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Cyclic GMP, sodium nitroprusside and sodium azide reduce aqueous humour formation in the isolated arterially perfused pig eye

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1 The effect of nitric oxide (NO) on aqueous humour formation (AHF) and intraocular pressure (IOP) was studied using NO donors, sodium azide (AZ) and sodium nitroprusside (SNP).

2 Using the porcine arterially perfused eye preparation, drug effects on AHF and IOP were measured by fluorescein dilution and manometry, respectively. Perfusion pressure of the ocular vasculature was also monitored using digital pressure transducer and pen recorder.

3 L-Arginine (1.0 mM), a precursor of NO, but not D-arginine (1.0 mM), the inactive analogue, produced a significant reduction in AHF (28.5%) and IOP (21.1%). L-NAME (L-nitro-L-arginine) (10–100 μM), an NO synthase inhibitor, had no effect on AHF and IOP. However, L-NAME (100 μM) completely reversed L-arginine's effect.

4 AZ and SNP reduced the AHF and IOP dose-dependently. AZ at 100 nM, 1 and 10 μM reduced AHF by 26.0, 39.7 and 51.7% and IOP by 10.8, 17.3 and 24.0%, respectively. SNP at 1, 10 and 100 μM reduced the AHF by 6.0, 24.2 and 35.4% and IOP by 3.5, 9.5 and 15.5%, respectively. 8-pCPT-cGMP (8-para-chlorophenyl-thioguanosine-3′,5′-cyclic guanosine monophosphate, 10 μM), a cGMP analogue, also reduced the AHF (34.9%) and IOP (15.9%).

5 The effects of AZ and SNP on the AHF and IOP were blocked by a soluble guanylate cyclase inhibitor ODQ (10 μM), whereas ODQ alone or combined with 8-pCPT-cGMP had no effect on the AHF and IOP.

6 None of the drugs had any significant effect on ocular vasculature.

7 The reduction of the AHF and IOP in the arterially perfused pig eye by nitrovasodilators is likely to involve the NO-cGMP pathway.

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Abbreviations: AH, aqueous humour; AHF, aqueous humour formation; AHS, aqueous humour substitute; AZ, sodium azide; cAMP, cyclic AMP; CE, ciliary epithelium; cGMP, cyclic GMP; DMSO, dimethyl sulphoxide; EDHF, endothelium-derived hyperpolarizing factor; IOP, intraocular pressure; K_{out}, rate constant for aqueous humour formation; L-NAME, L-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; 8-pCPT-cGMP, 8-para-chlorophenyl-thioguanosine-3′,5′-cyclic guanosine monophosphate; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside

Introduction

Nitric oxide (NO) is an important biomolecule responsible for many biological activities. It is widely believed that the biological effects of NO donors, such as nitrovasodilators, are due to the release of NO which activates soluble guanylate cyclase (sGC) (Feelisch & Noack, 1987). The biological functions of NO are so diverse that it has been described as a second messenger, autocrine, paracrine, neurotransmitter, hormonal (Murad, 1998), cytoprotective (Wang *et al*., 2002) and a cytotoxic (Kroncke *et al*., 1997) agent. The role of NO has been studied extensively in many tissues including the eye. (Chiou, 2001) Although many have reported that NO donors reduce intraocular pressure (IOP) in normal and glaucomatous animals (Nathanson, 1992; Schuman *et al*., 1994; Wang & Podos, 1995) and human (Chuman *et al*., 2000), the exact effect of NO on the aqueous humour (AH) flow remains unclear. Owing to the dominant role of NO in vasodilatation and relaxation of smooth muscle, attention has been given mostly to ocular blood flow (Schmetterer & Polak, 2001) or trabecular meshwork and ciliary muscle relaxation (Wiederholt *et al*., 1994) and AH outflow (Schuman *et al*., 1994; Behar-Cohen *et al*., 1996). In those studies that have reported on the role of NO on AH outflow and IOP, data are conflicting. Some studies showed that both topical and intraocular administration of NO donors reduced the IOP (Nathanson, 1992; Behar-Cohen *et al*., 1996; Kotikoski *et al*., 2003b), but others reported that topical NO donors increased IOP without affecting aqueous humour formation (AHF) (Larsson *et al*., 1995) or outflow facility (Krupin *et al*., 1977). Yet, other

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studies showed that NO donors has no effect on the IOP in rabbit (Taniguchi *et al*., 1998) or human (Kiss *et al*., 1999).

