.4 % oxybuprocain (Oftan® Obucain). Measurement was carried out using a pressure transducer (P-50, Gould/Statham, Bilthoven, the Netherlands) connected to a Grass Model 79-D polygraph (Quincy, MA, USA).

In human subjects (Study III) the IOP was measured by the applanation tonometry of Goldmann (Haag-Streit, Bern, Switzerland) after topical anesthesia with a combination of 0.3% oxybuprocain and 0.125% fluorescein (Oftan® Flurekain, Santen Oy).

4.3.2 Blood pressure

The systemic blood pressure in anesthetized rabbits was measured by cannulating a femoral artery with a polyethylene cannula containing heparinized isotonic saline (Study II). The cannula was connected to a pressure transducer (P-50, Gould/Statham) for blood pressure monitoring (Grass Model 79-D polygraph). An intravenous infusion of

hydroxyethylamylopectin (Plasmafusin® 60 mg/ml, Fresenius Kabi AB, Uppsala, Sweden) was used to sustain blood pressure when needed.

Blood pressure in human subjects (Studies III and V) was measured at the forearm with an automated blood pressure measuring device (Omron M5-I, Omron Matsusaka Co., Ltd., Kyoto, Japan).

4.3.3 Aqueous humor outflow facility

The outflow facility of aqueous humor in anesthetized rabbits was determined by the two-level constant pressure infusion method (Bárány 1964) (Study II). General anesthesia was initiated with an intramuscular injection and maintained by intravenous infusion of a combination of ketamine (Ketalar® 50 mg/ml, Parke-Davis Warner Lambert Nordic AB, Solna, Sweden) and xylazine (Rompun®Vet 20 mg/ml, Bayer AG, Leverkusen, Germany). The rabbits were pretreated with intravenous indomethacin at 10 mg/kg body weight (Confortid®, Dumex, Copenhagen, Denmark) to minimize the effect of the endogenous prostaglandins. The eyes of the animals were cannulated with three needles (27 G) connected to polyethylene cannulas after topical anesthesia. One cannula was used for continuous IOP monitoring, one for injection of the test compound or vehicle (5 μl) and one for the infusion of fluid for outflow facility measurements. IOP was increased 5 to 7 mmHg above the preinfusion level by infusing a mock solution of aqueous humor (Sperber and Bill 1984) into the anterior chamber. The infusion rate (F) and the increase in IOP (ΔP) were registered in steady-state conditions. Thereafter, the IOP was raised about 5 to 7 mmHg above the previous level, and the same procedure was repeated. The outflow facility (C) could then be calculated from the formula $C=F/\Delta P$. Infusion was carried out on both eyes simultaneously from separate reservoirs. The rabbits were euthanized by 300 mg pentobarbital (Mebunat®, Orion Oy, Espoo, Finland) after the last measurements.

4.3.4 Aqueous humor flow in man

A scanning computerized fluorophotometer (FM-2 Fluorotron Master, Ocumetrics, Mountain View, CA, USA) was used to assay cornea and anterior chamber fluorescein concentrations (Study III). Fluorescein was applied and rinsed approximately 6 hours (the experiments with oral ISMN or placebo) or 7 hours (the experiments with topical timolol) before the Fluorotron measurements. ISMN or placebo was administered 5 hours and

timolol was applied 6 hours after the rinsing of the eyes. Fluorescence in each eye was measured every 30 minutes at 6 to 8.5 hours after the rinsing and hourly from 8.5 to 10.5 hours after rinsing. In the timolol experiment the measurement was repeated every 30 minutes from 7 to 9.5 hours after rinsing. The readings obtained were used to calculate aqueous flow according to the mathematical model described by Brubaker (1989).

4.4 IRIS-CILIARY BODY INCUBATION METHOD

Porcine eyes were placed in a cold preoxygenated modified Krebs solution after enucleation. Tissue samples were prepared for the experiments within 3 hours of enucleation. The eyes were cut in half at the equator of the bulbus and vitreous and lens were removed. The ciliary body and iris were carefully detached from the sclera of the anterior bulbus by cutting the tissue with a cornea trepan. The tissue samples were pooled and placed in oxygenated modified Krebs solution.

After a preincubation of 60 minutes the multidish wells were cleared of the solution and fresh Krebs solution and test compounds or solvent were added. Two incubation periods, 30 and 60 minutes, were used. At the end of the experiment the tissues and incubation media were frozen in liquid nitrogen and stored at -80°C until measurement of cyclic GMP (tissue) and nitrate + nitrite (NO_x) (incubation media).

4.5 COLLECTION OF SAMPLES FOR BIOCHEMICAL ASSAYS

In the animal experiments for the biochemical assays (Study I), blood samples were taken before the rabbits were euthanized. After euthanization with 300 mg pentobarbital (Mebunat®, Orion Oy) aqueous humor was collected (Studies I and II) and iris and ciliary body separated (Study I). The samples were stored at -70°C until assayed for NO_x, nitrite, cyclic GMP, NO synthases (Study I) and protein concentration (Study II).

In the human experiment for the biochemical assays (Study V), blood samples were collected from the venous cannule immediately prior to cataract surgery and aqueous humor samples were collected through the first clear corneal incision of the eye. The samples were stored at -70°C until assayed for NO_x, nitrite and cyclic GMP.

4.6 BIOCHEMICAL DETERMINATIONS

4.6.1 Nitrite and nitrate

The concentrations of nitrite + nitrate (NO_x) in aqueous humor (Studies I and V), plasma (Study I) and serum (Study V) were measured by ozone-chemiluminescence. Vanadium chloride (VCl_3) in hydrochloric acid was used to convert nitrite and nitrate to NO, which was then quantitated by the ozone-chemiluminescence method (Braman and Hendrix 1989). NO was measured by NO analyzer NOA 280 (Sievers Instruments Inc., Boulder, CO, USA) using sodium nitrate as standard. When nitrite concentrations were measured, sodium iodide in acetic acid was used to convert nitrite in the deproteinized samples to NO, which was measured by NOA 280. The concentration of NO_x in the incubation medium (Study IV) was determined spectrophotometrically with the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA).

4.6.2 Cyclic GMP

The concentrations of cyclic GMP in aqueous humor (Studies I and V), plasma (Study I), serum (Study V) and porcine iris-ciliary body (Study IV) were measured by radioimmunoassay. During homogenization of the tissue samples, zaprinast was added to prevent the destruction of cyclic GMP. The iris-ciliary body samples were homogenized with an Ultra-turrax tissue homogenizer (Ultra-Turrax T8, Ika Labortechnik, Janke & Kunkel GmbH & Co KG, Staufen, Germany). The homogenates were centrifuged and the precipitated proteins dissolved in NaOH at +37°C overnight and assayed (Lowry et al. 1951). The supernatants (Study IV) and the samples of aqueous humor, plasma and serum (Studies I and V) were acidified with HCl and extracted before lyophilization. Acetylated cyclic GMP in the samples was assayed with the [^{125}I]-cyclic GMP RIA kit (Amersham International, Little Chalfont, Buckinghamshire, UK), except for Study V, where cyclic GMP concentrations in serum were measured with the Cyclic GMP (low pH) Immunoassay Kit (R&D Systems Europe, Abington, UK).

4.6.3 Nitric oxide synthases

The expression of NO synthases (eNOS, nNOS and iNOS) in the iris and ciliary muscle of rabbits (Study I) was measured by Western blot. The samples were homogenized using an Ultra-turrax homogenizer. Homogenates were centrifuged and the protein content of the

supernatants measured (Lowry et al. 1951). Equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (Minigel apparatus, Bio-Rad, Bio-Rad Laboratories, Hercules, CA, USA) (Laemmli 1970). After electrophoresis, the separated proteins were transferred to nitrocellulose membranes and incubated overnight with the appropriate primary antibody at +4°C. The antibodies were mouse monoclonal anti-eNOS IgG_1 (1:2500), anti-nNOS IgG_{2a} (1:3000) and anti-iNOS IgG_{2a} (1:2500) (Transduction Laboratories, Lexington, KY, USA). Membranes were washed and incubated with horseradish peroxidase-coupled anti-mouse IgG_1 or IgG_{2a} (Zymed Laboratories, San Francisco, CA, USA). Finally, the bound antibodies were detected using an enhanced chemiluminescence reagent (Amersham) and exposed to X-omat film (Kodak, Paris, France). Each band was quantified with computer programs (Gene Snap and Gene Tools, Synoptics, Cambridge, UK).

4.6.4 Proteins

The aqueous humor protein concentration used as an indicator of increased vascular permeability possibly induced by the test compounds was measured according to Lowry and co-workers (1951) (Study II).

4.7 TEST COMPOUNDS

The test compounds used in Studies I and II were dissolved in saline, phosphate buffer or dimethylsulfoxide (DMSO, Sigma Chemical Co., St. Louis, MO, USA) and in Study IV in Krebs solution. Sodium nitroprusside (SNP), zaprinast, L-arginine, 8-Bromo-cGMP, atriopeptin II and III, NOR-3 ((E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-2-hexenamide) and captopril were purchased from Sigma Chemical Co., spermine NONOate (N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine) and S-nitrosothiol (SNOG) from Tocris Cookson Ltd. (Bristol, UK), S-nitrosocaptopril from Calbiochem-Novabiochem Co. (La Jolla, CA, USA), ODQ (1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one) from Alexis Biochemicals (San Diego, CA, USA) and S-nitroso-N-acetylpenicillamine (SNAP) from Research Biochemicals International (Natick, MA, USA). YC-1 (3-(5'-hydroxymethyl-2'furyl)-1-benzylindazole) was a generous gift from Professor C. M. Teng (Taipei, Taiwan). The chemical structures of NO donors, GC-activators and a cyclic GMP analog are shown in Figure 3.

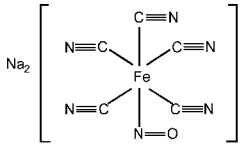
4.8 STATISTICAL ANALYSIS

The results are expressed as mean ± standard deviation (SD). Statistical analysis was carried out by analysis of variance for repeated measurements (ANOVA) (Study I), paired samples t-test (Studies I, II and V), area under curve (AUC) (Study III), Wilcoxon signed ranks test (Studies II and V), Mann-Whitney test (Studies II and V), permutation test (Study IV), Odd's Ratio (CIA) (Study V) and Kruskal-Wallis test (Study V). A p-value less than 0.05 was considered significant.

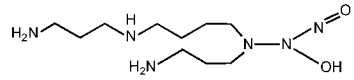
4.9 ETHICS

The clinical studies were conducted in accordance with the Helsinki Declaration. The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) were adhered to in the protocols of the animal studies and the protocols were approved by the local Animal Experimentation Committee (Studies I and II). The study protocols in the human studies were approved by the Ethical Committee of Tampere University Hospital (Studies III and V) and the National Agency for Medicines (Study III).

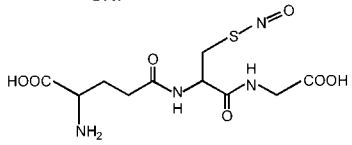
NO donors



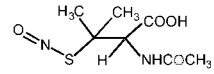
SNP



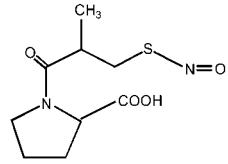
Spermine NONOate



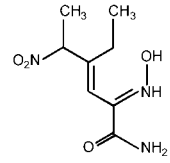
SNOG



SNAP

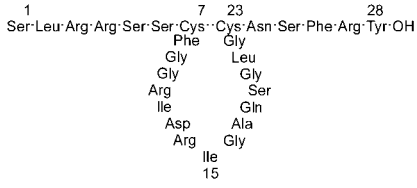


S-nitrosocaptopril

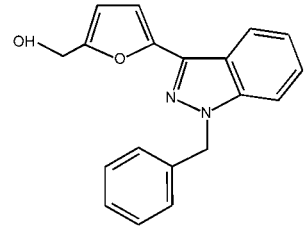


NOR-3

Guanylate cyclase activators

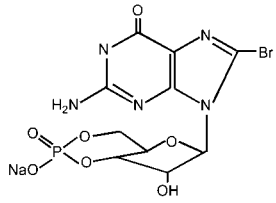


atriopeptin



YC-1

Cyclic GMP analog



8-Br-cGMP

Figure 3. Chemical structures of NO donors, guanylate cyclase activators and a cyclic GMP analog used in the present study.

5 RESULTS

5.1 NITRIC OXIDE AND CYCLIC GMP IN AQUEOUS HUMOR DYNAMICS (Studies I-IV)

5.1.1 Effect of nitric oxide donors and cyclic GMP on intraocular pressure and biochemical markers of NO (Study I)

Compounds affecting the L-arginine-NO-cyclic GMP pathway lowered IOP in ocular normotensive rabbits (Table 5). The precursor of NO synthesis, L-arginine, NO donors, SNP, spermine NONOate, SNOG, nitrosocaptopril, and a soluble guanylate cyclase activator, YC-1, lowered IOP equally, 20-29%. A cyclic GMP analog, 8-Br-cGMP, lowered IOP by 37%. Particulate guanylate cyclase activators, atriopeptin II and III, lowered IOP up to 50% after intravitreal injection. The maximal decrease in IOP was observed 1 - 2 hours after administration, except after intravitreal injections, when the maximal response was measured up to 24 hours. The decrease in IOP lasted only a few hours, but after atriopeptin III lowered values remained for approximately 2 days. Zaprinast, a cyclic nucleotide phosphodiesterase (PDE5/6) inhibitor, alone had no effect on IOP, but in combination with SNP it prolonged the response, supporting the conception of a role of cyclic GMP in IOP reduction. Slight or pronounced conjunctival or iridal hyperemia was detected after administration of L-arginine, SNP, nitrosocaptopril, 8-Br-cGMP, YC-1 and atriopeptin II and III. All NO donors and GC-activators tested increased the NO_x concentration in aqueous humor; cyclic GMP levels in 8-Br-cGMP- and atriopeptin III-treated eyes were higher than in the control eyes.

In conclusion, an association was observed between increased activity of the L-arginine-NO-cyclic GMP pathway and lowered IOP in ocular normotensive rabbits.

Table 5. Effects of L-arginine, NO donors, a cyclic GMP analog and GC-activators on IOP and NO metabolites of aqueous humor in rabbits.

Compound	Concentration	Administration	Decrease in IOP (from basal level)
L-Arginine	1 mM	Topical	29 %
SNP	40 mM	Topical	28 %
Spermine NONOate	100 mM	Topical	20 %
SNOG	10 mM	Intravitreal	27 %
Nitrosocaptopril	100 mM	Topical	28 %
8-Br-cGMP	90 mM	Topical	37 %
YC-1	10 μM	Topical	25 %
Atriopeptin II	84 μM	Intravitreal	37 %
Atriopeptin III	78 μM	Intravitreal	50 %

8-Br-cGMP, 8-bromo-cyclic guanosine 3',5'-monophosphate; cGMP, cyclic guanosine 3',5'-monophosphate; GC, guanylate cyclase; IOP, intraocular pressure; SNP, sodium nitroprusside; SNOG, S-nitrosothiol; Spermine NONOate, N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine; YC-1, 3-(5'-hydroxymethyl-2'furyl)-1-benzylindazole

5.1.2 Effect of nitric oxide donors and cyclic GMP on aqueous humor outflow facility (Study II)

The outflow facility was measured at lower and higher IOP pressure levels. The outflow facility was increased after the intracameral injection of SNP (10 mM), 8-Br-cGMP (10 mM) and nitrosocaptopril (10 mM), while captopril (10 mM) had no effect (Table 6).

It is thus evident that intracamerally administered NO donors and a cyclic GMP analog increase aqueous humor outflow facility in rabbits.

Table 6. Effects of SNP, 8-Br-cGMP, nitrosocaptopril and captopril on aqueous humor outflow facility measured 20 min after administration to anesthetized rabbits (increase in experimental eye as compared to control, n = 6).

Compound	Dose	C_1	C_2
SNP	13.1 µg	80 %	74 %
8-Br-cGMP	22.3 µg	35 %	33 %
Nitrosocaptopril	12.3 µg	69 %	64 %
Captopril	10.9 µg	-12 %	2 %

8-Br-cGMP, 8-bromo-cyclic guanosine 3',5'-monophosphate; C_1, outflow facility at lower pressure level; C_2, outflow facility at higher pressure level; SNP, sodium nitroprusside

5.1.3 Effect of isosorbide-5-mononitrate on aqueous humor flow (Study III)

The rate of aqueous humor flow was not significantly altered after a single oral dose of the NO donor isosorbide-5-mononitrate (ISMN) (10 mg), as compared to placebo in healthy volunteers. Locally given timolol, a positive control, reduced the aqueous humor flow and IOP as compared to contralateral control eyes. IOP measured about 6 hours after drug administration did not differ between placebo and ISMN sessions. ISMN had no effect on systolic blood pressure, but diastolic blood pressure was reduced.

In conclusion, a single oral dose of 10 mg ISMN had no effect on aqueous humor flow. Since IOP after placebo and ISMN intake was at the same level, the rate of aqueous humor flow can be regarded as an indicator of the formation of aqueous humor.

5.1.4 Cyclic GMP production in iris-ciliary body (Study IV)

This study evaluated the possibility of using cyclic GMP production in the porcine iris-ciliary body as a screening criterion for correlation of IOP-lowering effects of NO donors

and GC activators. Cyclic GMP production was increased in the iris-ciliary body incubation by nitrosocaptopril, SNP, SNOG, spermine NONOate, NOR-3 and SNAP (Table 7). ODQ, the inhibitor of GC, totally inhibited the production of cyclic GMP after the administration of SNP and nitrosocaptopril. Captopril had no influence on cyclic GMP production. Activators of GC, YC-1 and atriopeptin III, increased production of cyclic GMP in a dose-dependent manner.

To summarize, various NO donors and GC activators increased cyclic GMP production in a tissue incubation experiment with the porcine iris-ciliary body roughly parallel to the IOP decrease seen in Study I.

Table 7. Effect of various NO-donors, guanylate cyclase activators and captopril on cyclic GMP production in the porcine iris-ciliary body 60 minutes after administration (mean ± SD, n = 6).

Compound	Concentration	Zaprinast (10 μM)	Cyclic GMP (pmol/mg protein)
Control		-	0.4 ± 0.09
		+	0.4 ± 0.1
Nitrosocaptopril	10 μM	-	0.9 ± 0.4 *
		+	1.9 ± 0.7 *
Captopril	10 μM	-	0.3 ± 0.1
		+	0.3 ± 0.2
SNP	10 μM	-	3.0 ± 1.4 *
		+	3.1 ± 1.1 *
SNOG	10 μM	-	3.6 ± 1.5 *
Spermine NONOate	10 μM	-	3.7 ± 1.8 *
NOR-3	10 μM	-	7.1 ± 3.1 *
SNAP	10 μM	-	5.0 ± 2.1 *
YC-1	100 μM	+	1.6 ± 0.3 *
Atriopeptin III	100 μM	+	0.8 ± 0.2 *

NOR-3, (E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-2-hexenamide; SNAP, S-nitroso-N-acetylpenicillamine; SNOG, S-nitrosothiol; SNP, sodium nitroprusside; Spermine NONOate, N-[4-[1-(3-aminopropyl)-2-hydroxy-2-nitrosohydrazino]butyl]-1,3-propanediamine; YC-1, 3-(5'-hydroxymethyl-2'furyl)-1-benzylindazole;
*, $p < 0.01$

5.2 BIOCHEMICAL MARKERS OF THE NITRIC OXIDE-CYCLIC GMP PATHWAY IN GLAUCOMA PATIENTS (STUDY V)

Study V involved 28 glaucoma patients undergoing unilateral cataract surgery in the glaucomatous eye. The concentrations of NO metabolites (NO_x, nitrite and cyclic GMP) in aqueous humor were slightly higher in glaucoma patients than in matched controls, but the difference was not statistically significant. The subgroup of glaucoma patients with pseudoexfoliation (n = 6) had lower NO_x and nitrite levels in the aqueous humor than the matched controls, while cyclic GMP concentrations were higher. Patients who used oral nitroglycerin (n = 18) had higher levels of NO_x and nitrite in the aqueous humor than patients without this medication (n = 58).

Taken together, in patients appropriately treated to obtain normal IOP no significant differences were found in NO metabolites in aqueous humor and serum between glaucoma and control patients. Glaucoma medications, keeping the IOP in balance, may have interfered with the results.

6 DISCUSSION

NO is beneficial as a messenger or modulator and for immunologic self-defence, but on the other hand, as it or its metabolites are highly reactive it is potentially toxic. There is accumulating evidence suggesting a role for NO in the control of IOP, but no current antiglaucomatous drugs are based on such a system. The present study clarified the effects of NO and cyclic GMP on IOP and their association with glaucoma. IOP was found to decrease after the administration of NO donors and cyclic GMP due to increased aqueous humor outflow facility, but aqueous humor flow was unaffected. NO donors increased cyclic GMP production in the porcine iris-ciliary body, which is an important site in IOP regulation. Appropriately treated glaucoma patients and their controls evinced no significant differences in NO metabolites in aqueous humor and serum.

6.1 METHODOLOGICAL ASPECTS

Rabbits are widely used in ophthalmological studies by reason of their convenient size, ease of handling and large eyes suitable for different kinds of ophthalmological examinations. The topical ocular route of administration corresponds well to a proposed human therapeutic route. However, there are species differences in drug effects, which poses a challenge to drug discovery. Albino rabbits are most often used, but some compounds bind to the pigment of the iris, i.e. drug distribution in pigmented eyes is different from that in albino eyes, and in these cases pigmented rabbits are preferred. Rabbits are found to provide a better model for drugs which suppress aqueous humor inflow than for drugs which enhance outflow facility (Dinslage et al. 1998). This is possibly due to the anatomical structures of the rabbit eye. The trabecular endothelial layer transports aqueous humor efficiently from the anterior chamber to the trabecular meshwork, rendering the uveoscleral outflow an insignificant pathway. The variation between humans and rabbits in responses to pharmacological agents may be explained by the presence of this trabecular endothelial layer in rabbits (Bergmanson 1985). However, most currently used antiglaucomatous drugs reduce IOP also in rabbits.

The optimal way to investigate the effects of new potential glaucoma drugs would be to use animals with naturally occurring glaucoma. Genetically predisposed animals (such as

beagles and rabbits) have been shown to develop spontaneous glaucoma (Kolker 1963, Gelatt et al. 1976). Gaasterland and Kupfer (1974) reported the first experimental model of glaucoma consequent upon laser treatment of the trabecular meshwork in rhesus monkeys. Laser-induced glaucoma in rabbits was developed by Gherezghiher and colleagues in 1986. In the present study we used albino rabbits with normal IOP. Indisputably, rabbits with ocular hypertension or glaucoma or monkeys with glaucoma would have been an appropriate object of investigation. Nevertheless, IOP in these rabbits was reduced by NO donors or guanylate cyclase stimulators equally to the response to currently used antiglaucomatous drugs. The decrease in IOP might have been even greater, if glaucoma rabbits had been used in the present study. In the future gene-manipulated glaucoma mice or rats may be adopted for glaucoma research, and NOS knockout mice would be useful in further studies of NO and glaucoma.