Recently sodium azide (AZ), a nitrovasodilator, was shown to be able to lower the IOP in an isolated bovine eye by acting on the ciliary epithelial cells (CE) but not by relaxing the vascular smooth muscles (Millar *et al*., 1997; 2001). The significance of NO on AHF was further highlighted by the fact that L-NAME (L-nitro-L-arginine), a nitric oxide synthase (NOS) inhibitor, reduced the aqueous humour formation (AHF) in anaesthetised rabbit (Kiel *et al*., 2001). However, it was postulated that this reduction in the AHF was due to ciliary vasoconstriction, that is, blood-flow dependent. To complicate matter further, it was later reported that isosorbate-5-mononitrate, a NO donor, did not reduce AHF in healthy human volunteers (Kotikoski *et al*., 2003a). Since the AHF is an important determinant of IOP, we study the role of NO donors on the AH dynamics. Our hypothesis is that NO may directly regulate the AHF in addition to its effects on ocular vasculature and AH outflow. Indirect support to our hypothesis is now emerging. For instance, constitutive NOS (cNOS) activity has been detected in the bovine (Geyer *et al*., 1997) and porcine (Meyer *et al*., 1999) ciliary processes; basal nitric oxide production in the human and porcine ciliary processes has been shown to be inhibited by L-NAME (Haufschild *et al*., 2000); carbachol and NO can inhibit Na-K-ATPase activity of the bovine ciliary processes (Ellis *et al*., 2001) and stimulation of NO/cGMP pathway was shown to depolarise the epithelial transmembrane potential in porcine ciliary processes (Fleischhauer *et al*., 2000). In addition, NO has also been shown to be involved in fluid transport in other epithelia including kidney (Ortiz & Garvin, 2002) and salivary gland (Lomniczi *et al*., 1998).

The aim of the present study was to investigate the effect of NO donors on the AHF and IOP in isolated arterially perfused pig eyes.

Methods

Preparation of isolated eye

Fresh pig eyes were obtained from an abattoir and transported to the laboratory on ice-cold Krebs' solution. Perfusion of the eyes was started immediately after arrival. The postmortem time ranged from 9 to 35 min with an average of 23.0 ± 0.5 min ($n = 226$). The detailed methods of cannulation and perfusion of the eyes, as well as the estimation of the AHF rate have been described earlier (Wilson *et al*., 1993; Shahidullah *et al*., 2003). Briefly, excess adnexal tissues were trimmed away and care was taken not to damage the blood vessels running over the posterior surface of the globe and along the optic nerve. The ophthalmic artery was cleared of fat and cannulated distal to the point where it divides into the two long posterior ciliary arteries. The eye was placed in a circulating warming jacket at 37°C and covered with an insulated plastic cup. The ophthalmic artery was perfused with Krebs' solution at 37°C, which contained (mM): NaCl, 118; KCl, 4.0; $MgSO_4$, 1.2; $CaCl_2$, 2.0; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 10.0; ascorbate 0.05; glutathione, 1.0 and allopurinol, 1.8. The pH of the Krebs' solution was adjusted to 7.4 by bubbling with O_2 containing 5% CO_2. Allopurinol, a xanthine oxidase inhibitor, has been added to the perfusate to combat oxidative damage and reperfusion injury.

The eyes were perfused with a digital peristaltic pump (Watson Marlow, 505S) to induce flow through the vasculature. The arterial pressure was recorded with a pressure transducer (Harvard Apparatus, 60–3003) and pen recorder (Kipp & Zonen, BD 112). Flow was commenced at $0.2\,ml\,min^{-1}$ and increased to $1.5\,ml\,min^{-1}$ in the first hour. Flow rate was increased in 10–20 steps, one increment in every 2.5–5 min. The number of steps and size of the increment to reach the optimum flow rate ($1.5\,ml\,min^{-1}$) depended on the arterial pressure of the eye, which was monitored continuously. The preparations were said to be valid if the pressure was below 140 mm Hg.