IOP was measured with a pneumatonometer calibrated to the eye of the rabbit. Pneumatonometry is the most accurate approach in IOP determination of anesthetized eyes as compared to applanation tonometry and TonoPen (Eisenberg et al. 1998). The limitation of this method is that it slightly disturbs normal aqueous humor dynamics. It has been found that immediately after pneumatonometry IOP increases by approximately 3 mmHg and remains elevated for about 1 hour and then decreases about 2 mmHg below normal pressure for approximately 1 hour (Dinslage et al. 1998). There are certain precepts applying to tonometry; the tonometer should be accurately calibrated, corneal bending forces or tear attractive forces must be negligible or counterbalanced by other forces, pressure during measurement must be the same as that immediately prior to tonometry and the endpoint of the measurement must be precise and objective (Brubaker 2001). To minimize the disturbance involved in pneumatonometry, IOP measurements in the present study were made simultaneously for the experimental and control eyes of the rabbit and measurement was carried out every hour, except at the beginning of the day, to ensure the recovery of IOP. In addition, the same person made measurements at the same time of day. IOP in human subjects was measured by Goldmann applanation tonometry, which is the most common clinical measurement system.

Outflow facility of aqueous humor has to be determined by indirect methods or calculated, because fluid drained via the trabecular or uveoscleral routes cannot be collected. In the present study, outflow facility was measured by the two-level constant

pressure infusion method. This approach is based on the assumption of pressure-independent outflow facility and secretion rate. On an average, the indirect method gives correct values, but at very low pressure levels the variation is considerable. Here, the explanation might be that the outflow facility is not constant under changing pressure, because the outflow channels are not always filled with aqueous humor in a normal manner (Bárány 1964). This method is accurate and even small changes in the outflow facility can be detected. A further advantage is that the test compound can be administrated direct to the anterior chamber. One disadvantage is that the trabecular or uveoscleral outflow cannot be distinguished. In addition, the method is somewhat complicated and the person conducting the experiment must be practised. Outflow facility may also be measured by tonography.

Aqueous humor flow can be determined noninvasively by measuring the disappearance of topically applied fluorescein from the cornea and anterior chamber, a technique first described by Jones and Maurice (1966). The measurements are carried out by fluorophotometer. Stipulations for fluorophotometry are that the fluorophotometer is accurately calibrated and focused within the corneal stroma, the tracer is uniformly distributed in cornea and anterior chamber, no tracer is lost from the anterior segment of the eye except for bulk outflow, the tracer does not sieve or pool and the time-span of the measurement is adequate for the rate of loss of tracer (Brubaker 2001). Fluorescein should be administrated several hours before the measurements to allow it to distribute evenly in the cornea. In several studies fluorescein has been applied in the evening before testing, or the test subjects have been instructed to wake at 1 - 2 a.m. on the day of the study, apply fluorescein into the eye and sleep on. However, the eyes should be thoroughly rinsed in order to remove excess fluorescein, which may disturb the measurements. In the present study fluorescein was applied and rinsed by the same person before the measurements made at the same time of day. The advantage of fluorophotometry is that aqueous humor flow can be measured in the unanesthetized eye *in vivo* without disturbing IOP, but especially in glaucoma research it would nonetheless be interesting to correlate IOP to aqueous humor flow. Fluorophotometry is currently used most frequently for measuring aqueous humor flow as an index of aqueous production (Brubaker 1991). Disadvantages of the technique are its relative complexity and the tedium it involves for the test subject and sources of errors such as slight haziness of the

cornea or epithelial scattering following traumatic tonometry (Brubaker and McLaren 1985).

The activation of guanylate cyclase can be determined in the porcine eye by a simple incubation method by measuring the production cyclic GMP that introduced in the present study. The iris-ciliary body is the target tissue of NO donors in the modulation of aqueous humor flow and thus this novel method might be useful in screening new molecules designed to stimulate the production of cyclic GMP and further reduce IOP. There are several advantages in this method: porcine eyes are easily available from an abattoir, the measurements are well repeatable and the cyclic GMP production by both rapid and slow NO donors can be measured. In addition, with this method it is possible to measure the activation of soluble GC, which cannot be determined in broken cell preparations. However, this incubation method is somewhat crude, in that the tissue samples are cut with a cornea trepan and detached with tweezers and the tissue samples may also contain other tissues (such as those from the anterior chamber angle) in addition to iris and ciliary body.

6.2 EFFECTS OF NO DONORS ON THE MODULATION OF INTRAOCULAR PRESSURE

In the present study, several NO-donating compounds were found to reduce IOP in rabbits, in accord with previous findings in both rabbits (Nathanson 1992, Behar-Cohen 1996, Sugrue 1997) and monkeys (Schuman 1994). L-arginine, the precursor of NO synthesis, lowered IOP at a fairly low concentration, as also found by groups under Chiou (1995) and Chuman (2000) in rabbits and in humans. L-arginine is a non-toxic natural amino-acid, being thus a convenient compound for an IOP-lowering drug through the formation of NO. A lipophilic derivative of L-arginine has been shown to increase the blood flow significantly by relaxing blood vessels in the ciliary body, retina and choroid, suggesting that arginine could be used in the treatment of ocular hypertension (Chiou et al. 1995). SNP, a widely used NO-releasing reference compound, lowered IOP for a few hours in the present study as also noted by Nathanson (1992). However, in one earlier study, topical administration of SNP was seen to increase IOP in a dose-response manner (Krupin et al. 1977). The ocular hypotensive effect of nitrosocaptopril, spermine NONOate and SNOG has not previously been reported. In the present study, nitrosocaptopril was

found to reduce IOP equally to SNP. Nitrosocaptopril would be a potential IOP-lowering drug, because it is both a NO donor and an angiotensin-converting enzyme (ACE) inhibitor which inhibits the breakdown of bradykinin, leading to the stimulation of NO synthesis. We observed no effect of topical captopril on IOP in rabbits. However, topically administered captopril and other ACE inhibitors have been shown to reduce IOP in rabbits (Watkins et al. 1987, Watkins et al. 1988, Vogh and Godman 1989), whereas oral captopril has been reported to have no significant effect on IOP in human volunteers (Al-Sereiti and Turner 1989, Costagliola et al. 1995). Spermine NONOate and SNOG had more marked effects on IOP when administered intravitreally, indicating poor penetration. The traditional NO-donating compound nitroglycerin has been shown in previous studies to reduce IOP (Wizemann and Wizemann 1980, Nathanson 1988, Nathanson 1992, Schuman et al. 1994), even though Wang and Podos (1995) found no significant IOP-lowering effect after topical administration in monkeys. In the present study, the IOP-lowering effect of NO donors was fairly short, lasting only a few hours. Combination of SNP and zaprinast, a PDE5/6-inhibitor specific for the breakdown of cyclic GMP, prolonged the IOP-lowering effect by an hour. The combination of NO donors and PDE inhibitor might be one solution in the development of new antiglaucomatous drugs with administration once or twice a day. Elevated levels of NO metabolites were also found in the control eyes, indicating systemic distribution of metabolites or these test compounds with short half-life. The samples were taken 2 hours after administration (except 24 hours after intravitreal injection of atriopeptin III), when IOP was lowest. This time period might have been too long to show differences between the treated and control eyes.

The literature is inconsistent regarding the mechanism by which NO lowers IOP. In order to elucidate this mechanism aqueous humor outflow facility and aqueous humor flow after NO-donating compounds were measured in the present study. Further, the action of these compounds on the ciliary body, the site of aqueous humor production and outflow, was investigated.

NO-donating compounds have been shown to lower IOP in rabbits or monkeys, but whether the underlying mechanism is an enhancement of aqueous humor outflow has not previously been clearly confirmed *in vivo*. In the present study, we found that SNP and nitrosocaptopril enhanced aqueous humor outflow facility, while the control compound captopril did not. This would indicate a significant role of NO in the regulation of IOP by

aqueous humor outflow. Unfortunately, it is not possible to distinguish between trabecular and uveoscleral outflow in the two-level constant pressure infusion method used in the current study. The nitrovasodilator nitroglycerin and hydralazine have been shown to reduce IOP and increase outflow facility at certain drug doses, but not others, after intracameral administration in monkeys (Schuman et al. 1994). Topically applied nitroglycerin increased outflow facility in rabbits (Nathanson 1992). Inhibition of endogenous NOS activity resulted in a significant reduction of aqueous humor outflow through the human trabecular meshwork in the anterior segment perfusion model. In this model, application of SNP resulted in a significant increase in flow rate (Schneemann et al. 2002). In the present study, the possible effect of pseudofacility caused by the breakdown of the blood-aqueous barrier was investigated by determining the aqueous humor protein concentration after intracameral administration of nitrosocaptopril. No difference between treated and control eyes was found.

Once it emerged that NO-donating compounds lower IOP and enhance aqueous humor outflow facility, the next step was to clarify their effect on aqueous humor production by measuring the aqueous humor flow. It would have been interesting to investigate the effects of e.g. nitrosocaptopril or other NO donors, but in this measurement system, i.e. fluorophotometry, the test subjects were humans and thus, in view of the lack of toxicological data or for ethical reasons, use of these compounds was not permissible. Organic nitrates contain one or more nitrate functional group in their structure and their pharmacological effects are due to metabolic transformation of the nitrate group to NO (for review, see Vapaatalo 1994). In the present study we used a single oral dose of 10 mg isosorbide-5-mononitrate (ISMN), which had no significant effect on aqueous humor flow. To our knowledge, this is the first study investigating the effects of oral ISMN on aqueous humor flow. Higher doses of ISMN were not used because undesirable adverse effects shown in the pilot study might have disturbed the measurements. Since IOP after placebo and ISMN intake was at the same level, the rate of aqueous humor flow can be regarded as an indicator of the formation of aqueous humor. However, ISMN may have enhanced aqueous humor outflow facility and thus increased aqueous humor formation, since IOP remained stable. Studies on the effect of ISMN on aqueous humor outflow facility have not been reported. Diestelhorst and co-workers (1991) showed an increase in aqueous humor flow after topical ISMN, but since this increase was also found in control eyes the result was statistically not significant. Topical nitroglycerin has been shown to cause a decrease

in aqueous humor production, but to such a minor extent that it could not explain the ocular hypotension observed (Nathanson 1992).