Estimation of AHF rate

After the perfusion was set up and the eye appeared firm, the anterior chamber was cannulated with three 23G needles. The first 23G needle was connected by silicon rubber tubing (i.d., 0.5 mm; wall thickness 1.8 mm) through a Watson–Marlow peristaltic pump to a cuvette in a spectrophotometer (Pharmacia Biotech, Spectronic 2000). It returned to the anterior chamber via a second 23G needle to constitute the anterior chamber circulating system. The third needle connected the anterior chamber to a water manometer for measuring the IOP. The two circulating needles in the anterior chamber were kept wide apart to optimise mixing (Figure 1).

The cuvette and the tubing system were prefilled with 1.04 ml of an AH substitute (AHS) comprising (mM): NaCl, 110; KCl, 3; $CaCl_2$, 1.4; $MgCl_2$, 0.5; KH_2PO_4, 0.9; $NaHCO_3$, 30; glucose, 6; ascorbate, 3 and fluorescein sodium, 0.0186. The pH of this solution was adjusted to 7.6 by bubbling with 95% O_2 and 5% CO_2. This fluid was circulated through the anterior chamber at a rate of $0.2\,ml\,min^{-1}$. The absorbance was recorded at 490 nm every 5 min using a computer-controlled spectrophotometer. A steady state, that is, optimal mixing of fluorescein in the anterior chamber, was achieved within 40–60 min.

The AHF rate was estimated from the rate of fluorescein dilution (decrease in absorbance) of the anterior chamber circulating system. As the AH was continuously secreted into and drained from the anterior chamber, the initial concentration of fluorescein in the anterior chamber circulating system was diluted with time. At constant AHF rate, a plot of $\log_e$ (absorbance of fluorescein) against time (min) gave a straight line whose slope was taken as the rate constant for AHF ($K_{out}\,min^{-1}$). Regression lines representing the dilution of fluorescein in the AH were constructed using the ANOVA statistical package by Minitab. The K_{out} value for the initial 30 min, that is, before the addition of drugs, was used as the control value to compare against that for the subsequent 40 min to an hour of drug-induced conditions.

The calculation of the AHF rate (in $\mu l\,min^{-1}$) of the *in vitro* eye has been described previously (Wilson *et al*., 1993). In order to calculate the AHF rate, the volume of the anterior chamber is needed. The AHF rate can then be calculated as the product of ($V_{ac} + V_s$) and $K_{out}\,min^{-1}$ ($\mu l\,min^{-1}$).

The relationship which allows this calculation is: $F_0 \times V_s = F_m\ (V_s + V_{ac})$, where F_0 represents the initial fluorescein concentration in the cuvette and tubing system prior to cannulation of the anterior chamber; F_m is the fluorescein

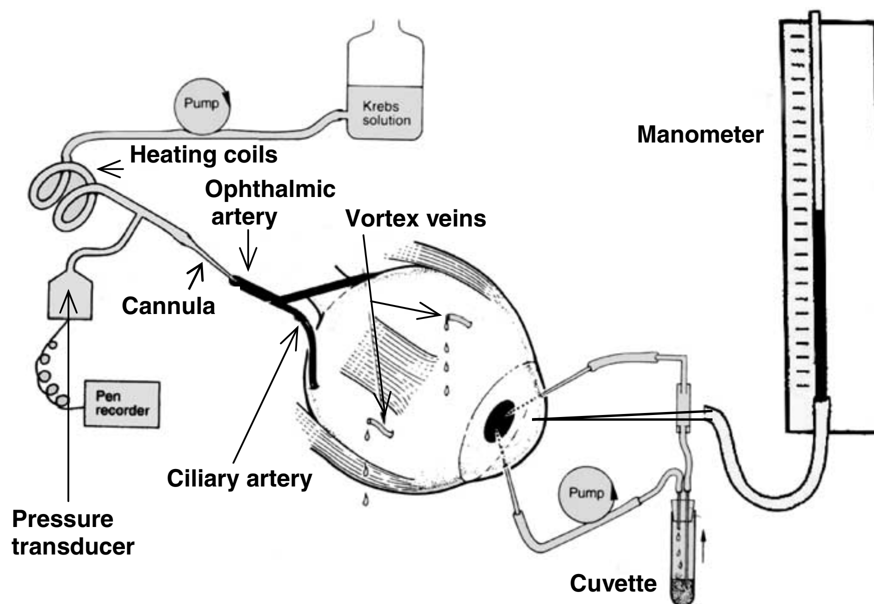

Figure 1 Schematic diagram of the *in vitro* whole eye preparation for simultaneous measurement of AHF, IOP, arterial flow and arterial pressure.