NO has been shown to be a transmitter of smooth muscle relaxation in the chamber angle and it might thus be involved in the regulation of aqueous humor dynamics. This relaxation of the ciliary muscle and trabecular meshwork results from an increase in intracellular cyclic GMP (Wiederholt et al. 1994). On the other hand, eNOS and nNOS have been shown to be present in the ciliary processes, indicating a role of NO in aqueous humor production (Osborne et al. 1993, Meyer et al. 1999). The ciliary body is an important tissue in the modulation of IOP in that it may regulate both aqueous humor formation and outflow. In the present study, it was found that NO donors with IOP-lowering ability increased cyclic GMP production in the porcine iris-ciliary body. SNP increased the cyclic GMP concentration up to ten times as compared to control. This is consistent with previous findings; SNP has been shown to increase cyclic GMP formation in bovine (Ding and Abdel-Latif 1997, Masuda et al. 1997, Kamikawatoko et al. 1998) and porcine (Fujimoto et al. 1998) ciliary muscle and to induce cyclic GMP-dependent ciliary muscle or trabecular meshwork relaxation in cats, bovines, dogs, monkeys and humans (Wiederholt et al. 1994, Goh et al. 1995, Azuma et al. 1997, Ding and Abdel-Latif 1997, Masuda et al. 1997, Kamikawatoko et al. 1998). Other NO donors, nitrosocaptopril, SNAP, SNOG, spermine NONOate and NOR-3, clearly increased the cyclic GMP concentration in the porcine iris-ciliary body. SNAP has also been found to relax ciliary muscle in bovines and cats (Wiederholt et al. 1994, Goh et al. 1995). In the present study a guanylate cyclase (GC)-inhibitor, ODQ, totally abolished the production of cyclic GMP after administration of SNP and nitrosocaptopril. These data indicate that this incubation method is valid.

The effect of NO production can be beneficial to the retina, where it can increase the blood circulation and facilitate the flow of metabolites. SNP has been shown to have a dose-dependent neuroprotective effect on retinal ganglion cells (Nakazawa et al. 2002). In a previous study, L-arginine and SNAP blocked cell death induced by anisomycin in the neuroblastic layer of retina in newborn rats (Guimarães et al. 2001). Excessive NO, however, may damage retinal tissues in a free radical oxidative mechanism by forming peroxynitrite. Two studies have demonstrated *in vivo* retinal neuronal death by intraocular administration of NO donors (Oku et al. 1997, Takahata et al. 2003). Induction of NOS in the optic nerve leads to glaucomatous damage (for review, see Neufeld 1999). A large

amount of the radical form of NO adequate to interact with oxygen radicals, is required to induce retinal damage, because NO has also pharmacologic functions such as lowering IOP and increasing retinal blood flow.

6.3 EFFECTS OF GUANYLATE CYCLASE ACTIVATORS AND CYCLIC GMP ON THE MODULATION OF INTRAOCULAR PRESSURE

Recent studies have demonstrated that elevation of ocular levels of cyclic GMP by GC activators or cyclic GMP analogs is associated with a reduction in IOP (Sugrue and Viader 1986, Mittag et al. 1987, Nathanson 1987, Diestelhorst and Krieglstein 1989, Korenfeld and Becker 1989, Becker 1990, Samuelsson-Almén et al. 1991, Busch et al. 1992, Stein and Clack 1994, Takashima et al. 1996). In accord with this, we found in the present study that the GC activators, atriopeptin and YC-1, and a cyclic GMP analog, 8-Br-cGMP, lowered IOP in rabbits. Atriopeptin, the activator of particulate GC, had a long-lasting effect with duration of up to two days. This could be due to persistent levels of atriopeptin in the ciliary processes (Nathanson 1987). YC-1, a NO-independent activator of soluble GC, induced a short-lasting decrease in IOP. This is to our knowledge the first study to show IOP effects of YC-1. As YC-1 potentiates the stimulatory effect of NO (Friebe et al. 1996), a combination of YC-1 and a NO donor, e.g. SNP, would be effective in lowering IOP. Due to direct synergistic action of YC-1 and NO on the soluble GC, low doses of YC-1 might be of therapeutic value in permitting a reduction of NO donor dosage (Mülsch et al. 1997). Decreased IOP after administration of 8-Br-cGMP confirmed the conception that increased cyclic GMP is associated with lowering of IOP. Cyclic GMP concentrations in aqueous humor after atriopeptin and 8-Br-cGMP were high compared to control eyes, suggesting no significant systemic absorption.

It has been proposed that atriopeptin lowers IOP by reducing aqueous humor formation (Mittag et al. 1987, Millar et al. 1997, Shahidullah and Wilson 1999). Samuelsson-Almén and co-workers (1991) found aqueous humor flow to be increased by approximately 50% after intracameral administration of atriopeptin in monkeys and uveoscleral outflow also tended to increase. Intravitreal injection of other endogenous ligands of natriuretic peptides, brain natriuretic peptide and C-type natriuretic peptide, reduced IOP in rabbits due to an increase in the outflow facility, this effect being associated with an increase in cyclic GMP concentration in the aqueous humor (Takashima et al. 1996, Takashima et al.

1998). In the present study, 8-Br-cGMP clearly increased aqueous humor outflow facility, which supports the earlier findings of Kee and co-workers (1994), even though they observed the positive effect on outflow facility only after intravitreal administration of 8-Br-cGMP. However, Becker (1990) showed that a decrease in IOP after 8-Br-cGMP was not due to enhanced outflow facility as measured by a different system, tonography. Exogenously applied 8-Br-cGMP has been shown to relax the ciliary muscle strips (Masuda 1997, Kamikawatoko 1998), which would suggest a role for cyclic GMP in increasing outflow.

In the present study, atriopeptin and YC-1 increased cyclic GMP production in the porcine iris-ciliary body dose-dependently, though their effect on cyclic GMP synthesis was smaller than with most of the NO donors. In the eye, the number of specific binding sites for atriopeptin is high in the ciliary body (Mantyh et al. 1987). Atriopeptin receptors, coupled to the activation of GC, are found to be present in the ciliary processes (Bianchi et al. 1986, Nathanson 1987). An increase in cyclic GMP concentration has been found in iris-ciliary body preparations after exposure to physiological concentrations of atriopeptin *in vitro* (Korenfeld and Becker 1989, Millar et al. 1997).

6.4 NITRIC OXIDE AND CYCLIC GMP IN GLAUCOMA PATIENTS

In order to investigate the clinical association between NO and glaucoma, the endogenous metabolites of NO were measured in glaucoma patients undergoing cataract surgery. A deficit in NOS-like reactivity has been shown in the ciliary muscle and outflow pathway in patients with primary open-angle glaucoma (Nathanson and McKee 1995b). The hypothesis in the present study was that NO metabolites would be lower in glaucoma patients as compared to control patients, this in view of the findings of Nathanson and McKee and our earlier results showing reduced IOP after administration of NO donors. However, no significant difference in NO metabolites in aqueous humor and serum were found between glaucoma and control patients. Glaucoma patients were further divided into subgroups according to the number of glaucoma drugs used, on the assumption that this would correlate with the degree of severity of glaucoma. The levels of NO metabolites in aqueous humor were fairly consistent with the conception that the more difficult it is to achieve the targeted IOP, the lower NO_x and nitrite concentrations will be. Earlier studies have shown considerable variability in NO levels in the aqueous humor of glaucoma

patients. Aqueous humor NO levels in primary open-angle and acute angle closure glaucoma have been found to be higher than in control patients (Chang et al. 2000, Tsai et al. 2002). In another study, decreased nitrite levels were found in the aqueous humor of primary open-angle glaucoma patients (Doganay et al. 2002). Cyclic GMP levels in aqueous humor in normal-tension glaucoma patients have been shown to be lower than those in control patients (Galassi et al. 2000). It is not possible to say whether the alterations in NOS and NO metabolites in primary open-angle glaucoma are causally related to glaucoma or secondary manifestations of the condition. In the present study, 92% of glaucoma patients were taking glaucoma medication and this may have masked the real changes in NO metabolites, which are possibly unbalanced in untreated glaucoma patients. It is not known whether the antiglaucomatous drugs currently used have a direct effect on the L-arginine-NO-cyclic GMP pathway. The control patients were matched for sex, age, smoking habits and organic nitrate medication to eliminate the potential confounding factors.

An interesting finding in the present study was that systemic hypertension was diagnosed twice as often in glaucoma patients as in the controls. Systolic and diastolic blood pressures were higher in glaucoma patients, the difference in diastolic blood pressure being statistically significant. In previous studies, systemic hypertension has been found to be associated with glaucoma (Tielsch et al. 1995, Leske et al. 1996). On one hand systemic hypertension may damage small vessels of the optic disc, on the other, low blood pressure might impair perfusion of the optic nerve. The relationship between blood pressure and glaucoma is complex and there is therefore some variability in results of different studies. Hypertension has been found significantly more frequently in persons with high-tension open-angle glaucoma (Dielemans et al. 1995, Bonomi et al. 2000) and these patients also have high diastolic blood pressure (Leske et al. 1996). Results of a previous study suggest that systemic hypertension does not increase the risk of open-angle glaucoma, while low perfusion pressure (blood pressure – IOP) approximately triples the risk. In addition, antihypertensive treatment did not increase the risk of open-angle glaucoma (Leske et al. 2002). Disturbed circulation and a systemic tendency to vasospasm may reflect endothelial dysfunction with decreased NO production in glaucoma patients (Drance et al. 1988, Haefliger et al. 1994, Flammer et al. 1999).

In conclusion, current glaucoma therapy remains that of lowering IOP. NO has a dual role in the pathogenesis of glaucoma; this role is dose-dependent. The overproduction of NO leads to retinal ganglion cell degeneration, but on the other hand NO has an important role in the regulation of regional blood flow in retina. NO donors have all the features of an optimal medical treatment for glaucoma; they reduce IOP, may provide additional vasodilation and may also offer neuroprotection.

7 SUMMARY AND CONCLUSIONS

The present study was set out to establish the roles of NO and cyclic GMP in the modulation of IOP, the mechanism of their IOP-lowering effects and the possible clinical relevance of NO in aqueous humor dynamics in glaucoma patients.

The main findings were the following:

1. Chemically different NO donors and particulate and soluble guanylate cyclase-activating compounds enhanced cyclic GMP production in iris-ciliary body and they lowered IOP in ocular normotensive rabbits. In addition, a cyclic GMP analog lowered IOP, suggesting the IOP-regulatory role of cyclic GMP.

2. NO donors and a cyclic GMP analog increased aqueous humor outflow facility in rabbits after intracameral administration, indicating the significance of that mechanism in the IOP-lowering effect of the NO/cyclic GMP pathway.

3. A single oral dose of isosorbide-5-mononitrate had no effect on aqueous humor flow in healthy volunteers. Since IOP after placebo and ISMN intake was at the same level, the rate of aqueous humor flow can be regarded as an indicator of the formation of aqueous humor, which may indicate that the NO/cyclic GMP pathway has no significant effect on aqueous humor production.

4. No significant differences in NO metabolites in aqueous humor and serum were found between treated glaucoma and control patients. Glaucoma medications may have interfered with the results.

In conclusion, it is well-founded to suggest that NO plays a role in the regulation of IOP. The evidence obtained in this study points to a contribution of cyclic GMP in this process. A non-toxic NO-donating or guanylate cyclase-activating compound would represent a new class of antiglaucomatous treatment.

8 ACKNOWLEDGEMENTS

The present study was carried out at the Institute of Biomedicine, Pharmacology, at the University of Helsinki, the Medical School at the University of Tampere and Tampere University Hospital during the years 1999-2003.

I wish to express my respectful gratitude to my supervisor Professor Heikki Vapaatalo. It has been a privilege to work under his excellent guidance. His wide experience in scientific work, his unfailing optimism and continuous encouragement have been very invaluable to me. I would also like to thank Professor Vapaatalo and his successor Professor Esa Korpi for placing the research facilities of the Department of Pharmacology at my disposal during this work. I am greatly indebted to my other supervisor, Professor Eeva Moilanen, for her advice and for sharing her profound knowledge of nitric oxide research.