concentration immediately after anterior chamber perfusion starts if instantaneous mixing was achieved; V_s represents the volume of the cuvette and the tubing system and V_{ac} is the volume of the anterior chamber. F_m was obtained by the extrapolation of the control decay curve to time zero, that is, when the fluorescein solution was first circulated into the anterior chamber. V_{ac} was obtained from the above relationship as

$$V_{ac} = \frac{V_s(F_0 - F_m)}{F_m}$$

Measurement of IOP

IOP was measured with a water manometer which was connected to the anterior chamber *via* a 23G needle. The silicon rubber tubing (diameter, 0.5 mm) connecting the needle and the manometer was filled with AHS. Eyes showing IOP above 20 mmHg (270 mmH_2O) were excluded from this study.

Measurement of vascular pressure

The arterial pressure was continuously monitored *via* a digital pressure transducer (Harvard Apparatus, 60–3003) and a dual channel chart recorder (Kipp and Zonen, BD 112). Eyes showing vascular perfusion pressure above 140 mmHg were excluded from the study.

A schematic diagram of the *in vitro* whole eye preparation for simultaneous measurement of AHF, IOP and vascular pressure is shown in Figure 1.

Drugs and chemicals

AZ, sodium nitroprusside (SNP), L-NAME, L-arginine, D-arginine, glutathione, 8-para-chlorophenyl-thioguanosine-3′,5′-cyclic guanosine monophosphate (8-pCPT-cGMP), 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), allopurinol and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals used in the Krebs' solution and AHS were of analytical grade and were obtained either from Sigma Chemical Co. or from Fisher Scientific Products (Pittsburgh, PA, U.S.A.). Stock solutions of drugs were prepared by dissolving either in distilled water or in DMSO according to their solubility. Stock solutions of drugs were added to the perfusate to obtain intended final concentration.

Statistical analysis of data

Results were expressed as the mean $\pm$ s.e.m. of separate experiments. Statistical comparisons were made by paired two-tailed Student's *t*-test and analysis of variance followed by the Bonferroni *post hoc* multiple comparison tests. A probability (P) value of <0.05 was considered significant.

Results

Baseline AHF rate, IOP and arterial pressure

The mean baseline AHF rate, IOP and arterial perfusion pressure for all the eyes used in this investigation were 2.7 ± 0.5 ($n = 100$) $\mu l\,min^{-1}$; 11.8 ± 0.2, ($n = 100$) mmHg and 53.52 ± 2.26 ($n = 100$) mmHg, respectively. The mean baseline as well as drug-treated values for AHF rate, IOP and arterial pressure for the various treatment groups are shown in Tables 1, 2 and 3, respectively.

Effect of DMSO on AHF and IOP

In order to see if DMSO used in dissolving the drugs had any effect on the AHF or IOP, we perfused the eye with Kreb's solution containing DMSO alone. The data showed that

Table 1 Effects of drugs on AHF in isolated arterially perfused pig eye

Drug/vehicle	*Concentration* (μM)	*AHF* (K_{out}.min^{-1} × 10^{-4})[a] (μl min^{-1})[b] (*mean ± s.e.m.*) *Baseline/control*	*Drug treated*	n	P (*ns = not significant*)	*% Change*
DMSO	0.05%	26.1 ± 1.8 (2.7 ± 0.2)	28.2 ± 2.0 (2.9 ± 0.2)	6	0.38 (ns)	−8.0
L-NAME	100	24.0 ± 2.3 (2.7 ± 0.2)	22.6 ± 2.4 (2.4 ± 0.2)	10	0.33 (ns)	5.8
L-NAME	10	36.8 ± 4.7 (3.7 ± 0.5)	33.7 ± 0.5 (3.5 ± 0.5)	4	0.53(ns)	8.4
L-Arginine	1000	28.1 ± 3.6 (2.9 ± 0.4)	20.1 ± 4.4 (2.1 ± 0.4)	5	< 0.01	28.5
D-Arginine	1000	24.2 ± 1.8 (2.5 ± 0.2)	22.4 ± 1.6 (2.3 ± 0.2)	6	0.52 (ns)	8.3
L-Arginine + L-NAME	1000 + 100	26.5 ± 1.8 (2.7 ± 0.2)	26.5 ± 2.0 (2.7 ± 0.2)	6	0.98 (ns)	0.0
Sodium azide (AZ)	10	25.5 ± 1.3 (2.6 ± 0.1)	12.3 ± 1.3 (1.2 ± 0.1)	5	< 0.001	51.7
AZ	1.0	23.4 ± 0.9 (2.4 ± 0.1)	14.3 ± 1.7 (1.4 ± 0.2)	6	< 0.01	39.7
AZ	0.1	29.2 ± 2.7 (3.0 ± 0.3)	21.6 ± 1.6 (2.2 ± 0.2)	5	< 0.01	26.0
AZ + ODQ	10 + 10	21.6 ± 0.6 (2.2 ± 0.1)	20.5 ± 0.7 (2.1 ± 0.1)	6	0.38 (ns)	5.1
SNP	100	27.4 ± 1.5 (2.8 ± 0.2)	17.6 ± 2.0 (1.8 ± 0.2)	7	< 0.01	35.4
SNP	10	23.1 ± 1.2 (2.4 ± 0.1)	17.5 ± 1.0 (1.8 ± 0.1)	7	< 0.05	24.2
SNP	1.0	28.3 ± 2.2 (2.9 ± 0.2)	26.6 ± 3.4 (2.7 ± 0.3)	6	0.61 (ns)	6.0
SNP + ODQ	100 + 10	29.7 ± 2.8 (3.0 ± 0.3)	26.0 ± 3.0 (2.7 ± 0.3)	5	0.07 (ns)	12.45
8-pCPT-cGMP	10	25.2 ± 1.2 (2.6 ± 0.1)	16.4 ± 1.1 (1.7 ± 0.1)	6	< 0.01	34.9
8-pCPT + ODQ	10 + 10	27.1 ± 1.9 (2.8 ± 0.2)	17.0 ± 1.0 (1.7 ± 0.2)	6	< 0.01	37.3
ODQ	10	27.6 ± 1.9 (2.8 ± 0.2)	24.3 ± 1.0 (2.5 ± 0.1)	4	0.25 (ns)	11.9

[a]AHF was expressed as the rate constant (K_{out} min^{-1} × 10^{-4}), defined as the slope of regression line drawn on LN (Log_e) of the changes of absorbance with time (min) and was shown as mean ± s.e.m.
[b]Values enclosed between parentheses indicates mean AHF rate in terms of μl min^{-1}. AHF rate measured during the first 30 min prior to drug addition was taken as the control values. AHF rate for the subsequent 40–60 min after establishment of drug effects was taken as treated values. Note that after the addition of drug, 20 min stabilisation period was allowed to establish drug effect. A probability (P) of 0.05 or less was considered significantly different from control.