I take this opportunity to thank Professor Leila Laatikainen and Docent Pauli Vuorinen for their critical and constructive revision of the manuscript.

I wish to thank my co-authors in the original studies for kind and fruitful collaboration. I am especially grateful to Olli Oksala, PhD for a number of enlightening and interesting conversations and patient advice. His expertise in the field of experimental ophthalmology has impressed me deeply. I sincerely thank Esko Kankuri, MD, PhD for his important contribution with the laboratory techniques and for guidance in practical matters there. My thanks are also due to Pertteli Salmenperä, BSc for sharing his special knowledge of nitric oxide synthases. I warmly acknowledge the contribution of Päivi Alajuuma, MSc in inspiring and constructive discussions in and outside of the scientific world. I also owe a debt of gratitude to late Professor Pekka Laippala who offered me invaluable statistical advice.

The considerable support accorded me by Santen Oy is most gratefully acknowledged. I would especially warmly like to thank Santen Oy, President Jyrki Liljeroos and Vice President, Research and Development, Kari Lehmussaari for their valuable support and positive attitude towards my study. I also wish to express my gratitude to Professor Pertti Neuvonen and Docent Esko Pohjala for offering their support.

My sincere gratitude goes to Ms Mari Hämäläinen, MSc, Ms Niina Ikonen, Ms Hanna-Mari Kankaanpää, Ms Pirkko Lahti, MSc, Ms Jaana Laine, Ms Marja-Leena Lampén, Ms Pirkko Möller, Ms Riina Nieminen, MSc, Ms Eija Nissinen, Ms Minna Riekkinen, Ms Leena Seppo, MSc, Ms Anne Tuhkanen, Ms Jaana Tuure and Ms Kirsti Vähätupa, for their competent technical assistance. Especially I would like to thank Ms Marja Mali for teaching me the animal experimental techniques and for her friendly help in all kinds of practical questions. I am very grateful to Ms Aira Säisä for indispensable laboratory work. I wish to thank Ms Eeva Harju and Ms Heli Määttä for skilful secretarial assistance. Statistical advice from Heini Huhtala, MSc and Hannu Kautiainen, MSc is greatly appreciated. The help of Robert MacGilleon, MA is gratefully acknowledged in the linguistic revision of this thesis.

I express my profound appreciation to my parents Liisa and Esko Aine for their care and encouragement throughout my life. They have contributed to the preparation of this thesis in numerous ways. I wish to thank with all my heart my father for introducing me initially to scientific research and for standing by me throughout this work. I would also like to thank my brother Antti and my sister Leena for backing me up during these years. We three have always been a great team together! I am deeply indebted to my mother-in-law, Pirkko Kotikoski, who took good care of Emil and let me accomplish this thesis. My warm thanks go to all my friends for their support and interest towards my work.

Finally, I would like to thank my dear husband Mikko for loving care and unfailing support. I am happy that we have had a chance to share the delights and drawbacks of scientific work together. My warmest thoughts are devoted to our sweet little son Emil. He has filled my days with sunshine and joy during these last nine months.

I am grateful for the financial support of the National Technology Agency (TEKES), the Clinical Drug Research Graduate School, the Academy of Finland (to Professor Moilanen), the Medical Research Fund of Tampere University Hospital, the Eye Foundation (Silmäsäätiö), the Eye and Tissue Bank Foundation (Silmä- ja kudospankkisäätiö), the Glaucoma Research Foundation (Glaukooma Tukisäätiö Lux) and a grant from the Meeri Sinivuori Testament.

Tampere, August 2003

Hanna Kotikoski

9 REFERENCES

Alderton WK, Cooper CE and Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001;357:593-615.

Allingham RR, Loftsdottir M, Gottfredsdottir MS, Thorgeirsson E, Jonasson F, Sverisson T, Hodge WG, Damji KF and Stefánsson E. Pseudoexfoliation syndrome in Icelandic families. Br J Ophthalmol 2001;85:702-707.

Al-Sereiti MR and Turner P. Effect of captopril (an angiotensin-converting enzyme inhibitor) on intraocular pressure in healthy human volunteers. J Ocul Pharmacol 1989;1:1-5.

Änggård E. Nitric oxide: mediator, murderer, and medicine. Lancet 1994;343:1199-1206.

Araie M, Sekine M, Suzuki Y and Koseki N. Factors contributing to the progression of visual field damage in eyes with normal tension glaucoma. Ophthalmology 1994;101:1440-1444.

Arnold WP, Katsuki S and Murad F. Nitric oxide activates guanylate cyclase and increases guanosine 3':5'- cyclic monophosphate levels in various tissue preparations. Proc Natl Acad Sci USA 1977;74:3203-3207.

Astin M, Stjernschantz J and Selen G. Role of nitric oxide in PGF2 alpha-induced ocular hyperemia. Exp Eye Res 1994;59:401-407.

Azuma H, Masuda H, Sato J, Niwa K and Tokoro T. A possible role of endogenous inhibitor for nitric oxide synthesis in the bovine ciliary muscle. Exp Eye Res 1997;64:823-830.

Bárány EH. Simultaneous measurement of changing intraocular pressure and outflow facility in the vervet monkey by constant pressure infusion. Invest Ophthalmol Vis Sci 1964;3:135-143.

Bathija R, Gupta N, Zangwill L and Weinreb RN. Changing definition of glaucoma. J Glaucoma 1998;7:165-169.

Beck RW, Messner DK, Musch DC, Martonyi CL and Lichter PR. Is there a racial difference in physiologic cup size? Ophthalmology 1985;92:873-876.

Becker B. Topical 8-bromo-cyclic GMP lowers intraocular pressure in rabbits. Invest Ophthalmol Vis Sci 1990;31:1647-1649.

Beckman JS and Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly. Am J Physiol 1996;271:C1424-C1437.

Becquet F, Courtois Y and Goureau O. Nitric oxide in the eye: multifaceted roles and diverse outcomes. Surv Ophthalmol 1997;42:71-82.

Behar-Cohen FF, Goureau O, D'Hermies F and Courtois Y. Decreased intraocular pressure induced by nitric oxide donors is correlated to nitrite production in the eye. Invest Ophthalmol Vis Sci 1996;37:1711-1715.

Bergmanson JPG. The anatomy of the rabbit aqueous outflow pathway. Acta Ophthalmol 1985;63:493-501.

Bianchi C, Anand-Srivastava MB, De Lean A, Gutkowska J, Forthomme D, Genest J and Cantin M. Localization and characterization of specific receptors for atrial natriuretic factor in the ciliary processes of the eye. Curr Eye Res 1986;5:283-293.

Biel M, Sautter A, Ludwig A, Hofmann F and Zong X. Cyclic nucleotide-gated channels – mediators of NO:cGMP-regulated processes. Naunyn Schmiedeberg's Arch Pharmacol 1998;358:140-144.

Bill A. Further studies on the influence of the intraocular pressure on aqueous humor dynamics in cynomolgus monkeys. Invest Ophthalmol 1967;6:364-372.

Bill A. Some aspects of aqueous humor drainage. Eye 1993;7:14-19.

Bonomi L, Marchini G, Marraffa M, Bernardi P, Morbio R and Varotto A. Vascular risk factors for primary open angle glaucoma. Ophthalmology 2000;107:1287-1293.

Braman RS and Hendrix SA. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium (III) reduction with chemiluminescence detection. Anal Chem 61:2715-2718, 1989.

Bredt DS and Snyder SH. Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem 1994;63:175-195.

Brubaker RF. Measurement of aqueous humor flow by fluorophotometry. In: The Glaucomas, Eds. Ritch R, Shields MB and Krupin T, The C.V. Mosby Company, St. Louis, 1989, pp. 337-344.

Brubaker RF. Clinical evaluation of the circulation of aqueous humor. In: Duane's Clinical Ophthalmology, Eds. Tasman W and Jaeger EA, J.B. Lippincott Company, Philadelphia, 1994, Vol. 3, Chap. 46, pp. 1-11.

Brubaker RF. Flow of aqueous humor in humans. Invest Ophthalmol Vis Sci 1991;32:3145-3166.

Brubaker RF. Measurement of uveoscleral outflow in humans. J Glauc 2001;10:S45-S48.

Brubaker RF and McLaren JW. Uses of fluorophotometry in glaucoma research. Ophthalmology 1985;92:884-890.

Busch MJWM, van Oosterhout EJGM and Hoyng PFJ. Effects of cyclic nucleotide analogs on intraocular pressure and trauma-induced inflammation in the rabbit eye. Curr Eye Res 1992;11:5-13.

Butler AR and Rhodes P. Chemistry, analysis, and biological roles of S-nitrosothiols. Anal Biochem 1997;249:1-9.

Caprioli J. The ciliary epithelia and aqueous humor. In: Adler's Physiology of the Eye, Eds. Hart WM, Mosby-Year Book Inc., St. Louis, 1992, Chap. 7, pp. 228-247.

Chang C-J, Chiang C-H, Chow JC-K and Lu D-W. Aqueous humor nitric oxide levels differ in patients with different types of glaucoma. J Ocul Pharmacol Ther 2000;16:399-406.

Chiou GCY. Review: effects of nitric oxide on eye diseases and their treatment. J Ocul Pharmacol Ther 2001;17:189-198.

Chiou GCY, Liu SXL, Li BHP and Varma RS. Ocular hypotensive effects of L-arginine and its derivatives and their actions on ocular blood flow. J Ocul Pharmacol Ther 1995;11:1-10.

Chuman H, Chuman T, Nao-i N and Sawada A. The effect of L-arginine on intraocular pressure in the human eye. Curr Eye Res 2000;20:511-516.

Coleman AL and Brigatti L. The glaucomas. Minerva Med 2001;92:365-379.

Costagliola C, Di Benedetto R, De Caprio L, Verde R and Mastropasqua L. Effect of oral captopril (SQ 14225) on intraocular pressure in man. Eur J Ophthalmol 1995;1:19-25.

Damji KF, Bains HS, Stefansson E, Loftsdottir M, Sverrisson T, Thorgeirsson E, Jonasson F, Gottfredsdottir M and Allingham RR. Is pseudoexfoliation syndrome inherited? A review of genetic and nongenetic factors and a new observation. Ophthalmic Genet 1998;19:175-185.

Davis KL, Martin E, Turko IV and Murad F. Novel effects of nitric oxide. Annu Rev Pharmacol Toxicol 2001;41:203-236.

Dielemans I, Vingerling JR, Algra D, Hofman A, Grobbee DE and de Jong PT. Primary open-angle glaucoma, intraocular pressure, and systemic blood pressure in the general elderly population: the Rotterdam Study. Ophthalmology 1995;102:54-60.

Diestelhorst M, Hinzpeter B and Krieglstein GK. The effect of isosorbide-mononitrate eye drops on the human intraocular pressure and aqueous humor dynamics. Int Ophthalmol 1991;15:259-262.

Diestelhorst M and Krieglstein GK. The intraocular pressure response of human atrial natriuretic factor in glaucoma. Int Ophthalmol 1989;13:99-101.

Ding K-H and Abdel-Latif AA. Actions of C-type natriuretic peptide and sodium nitroprusside on carbachol-stimulated inositol phosphate formation and contraction in ciliary and iris sphincter smooth muscles. Invest Ophthalmol Vis Sci 1997;38:2629-2638.

Dinslage S, McLaren J and Brubaker R. Intraocular pressure in rabbits by telemetry II: effects of animal handling and drugs. Invest Ophthalmol Vis Sci 1998;39:2485-2489.