Table 2 Effects of drugs on IOP in isolated arterially perfused pig eye

Drug/vehicle	*Concentration* (μM)	*IOP* (*slope* × 10^4)[a] (mmHg)[b] (*mean ± s.e.m.*) *Baseline/control*	*Drug treated*	n	P (*ns = not significant*)	*% Change*
DMSO	0.05%	−0.4 ± 2.0 (11.1 ± 0.7)	1.3 ± 3.0 (11.2 ± 1.0)	8	0.62 (ns)	0.4
L-NAME	100	−9.3 ± 6.0 (12.5 ± 1.1)	−4.0 ± 3.0 (12.1 ± 1.0)	10	0.27 (ns)	−0.5
L-NAME	10	−0.1 ± 4.0 (11.5 ± 1.4)	0.3 ± 2.0 (11.6 ± 1.3)	4	0.94 (ns)	3.2
L-Arginine	1000	6.2 ± 11.0 (13 ± 0.4)	−22.1 ± 3.0 (11.5 ± 0.4)	5	< 0.05	12.1
D-Arginine	1000	−7.7 ± 2.0 (12.0 ± 0.1)	5.6 ± 11.0 (11.6 ± 0.3)	6	0.33 (ns)	3.2
L-Arg + L-NAME	1000 + 100	0.1 ± 1.0 (10.4 ± 0.8)	−3.1 ± 1.0 (10.2 ± 0.7)	6	0.26 (ns)	2.3
Sodium azide (AZ)	10	1.4 ± 3.0 (12.4 ± 0.6)	−45.4 ± 2.0 (9.4 ± 0.5)	5	< 0.001	24.0
AZ	1.0	−1.0 ± 2.0 (11.8 ± 1.2)	−33.6 ± 4.0 (9.7 ± 1.0)	6	< 0.01	17.3
AZ	0.1	1.5 ± 3.0 (10.9 ± 0.8)	−19.2 ± 4.0 (9.7 ± 1.0)	5	< 0.01	10.8
AZ + ODQ	10 + 10	1.0 ± 2.0 (12.8 ± 0.7)	−3.9 ± 4.0 (12.3 ± 0.8)	6	0.27 (ns)	3.7
SNP	100	−3.6 ± 5.0 (13.0 ± 1.0)	−29.8 ± 3.0 (11.0 ± 0.8)	7	< 0.001	15.6
SNP	10	6.9 ± 5.0 (12.2 ± 0.8)	−2.2 ± 6.0 (11.1 ± 1.3)	7	< 0.01	9.5
SNP	1.0	−1.2 ± 1.0 (10.5 ± 0.8)	−5.4 ± 3.0 (10.2 ± 0.8)	6	0.16 (ns)	3.5
SNP + ODQ	100 + 10	−1.4 ± 1.0 (13.1 ± 1.5)	−0.7 ± 1.0 (13.0 ± 1.4)	5	0.64 (ns)	1.2
8-pCPT-cGMP	10	1.0 ± 2.0 (10.9 ± 1.0)	−27.8 ± 3.0 (9.2 ± 0.9)	6	< 0.001	15.9
8-pCPT + ODQ	10 + 10	1.4 ± 2.0 (11.0 ± 0.9)	−20.6 ± 3.0 (9.2 ± 1.0)	6	< 0.001	16.6
ODQ	10	−5.7 ± 2.6 (11.6 ± 0.7)	−1.2 ± 0.5 (11.3 ± 0.7)	4	0.18 (ns)	2.8

[a]IOP was expressed as the slope of regression line drawn on LN (Log_e) of the changes of IOP with time (min) (slope × 10^4) and was shown as mean ± s.e.m.
[b]Values enclosed between parentheses indicates mean IOP in terms of mmHg. IOP recorded during the first 30 min prior to drug addition was taken as the control values. IOP recorded for the subsequent 40–60 min after establishment of drug effects was taken as treated values. Note that after the addition of drug, 20 min stabilisation period was allowed to establish drug effect. A probability (P) of 0.05 or less was considered significantly different from control.

DMSO has no effect on the AHF and IOP (Tables 1 and 2) and the AHF rate of the whole experimental period did not change significantly.

Effect of L-NAME and L-arginine on AHF and IOP

We tested two concentrations of L-NAME (10 and 100 μM), an NOS inhibitor, and found no effect on either the AHF (Table 1) or IOP (Table 2). We then used L-arginine, a physiological precursor of NO, to investigate its effect on the AHF and IOP. L-Arginine (1.0 mM) produced significant reduction in AHF and IOP (Table 1) by 28.5, 12.1%, respectively. However, addition of D-arginine (1.0 mM), an inactive analogue of L-arginine, produced no significant effect on either the AHF or IOP (Tables 1 and 2). When L-NAME (100 μM) and L-arginine (1 mM) were used together, the L-arginine's effects on AHF and IOP was effectively abolished.