Doganay S, Evereklioglu C Turkoz Y and Er H. Decreased nitric oxide production in primary open-angle glaucoma. Eur J Ophthalmol 2002;12:44-48.

Drance SM, Douglas GR, Wijsman K, Schulzer M and Britton RJ. Response of blood flow to warm and cold in normal and low-tension glaucoma patients. Am J Ophthalmol 1988;105:35-39.

Dreyer EB, Zurakowski D, Schumer RA, Podos SM and Lipton SA. Elevated glutamate in the vitreous body of humans and monkeys with glaucoma. Arch Ophthalmol 1996;114:643-651.

Eisenberg DL, Sherman BG, McKeown CA and Schuman JS. Tonometry in adults and children. A monometric evaluation of pneumatonometry, applanation, and TonoPen in vitro and in vivo. Ophthalmology 1998;105:1173-1181.

Ekström C. Elevated intraocular pressure and pseudoexfoliation of the lens capsule as risk factors for chronic open-angle glaucoma. A population based five-year follow-up study. Acta Ophthalmol 1993;71:189-195.

Ellis D and Nathanson J. Nitric oxide in the human eye: sites of synthesis and physiologic actions on intraocular pressure, blood flow, sodium transport and neuronal viability. In: Nitric Oxide and Endothelin in the Pathogenesis of Glaucoma, Eds. Haefliger IO and Flammer J, Lippincott-Raven Publishers, Philadelphia, 1998, Chap. 16, pp. 178-204.

Ethier CR. The inner wall of Schlemm's canal. Exp Eye Res 2002;74:161-172.

Farrell AJ and Blake DR. Nitric oxide. Ann Rheum Dis 1996;55:7-20.

Feelisch M. The biochemical pathways of nitric oxide formation from nitrovasodilators: appropriate choice of exogenous NO donors and aspects of preparation and handling of aqueous NO solutions. J Cardiovasc Pharmacol 1991;17 Suppl 3:S25-S33.

Feelisch M. Biotransformation to nitric oxide of organic nitrates in comparison to other nitrovasodilators. Eur Heart J 1993;14 Suppl I:123-132.

Feelisch M. The use of nitric oxide donors in pharmacological studies. Naunyn Schmiedebergs Arch Pharmacol 1998;358:113-122.

Feelisch M, Ostrowski J and Noack E. On the mechanism of NO release from sydnonimines. J Cardiovasc Pharmacol 1989;14 Suppl 11:S13-S22.

Feelisch M, Schönafinger K and Noack E. Thiol-mediated generation of nitric oxide accounts for the vasodilator action of furoxans. Biochem Pharmacol 1992;44:1149-1157.

Fernandez-Durango R, Moya FJ, Ripodas A, de Juan JA, Fernandez-Cruz A and Bernal R. Type B and type C natriuretic peptide receptors modulate intraocular pressure in the rabbit eye. Eur J Pharmacol 1999;364:107-113.

Flammer J. Classification of the various types of glaucoma. In: Glaucoma, Eds. Flammer J, Verlag Hans Huber, Bern, 2001, Chap. 3, pp. 38-75.

Flammer J, Haefliger IO, Orgül S and Resink T. Vascular dysregulation: a principal risk factor for glaucomatous damage? J Glauc 1999;8:212-219.

Flammer J, Orgül S, Costa VP, Orzalesi N, Krieglstein GK, Serra LM, Renard JP and Stefansson E. The impact of ocular blood flow in glaucoma. Prog Retin Eye Res 2002;21:359-393.

Fleischhauer JC, Liu R, Elena PP, Flammer J and Haefliger IO. Topical ocular instillation of nitric oxide synthase inhibitors and intraocular pressure in rabbits. Klin Monatsbl Augenheilkd 2001;218:351-353.

Freddo TF. Shifting the paradigm of the blood-aqueous barrier. Exp Eye Res 2001;73:581-592.

Friebe A, Schultz G and Koesling D. Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. EMBO J 1996;15:6863-6868.

Fujimoto N, Hanawa T and Kimura T. Effect of cAMP- and cGMP-related drugs on porcine cultured ciliary muscle. J Ocul Pharmacol Ther 1998;14:247-252.

Furchgott RF and Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373-376.

Gaasterland D and Kupfer C. Experimental glaucoma in the rhesus monkey. Invest Ophthalmol 1974;13:455-457.

Galassi F, Sodi A, Ucci F, Renieri G, Pieri G and Masini E. Ocular dynamics and nitric oxide in normal pressure glaucoma. Acta Ophthalmol Scand 2000; Suppl 78:37-38.

Gasser P. Why study vascular factors in glaucoma? Int Ophthalmol 1999;22:221-225.

Gasser P and Flammer J. Blood-cell velocity in the nailfold capillaries of patients with normal-tension glaucoma and of healthy controls. Am J Ophthalmol 1991;111:585-588.

Gelatt KN, Pfeiffer RLJr, Gwin RM and Sauk JJJr. Glaucoma in the beagle. Trans Am Acad Ophthalmol Otolaryngol 1976;81:OP636-644.

Geyer O, Podos SM and Mittag T. Nitric oxide synthase activity in tissues of the bovine eye. Graefe's Arch Clin Exp Ophthalmol 1997;235:786-793.

Gherezghiher T, March WF, Nordquist RE and Koss MC. Laser-induced glaucoma in rabbits. Exp Eye Res 1986;43:885-894.

Glasser SP. Prospects for therapy of nitrate tolerance. Lancet 1999;353:1545-1546.

Goh Y, Hotehama Y and Mishima HK. Characterization of ciliary muscle relaxation induced by various agents in cats. Invest Ophthalmol Vis Sci 1995;36:1188-1192.

Gow AJ and Ischiropoulos H. Nitric oxide chemistry and cellular signaling. J Cell Physiol 2001;187:277-282.

Graham SL and Drance SM. Nocturnal hypotension: role in glaucoma progression. Surv Ophthalmol 1999;43 Suppl 1:S10-S16.

Grehn F. World health problem of glaucoma. J Glauc 2001;10 Suppl 1:S2-S4.

Greve EL, Ferdinand H, Duijm A and Geijssen HC. Risk factors in glaucoma. In: Nitric Oxide and Endothelin in the Pathogenesis of Glaucoma, Eds. Haefliger IO and Flammer J, Lippincott-Raven Publishers, Philadelphia, 1998, Chap. 1, pp. 1-13.

Grodum K, Heijl A and Bengtsson B. Refractive error and glaucoma. Acta Ophthalmol Scand 2001;79:560-566.

Guimarães C, Assreuy J and Linden R. Paracrine neuroprotective effect of nitric oxide in the developing retina. J Neurochem 2001;76:1233-1241.

Haefliger IO and Dettmann ES. Nitric oxide and endothelin in the pathogenesis of glaucoma: an overview. In: Nitric Oxide and Endothelin in the Pathogenesis of Glaucoma, Eds. Haefliger IO and Flammer J, Lippincott-Raven Publishers, Philadelphia, 1998, Chap. 3, pp. 22-33.

Haefliger IO, Dettmann E, Liu R, Meyer P, Prünte C, Messerli J and Flammer J. Potential role of nitric oxide and endothelin in the pathogenesis of glaucoma. Surv Ophthalmol 1999;43 Suppl 1:S51-S58.

Haefliger IO, Meyer P, Flammer J and Lüscher TF. The vascular endothelium as a regulator of the ocular circulation: a new concept in ophthalmology? Surv Ophthalmol 1994;39:123-132.

Hart WM. Intraocular pressure. In: Adler's Physiology of the Eye, Eds. Hart WM, Mosby-Year Book Inc., St. Louis, 1992, Chap. 8, pp. 248-267.

Haufschild T, Nava E, Meyer P, Flammer J, Lüscher TF and Haefliger IO. Spontaneous calcium-independent nitric oxide synthase activity in porcine ciliary processes. Biochem Biophys Res Comm 1996;222:786-789.

Haufschild T, Tschudi MR, Flammer J, Lüscher TF and Haefliger IO. Nitric oxide production by isolated human and porcine ciliary processes. Graefe's Arch Clin Exp Ophthalmol 2000;238:448-453.

Hayreh SS. The role of age and cardiovascular disease in glaucomatous optic neuropathy. Surv Ophthalmol 1999;43 Suppl 1:S27-S42.

Hecker M, Vorhoff W, Bara AT, Mordvintcev PI and Busse R. Characterization of furoxans as a new class of tolerance-resistent nitrovasodilators. Naunyn Schmiedeberg's Arch Pharmacol 1995;351:426-432.

Hendrickx KH, van den Enden A, Rasker MT and Hoyng PFJ. Cumulative incidence of patients with disc hemorrhages in glaucoma and the effect of therapy. Ophthalmology 1994;101:1165-1172.

Hinz B and Schröder H. Nitrate tolerance is specific for nitrate acid esters and its recovery requires an intact protein synthesis. Biochem Biophys Res Commun 1998;252:232-235.

Hinz B and Schröder H. The nitric oxide donor SIN-1 is free of tolerance and maintains its cyclic GMP stimulatory potency in nitrate-tolerant LLC-PK_1 cells. Pharm Res 1999;16:633-636.

Hirvelä H, Tuulonen A and Laatikainen L. Intraocular pressure and prevalence of glaucoma in elderly people in Finland: a population-based study. Int Ophthalmol 1995;18:299-307.

Hobbs AJ. Soluble guanylate cyclase: the forgotten sibling. Trends Pharmacol Sci 1997;18:484-491.

Hogg N. The biochemistry and physiology of S-nitrosothiols. Annu Rev Pharmacol Toxicol 2002;42:585-600.

Hoyng PJF and Kitazawa Y. Medical treatment of normal tension glaucoma. Surv Ophthalmol 2002;47:S116-S124.

Hughes MN. Relationship between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochim Biophys Acta 1999;1411:263-272.

Iannaccone A, DuPont J and Grunwald JE. Human retinal hemodynamics following administration of 5-isosorbide mononitrate. Curr Eye Res 2000;20:205-210.

Ignarro LJ. Nitric oxide, a novel transduction mechanism for transcellular communication. Hypertension 1990;16:477-483.

Ignarro LJ, Cirino G, Casini A and Napoli C. Nitric oxide as a signaling molecule in the vascular system: an overview. J Cardiovasc Pharmacol 1999;34:879-886.

Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. J Physiol Pharmacol 2002;53:503-514.

Ignarro LJ, Buga GM, Wood KS, Byrns RE and Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA 1987;84:9265-9269.

Infeld DA and O'Shea JG. Glaucoma: diagnosis and management. Postgrad Med J 1998;74:709-715.

Jones RF and Maurice DM. New methods of measuring the rate of aqueous flow in man with fluorescein. Exp Eye Res 1966;5:208-220.

Kaiser HJ, Flammer J, Graf T and Stümpfig D. Systemic blood pressure in glaucoma patients. Graefe's Arch Clin Exp Ophthalmol 1993;231:677-680.

Kamikawatoko S, Tokoro T, Ishida A, Masuda H, Hamasaki H, Sato J and Azuma H. Nitric oxide relaxes ciliary muscle contracted by carbachol through elevation of cyclic GMP. Exp Eye Res 1998;66:1-7.

Kardon RH and Weingeist TA. Anatomy of the ciliary body and outflow pathways. In: Duane's Clinical Ophthalmology, Eds. Tasman W and Jaeger EA, J.B. Lippincott Company, Philadelphia, 1994, Vol. 3, Chap. 43, pp. 1-26.