Table 3 Effects of drugs on ocular vasculature in isolated arterially perfused pig eye

Drug/vehicle	*Concentration* (μM)	*Arterial pressure (mmHg) (mean±s.e.m.)* Control	Treated	n	P	*% Change*
DMSO	0.05%	54.25±6.29	51.17±9.60	6	>0.05	2.07
L-NAME	100	51.00±15.64	53.25±15.43	10	>0.05	4.41
L-NAME	10	58.33±6.03	61.44±5.70	4	>0.05	5.33
L-Arginine	1000	64.60±13.98	67.20±15.77	5	>0.05	4.02
D-Arginine	1000	46.33±0.95	45.00±1.58	6	>0.05	2.88
L-Arg+L-NAME	1000 +100	56.50±9.33	58.83±9.20	6	>0.05	4.13
Sodium azide (AZ)	10	62.60±11.88	62.00±9.94	5	>0.05	0.96
AZ	1	48.67±10.14	50.33±8.85	6	>0.05	3.43
AZ	0.1	47.80±14.22	47.40±11.97	5	>0.05	0.84
AZ+ODQ	10+10	52.17±12.88	53.00±12.76	6	>0.05	1.60
SNP	100	53.53±7.14	52.00±6.16	7	>0.05	2.93
SNP	10	46.43±11.96	45.57±11.82	7	>0.05	1.85
SNP	1	48.50±7.24	51.00±7.38	6	>0.05	5.15
SNP+ODQ	100 +10	49.00±7.97	49.20±8.78	5	>0.05	0.41
8-pCPT-cGMP	10	48.67±7.15	49.00±8.19	6	>0.05	0.68
8-pCPT+ODQ	10+10	60.75±12.88	59.25±13.11	6	>0.05	2.47
ODQ	10	60.75±13.1	59.00±12.39	4	>0.05	2.90

The arterial pressure was recorded in mmHg and was shown as mean±s.e.m. The control arterial pressures represent the average arterial pressure of the eyes of each treatment group before addition of drug. The treated arterial pressures represent the average arterial pressure of the respective eyes at the end of 60 min of drug treatment. Note that for each group of eyes, arterial pressure recorded during the first 30 min prior to drug addition was taken as the control values. Pressure recorded for the subsequent 40–60 min after establishment of drug effects was taken as treated values.

Effect of AZ and SNP on AHF and IOP

The vasodilator drug AZ, which acts through the generation of NO, produced significant and concentration-dependent reduction of the AHF at the three concentrations used (10, 1 and 0.1 μM). The reductions with the three concentrations used were 51.7, 39.7 and 26.0%, respectively (Table 1). All three concentrations reduced the IOP significantly in a dose-dependent manner. The reductions were 24.0, 17.3 and 10.8%, respectively (Table 2).

The NO donor SNP also reduced AHF and IOP at concentrations of 10 and 100 μM. The AHF was reduced by 35.4 and 24.2% (Table 1), and the IOP was also depressed by 15.6 and 9.5%, respectively (Table 2). Lower concentration of SNP (1 μM) failed to affect both the AHF and IOP significantly.

The effect of AZ and SNP on AHF and IOP is inhibited by ODQ

We used ODQ, a specific inhibitor of sGC, to study its influence on the AZ and SNP-induced reduction of AHF and IOP. ODQ (10 μM) reversed both the AHF and IOP-reducing effect of AZ and SNP (Tables 1 and 2).

Effect of 8-pCPT-cGMP and/or ODQ on AHF and IOP

To further explore whether AZ and SNP acted through cGMP in the pig eye preparation, we studied the effect of 8-pCPT-cGMP, a cell-permeable and a stable analogue of cGMP, on the AHF and IOP. 8-pCPT-cGMP (10 μM) reduced both AHF and IOP by 34.9% (Table 1) and 15.9% (Table 2), respectively. However, the addition of ODQ (10 μM) failed to reverse the AHF and IOP-reducing effect of 8-pCPT-cGMP (Tables 1 and 2). ODQ (10 μM) alone had no effect on the AHF or IOP.

Thus, our data indicate that the reduction in AHF rate in the *in vitro* pig eye model may involve the NO/sGC/cGMO pathway. Figure 2 is a simplified diagram which shows the interaction between NO and cyclic GMP (cGMP).