Katsuki S, Arnold W, Mittal C and Murad F. Stimulation of guanylate cyclase by sodium nitroprusside, nitroglycerin and nitric oxide in various tissue preparations and comparison to the effects of sodium azide and hydroxylamine. J Cyclic Nucleotide Res 1977a;3:23-35.

Katsuki S, Arnold W and Murad F. Effects of sodium nitroprusside, nitroglycerin, and sodium azide on levels of cyclic nucleotides and mechanical activity of various tissues. J Cyclic Nucleotide Res 1977b;3:239-247.

Kaufman PL. Aqueous humor dynamics. In: Duane's Clinical Ophthalmology, Eds. Tasman W and Jaeger EA, J.B. Lippincott Company, Philadelphia, 1994, Vol. 3, Chap. 45, pp. 1-24.

Kee C, Kaufman PL and Gabelt BT. Effect of 8-Br cGMP on aqueous humor dynamics in monkeys. Invest Ophthalmol Vis Sci 1994;35:2769-2773.

Kiel JW, Reitsamer HA, Walker JS and Kiel FW. Effects of nitric oxide synthase inhibition on ciliary blood flow, aqueous humor production and intraocular pressure. Exp Eye Res 2001;73:355-364.

Klein BE and Klein R. Intraocular pressure and cardiovascular risk variables. Arch Ophthalmol 1981;99:837-839.

Klein BEK, Klein R and Jensen SC. Open-angle glaucoma and older-onset diabetes. Ophthalmology 1994;101:1173-1177.

Kolker AE, Moses RA, Constant MA and Becker B. The development of glaucoma in rabbits. Invest Ophthalmol 1963;2:316-321.

Korenfeld MS and Becker B. Atrial natriuretic peptides – effects on intraocular pressure, cGMP, and aqueous flow. Invest Ophthalmol Vis Sci 1989;30:2385-2392.

Korhonen R, Lahti A, Hämäläinen M, Kankaanranta H and Moilanen E. Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. Mol Pharmacol 2002;62:698-704.

Koss MC. Functional role of nitric oxide in regulation of ocular blood flow. Eur J Pharmacol 1999;374:161-174.

Krupin T, Weiss A, Becker B, Holmberg N and Fritz C. Increased intraocular pressure following topical azide or nitroprusside. Invest Ophthalmol Vis Sci 1977;16:1002-1007.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.

Lee BL, Bathija R and Weinreb RN. The definition of normal-tension glaucoma. J Glaucoma 1998;7:366-371.

Leopold IH. Perspectives in drug therapy of glaucoma. In: Glaucoma: Applied Pharmacology in Medical Treatment, Eds. Drance MS and Neufeld AH, Grune & Stratton, Inc., Orlando, 1984, pp. 1-22.

Leske MC. The epidemiology of open-angle glaucoma: a review. Am J Epidemiol 1983;118:166-191.

Leske MC, Heijl A, Hussein M, Bengtsson B, Hyman L, Komaroff E and Early Manifest Glaucoma Trial Group. Factors for glaucoma progression and the effect of treatment: the early manifest glaucoma trial. Arch Ophthalmol 2003;121:48-56.

Leske MC and Podgor MJ. Intraocular pressure, cardiovascular risk variables and visual field defects. Am J Epidemiol 1983;118:280-287.

Leske MC, Warheit-Roberts L, Wu S-Y and the Long Island Glaucoma Study Group. Open-angle glaucoma and ocular hypertension: the Long Island Glaucoma Case-Control Study Group. Ophthalmol Epidemiol 1996;3:85-96.

Leske MC, Wu S-Y, Nemesure B and Hennis A. Incident open-angle glaucoma and blood pressure. Arch Ophthalmol 2002;120:954-959.

Lichter PR. Genetics of the glaucomas. J Glauc 2001;10 Suppl 1:S13-S15.

Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ. Protein measurement with folin phenol reagent. J Biol Chem 1951;193:265-275.

Mantyh CR, Kruger L, Brecha NC and Mantyh PW. Localization of specific binding sites for atrial natriuretic factor in the central nervous system of rat, guinea pig, cat and human. Brain Res 1987;412:329-342.

Marletta MA, Hurshman AR and Rusche KM. Catalysis by nitric oxide synthase. Curr Opin Chem Biol 1998;2:656-663.

Masuda H, Tamaoki S, Goto M, Ishida A, Kamikawatoko S, Tokoro T and Azuma H. Nitric oxide-induced ciliary muscle relaxation during contraction with endothelin-1 is mediated through elevation of cyclic GMP. Curr Eye Res 1997;16:1245-1251.

McLeod SD, West SK, Quigley HA and Fozard JL. A longitudinal study of the relationship between intraocular pressure and blood pressures. Invest Ophthalmol Vis Sci 1990;31:2361-2366.

Meijer F, Van Delft JL, Garrelds IM, Van Haeringen NJ and Kijlstra A. Nitric oxide plays a role as a mediator of conjunctival edema in experimental allergic conjunctivitis. Exp Eye Res 1996;62:359-365.

Meyer P, Champion C, Schlötzer-Schrehardt U, Flammer J and Haefliger IO. Localization of nitric oxide synthase isoforms in porcine ocular tissues. Curr Eye Res 1999;18:375-380.

Migdal C. Primary open-angle glaucoma. In: Duane's Clinical Ophthalmology, Eds. Tasman W and Jaeger EA, J.B. Lippincott Company, Philadelphia, 1994, Vol. 3, Chap. 52, pp. 1-32.

Millar JC, Shahidullah M and Wilson WS. Atriopeptin lowers aqueous humor formation and intraocular pressure and elevates ciliary cyclic GMP but lacks uveal vascular effects in the bovine perfused eye. J Ocul Pharmacol Ther 1997;13:1-11.

Mitchell P, Smith W, Chey T and Healey PR. Open-angle glaucoma and diabetes. Ophthalmology 1997;104:712-718.

Mittag TW, Tormay A, Ortega M and Severin C. Atrial natriuretic peptide (ANP), guanylate cyclase, and intraocular pressure in the rabbit eye. Curr Eye Res 1987;6:1189-1196.

Moilanen E and Vapaatalo H. Nitric oxide in inflammation and immune response. Ann Med 1995;27:359-367.

Moilanen E, Whittle B and Moncada S. Nitric oxide as a factor in inflammation. In: Inflammation: Basic Principles and Clinical Correlates, Eds. Gallin JI and Snyderman R, Lippincott Williams & Wilkins, Philadelphia, 1999, Chap. 49, pp. 787-800.

Moncada S. Nitric oxide gas: mediator, modulator, and pathophysiologic entity. J Lab Clin Med 1992;120:187-191.

Moncada S. Nitric oxide in the vasculature: physiology and pathophysiology. Ann N Y Acad Sci 1997;15:60-67.

Moncada S and Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;30:2002-2012.

Moncada S and Higgs A. Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J 1995;9:1319-1330.

Moncada S, Palmer RMJ and Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-142.

Morley D and Keefer LK. Nitric oxide/nucleophile complexes: a unique class of nitric oxide-based vasodilators. J Cardiovasc Pharmacol 1993;22 Suppl 7:S3-S9.

Murad F. Discovery of some of the biological effects of nitric oxide and its role in cell signaling. Biosci Rep 1999;19:133-154.

Mülsch A, Bauersachs J, Schafer A, Stasch JP, Kast R and Busse R. Effect of YC-1, an NO-independent, superoxide-sensitive stimulator of soluble guanylyl cyclase, on smooth muscle responsiveness to nitrovasodilators. Br J Pharmacol 1997;120:681-689.

Münzel T and Harrison DG. Evidence for a role of oxygen-derived free radicals and protein kinase C in nitrate tolerance J Mol Med 1997;75:891-900.

Nakazawa T, Tomita H, Yamaguchi K, Sato Y, Shimura M, Kuwahara S and Tamai M. Neuroprotective effect of nipradilol on axotomized rat retinal ganglion cells. Curr Eye Res 2002;24:114-122.

Nathanson JA. Atriopeptin activated guanylate cyclase in the anterior segment – identification, localization, and effects of atriopeptins on IOP. Invest Ophthalmol Vis Sci 1987:28:1357-1364.

Nathanson JA. Direct application of a guanylate cyclase activator lowers intraocular pressure. Eur J Pharmacol 1988;147:155-156.

Nathanson JA. Nitrovasodilators as a new class of ocular hypotensive agents. J Pharm Exp Ther 1992;260:956-965.

Nathanson JA and McKee M. Identification of an extensive system of nitric oxide-producing cells in the ciliary muscle and outflow pathway of the human eye. Invest Ophthalmol Vis Sci 1995a;36:1765-1773.

Nathanson JA and McKee M. Alterations of ocular nitric oxide synthase in human glaucoma. Invest Ophthalmol Vis Sci 1995b;36:1774-1784.

Nemesure B, Leske MC, He Q and Mendell N. Analyses of reported history of glaucoma: a preliminary investigation. The Barbados Eye Study Group. Ophthalmic Epidemiol 1996;3:135-141.

Neufeld AH. Nitric oxide: A potential mediator of retinal ganglion cell damage in glaucoma. Surv Ophthalmol 1999;43 Suppl 1:S129-S135.

Neufeld AH, Das S, Vora S, Gachie E, Kawai S, Manning PT and Connor JR. A prodrug of a selective inhibitor of inducible nitric oxide synthase is neuroprotective in the rat model of glaucoma. J Glaucoma 2002a;11:221-225.

Neufeld AH, Hernandez MR and Gonzalez M. Nitric oxide synthase in the human glaucomatous optic nerve head. Arch Ophthalmol 1997;115:497-503.

Neufeld AH, Kawai S-I, Das S, Vora S, Gachie E, Connor JR and Manning P. Loss of retinal ganglion cells following retinal ischemia: the role of inducible nitric oxide synthase. Exp Eye Res 2002b;75:521-528.

Neufeld AH, Sawada A and Becker B. Inhibition of nitric oxide synthase-2 by aminoguanidine provide neuroprotection of retinal ganglion cells in a rat model of chronic glaucoma. Proc Natl Acad Sci USA 1999;96:9944-9948.

Nilsson SFE. The uveoscleral outflow routes. Eye 1997;11:149-154.

Nomura T and Smelser GK. The identification of adrenergic and cholinergic nerve endings in the trabecular meshwork. Invest Ophthalmol Vis Sci 1974;13:525-532.

Oku H, Yamaguchi H, Sugiyama T, Kojima S, Ota M and Azuma I. Retinal toxicity of nitric oxide released by administration of a nitric oxide donor in the albino rabbit. Invest Ophthalmol Vis Sci 1997;38:2540-2544.

Osborne NN, Barnett NL and Herrera AJ. NADPH diaphorase localization and nitric oxide synthetase activity in the retina and anterior uvea of the rabbit eye. Brain Res 1993;610:194-198.

Palmer RMJ, Ashton DS and Moncada S. Vascular endothelial cells synthetize nitric oxide from L-arginine. Nature 1988;333:664-666.

Palmer RMJ, Ferrige AG and Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524-526.

Peräsalo R, Peräsalo J and Raitta C. Electrocardiographic changes in institutionalized geriatric glaucoma patients. Graefe's Arch Clin Exp Ophthalmol 1992;230:213-217.

Phelps CD. Glaucoma: General concepts. In: Duane's Clinical Ophthalmology, Eds. Tasman W and Jaeger EA, J.B. Lippincott Company, Philadelphia, 1994, Vol. 3, Chap. 42, pp. 1-8.

Prünte C and Markstein R. Adrenergic regulation and its therapeutic aspects in the eye. In: Pharmacotherapy in Glaucoma, Eds. Orgül S and Flammer J, Verlag Hans Huber, Bern, 2000; pp. 73-78.

Quigley HA. Number of people with glaucoma worldwide. Br J Ophthalmol 1996;80:389-393.

Quigley HA, Nickells RW, Kerrigan LA, Pease ME, Thibault DJ and Zack DJ. Retinal ganglion cell death in experimental glaucoma and after axotomy occurs in glaucoma. Invest Ophthalmol Vis Sci 1995;36:774-786.

Ringvold A, Blika S, Elsås T, Guldahl J, Brevik T, Hessvedt P, Hoff K, Hoisen H, Kjorsvik S and Rossvold I. The middle-Norway eye-screening study. II. Prevalence of simple and capsular glaucoma. Acta Ophthalmol 1991;69:273-280.

Ritch R. Perspective on exfoliation syndrome. J Glaucoma 2001;10:S33-35.

Roodhooft JMJ. Leading causes of blindness worldwide. Bull Soc belge Ophthalmol 2002;283:19-25.

Ruskell GL and Griffith T. Peripheral nerve pathway to the ciliary muscle. Exp Eye Res 1979;28:277-284.

Samuelsson-Almén M, Nilsson SFE, Mäepea O and Bill A. Effects of atrial natriuretic factor (ANF) on intraocular pressure and aqueous flow in the cynomolgus monkey. Exp Eye Res 1991;53:253-260.

Schmidt HHHW, Lohmann SL and Walter U. The nitric oxide and cGMP signal-transduction pathway. Biochem Biophys Acta 1993;1178:153-175.

Schneemann A, Dijkstra B, van den Berg T, Kamphuis W and Hoyng PFJ. Nitric oxide/guanylate cyclase pathways and flow in anterior segment perfusion. Graefe's Arch Clin Exp Ophthalmol 2002;240:936-941.

Schulzer M, Drance SM, Carter CJ, Brooks DE, Douglas GR and Lau W. Biostatistical evidence for two distinct chronic open angle glaucoma populations. Br J Ophthalmol 1990;74:196-200.

Schuman JS, Erickson K and Nathanson JA. Nitrovasodilator effects on intraocular pressure and outflow facility in monkeys. Exp Eye Res 1994;58:99-105.

Shahidullah M and Wilson WS. Atriopeptin, sodium azide and cGMP reduce secretion of aqueous humour and inhibit intracellular calcium release in bovine cultured ciliary epithelium. Br J Pharmacol 1999;127:1438-1446.

Shareef S, Sawada A and Neufeld AH. Isoforms of nitric oxide synthase in the optic nerves of rat eyes with chronic moderately elevated intraocular pressure. Invest Ophthalmol Vis Sci 1999;40:2884-2891.

Shyjan AW, de Sauvage FJ, Gillett NA, Goeddel DV and Lowe DG. Molecular cloning of a retina-specific membrane guanylyl cyclase. Neuron 1992;9:727-737.

Siu AW, Leung MC, Ho To C, Siu FK, Ji JZ and Fai So K. Total retinal nitric oxide production is increased in intraocular pressure-elevated rats. Exp Eye Res 2002;75:401-406.

Skuta GL. Pseudoexfoliation syndrome, pigment dispersion syndrome, and associated glaucomas. In: Duane's Clinical Ophthalmology, Eds. Tasman W and Jaeger EA, J.B. Lippincott Company, Philadelphia, 1994, Vol. 3, Chap. 54B, pp. 1-17.

Smolenski A, Burkhardt AM, Eigenthaler M, Butt E, Gambaryan S, Lohmann SM and Walter U. Functional analysis of cGMP-dependent protein kinases I and II as mediators of NO/cGMP effects. Naunyn Schmiedeberg's Arch Pharmacol 1998;358:134-139.

Soltau JB and Zimmermann TJ. Changing paradigms in the medical treatment of glaucoma. Surv Ophthalmol 2002;47 Suppl 1:S2-S5.

Sperber G and Bill A. A method for near-continuous determination of aqueous humor flow: effects of anaesthetics, temperature and indomethacin. Exp Eye Res 1984;39:435-453.

Stein PJ and Clack JW. Topical application of a cyclic GMP analog lowers IOP in normal and ocular hypertensive rabbits. Invest Ophthalmol Vis Sci 1994;35:2765-2768.

Stoilova D, Child A, Trifan OC, Crick RP, Coakes RL and Sarfarazi M. Localization of a locus (GLC1B) for adult-onset primary open angle glaucoma to the 2cen-q13 region. Genomics 1996;36:142-150.

Stone EM, Fingert JH, Alward WLM, Nguyen TD, Polansky JR, Sunden SLF, Nishimura D, Clark AF, Nystuen A, Nichols BE, Mackey DA, Ritch R, Kalenak JW, Craven ER and

Sheffield VC. Identification of a gene that causes primary open angle glaucoma. Science 1997;275:668-670.

Stuehr DJ. Structure-function aspects in the nitric oxide synthases. Annu Rev Pharmacol Toxicol 1997;37:339-359.

Sugrue MF. New approaches to antiglaucoma therapy. J Med Chem 1997;40:2793-2809.

Sugrue MF and Viader M-P. Synthetic atrial natriuretic factor lowers rabbit intraocular pressure. Eur J Pharmacol 1986;130:349-350.

Takahata K, Katsuki H, Kume T, Nakata D, Ito K, Muraoka S, Yoneda F, Kashii S, Honda Y and Akaike A. Retinal neuronal death induced by intraocular administration of a nitric oxide donor and its rescue by neurotrophic factors in rats. Invest Ophthalmol Vis Sci 2003;44:1760-1766.

Takashima Y, Taniguchi T, Yoshida M, Haque MSR, Yoshimura N and Honda Y. Ocular hypotensive mechanism of intravitreally injected brain natriuretic peptide in rabbit. Invest Ophthalmol Vis Sci 1996;37:2671-2677.

Takashima Y, Taniguchi T, Yoshida M, Haque MSR, Igaki T, Itoh H, Nakao K, Honda Y and Yoshimura N. Ocular hypotension induced by intravitreally injected C-type natriuretic peptide. Exp Eye Res 1998;66:89-96.

Tamm ER and Lütjen-Drecoll E. Nitric oxide-synthesizing cells in the posterior eye segment. In: Nitric Oxide and Endothelin in the Pathogenesis of Glaucoma, Eds. Haefliger IO and Flammer J, Lippincott-Raven Publishers, Philadelphia, 1998a, Chap. 7, pp. 68-78.

Tamm ER and Lütjen-Drecoll E. Nitric oxide in the outflow pathways of the aqueous humor. In: Nitric Oxide and Endothelin in the Pathogenesis of Glaucoma, Eds. Haefliger IO and Flammer J, Lippincott-Raven Publishers, Philadelphia, 1998b, Chap. 14, pp. 158-167.

Tielsch JM, Katz J, Singh K, Quigley HA, Gottsch JD, Javitt J and Sommer A. A population-based evaluation of glaucoma screening: the Baltimore eye survey. Am J Epidemiol 1991;134:1102-1110.

Tielsch JM, Katz J, Sommer A, Quigley HA and Javitt JC. Family history and risk of primary open angle glaucoma: the Baltimore Eye Survey. Arch Ophthalmol 1994;112:69-73.

Tielsch JM, Katz J, Sommer A, Quigley HA and Javitt JC. Hypertension, perfusion pressure, and open-angle glaucoma: a population based assessment. Arch Ophthalmol 1995;113:216-221.

Tsai D-C, Hsu W-M, Chou C-K, Chen S-J, Peng C-H, Chi C-W, Ho LLT, Liu J-H and Chiou S-H. Significant variation of the elevated nitric oxide levels in aqueous humor from patients with different types of glaucoma. Ophthalmologica 2002;216:346-350.

Tsukahara S, Sasaki T, Yamabayashi S, Furuta M, Ushiyama M and Yamamoto T. Effect of alpha-human atrial natriuretic peptides on intraocular pressure in normal albino rabbits. Ophthalmologica 1988;197:104-109.

Vallance P and Leiper J. Blocking NO synthesis: how, where and why? Nat Rev Drug Discov 2002;1:939-950.

Vapaatalo H. NO-donors in cardiology. Pol J Pharmacol 1994;46:531-540.

Vapaatalo H. Pharmacology of agents for glaucoma. Vox 1995;17:19-28.

Vogh BP and Godman DR. Effects of inhibition of angiotensin converting enzyme and carbonic anhydrase on fluid production by ciliary process, choroid plexus, and pancreas. J Ocul Pharmacol 1989;5:303-311.

Wang JJ, Mitchell P and Smith W. Is there an association between migraine headache and open-angle glaucoma? Ophthalmology 1997;104:1714-1719.

Wang R-F and Podos SM: Effect of topical application of nitroglycerin on intraocular pressure in normal and glaucomatous monkeys. Exp Eye Res 1995;60:337-339.

Watkins RW, Baum T, Cedeno K, Smith EM, Yuin P-H, Ahn H-S and Barnett A. Topical ocular hypotensive effects of the novel, non-sulfhydryl angiotensin converting enzyme inhibitor SCH 33861 in conscious rabbits. J Ocul Pharmacol 1987;3:295-307.

Watkins RW, Baum T, Tedesco RP, Pula K and Barnett A. Systemic effects resulting from topical ocular administration of SCH 33861, a novel ACE inhibitor ocular hypotensive agent. J Ocul Pharmacol 1988;4:93-100.

Wax MB, Camras CB, Fiscella RG, Girkin C, Singh K and Weinreb RN. Emerging perspective in glaucoma: optimizing 24-hour control of intraocular pressure. Am J Ophthalmol 2002;133 Suppl:S1-10.

Weinreb RN. Uveoscleral outflow: the other outflow pathway. J Glauc 2000;9:343-345.

Weitzberg E and Lundberg JON. Nonenzymatic nitric oxide production in humans. Nitric oxide 1998;2:1-7.

Wiederholt M. Nitric oxide and endothelin in aqueous humor outflow regulation. In: Nitric Oxide and Endothelin in the Pathogenesis of Glaucoma, Eds. Haefliger IO and Flammer J, Lippincott-Raven Publishers, Philadelphia, 1998, Chap. 15, pp. 168-177.

Wiederholt M. New aspects in aqueous humor dynamics. In: Pharmacotherapy in Glaucoma, Eds. Orgül S and Flammer J, Verlag Hans Huber, Bern, 2000, pp. 65-71.

Wiederholt M, Sturm A and Lepple-Wienhues A. Relaxation of trabecular meshwork and ciliary muscle by release of nitric oxide. Invest Ophthalmol Vis Sci 1994;35:2515-2520.

Wilson MR, Hertzmark E, Walker AM, Childs-Shaw K and Epstein DL. A case-control study of risk factors in open angle glaucoma. Arch Ophthalmol 1987;105:1066-1071.

Wizemann AJS and Wizemann V. Organic nitrate therapy in glaucoma. Am J Ophthalmol 1980;90:106-109.

Wolfs RCW, Klaver CCW, Ramrattan RS, Van Duijn CM, Hofman A and de Jong PTVM. Genetic risk of primary open angle glaucoma. Population-based familial aggregation study. Arch Ophthalmol 1998;116:1640-1645.

Woodward DF, Dowling MC, Chen J, Padillo E and Ruiz G. Sustained decreases in systemic blood pressure do not cause ocular hypotension. Ophthalmic Res 1989;21:37-43.