Effects of drugs on the arterial pressure of the in vitro *pig eye*

The mean arterial pressures of the *in vitro* pig eyes ranged from 47±2 and 67±6 mmHg in the present study. The average arterial pressure for all the treatment groups was 53.52±2.26 mmHg ($n=100$). None of the drugs used in the present investigation had any significant effect on the arterial pressure of the *in vitro* pig eyes (Table 3).

Discussion

We have used an *in vitro* pig eye preparation to investigate two well-known NO donors – AZ and SNP – for their effects on the AHF, IOP and ocular vasculature. Pig eye are similar to the human eye both in terms of the size and anatomy and it has been considered as a good animal model for human eye (Beauchemin, 1974; Simoens *et al*., 1992). Therefore, we chose to use pig eyes to study the physiology of the AHF.

In addition to the NO donors, we have also used L-NAME, an inhibitor of NOS, to block the physiological production of NO and L-arginine, a nontoxic physiological precursor of NO to mimic the action of NO donors. Physiologically, NO is produced from L-arginine *via* various isoforms of NOS. Since it is widely believed that NO acts through the production of cGMP by activation of sGC, we used 8-pCPT-cGMP, a stable and cell-permeable analogue of cGMP and ODQ, a specific inhibitor of sGC, to simulate and to block the action of NO donors, respectively.

To the best of our knowledge, the present work represents the first attempt to study the effect of NO donors on the AHF

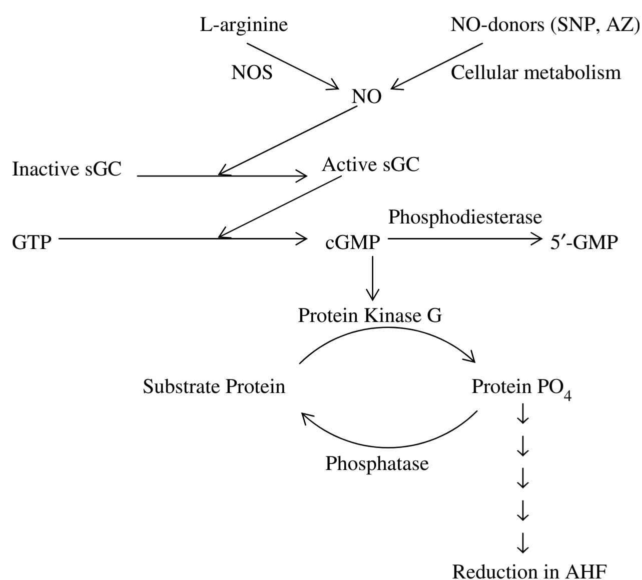

Figure 2 Physiological actions of NO in reducing AHF. Endogenous NO is produced by NOS isoforms from L-arginine. Exogenous NO is produced from a class of compounds called NO donors, when administered to biological system. Once produced, NO diffuses into the cytoplasm, interacts and activates the enzyme sGC. Activated sGC hydrolyses GTP to produce cGMP. cGMP activates cGMP-dependent protein kinase (PKG). PKG in turn phosphorylates specific substrate proteins. The substrate proteins may itself constitute the transporter or ion channel, phosphorylation of which causes to affect the physiological function. Phosphorylated substrate proteins may also interact in subsequent steps with the final functional protein to produce the ultimate physiological action. The actions of phosphoproteins are usually terminated by a class of enzymes called phosphatases, which remove the phosphate group from the serine/threonine or tyrosine residues of the substrate protein to bring it back to the resting state.

of pig eye. The AHF and IOP in the isolated pig eye preparation were reduced dose-dependently by both AZe and SNP. In earlier investigations, it had been reported that both sodium azide and atriopeptin reduced the AHF in isolated arterially perfused bovine eye (Millar *et al*., 1997; 2001). NO had also been shown to modulate the secretion in a number of other epithelial tissues. Thus, the present observation of reduction of the AHF and IOP by AZ and SNP is in agreement with previous studies in ocular and other tissues. However, a recent study has shown that a single 10 mg oral dose of isosorbide-5-nomonitrate did not produce any significant effect on the AHF or IOP in healthy volunteers (Kotikoski *et al*., 2003a). For oral uptake, this drug will be digested and metabolised in the blood circulation so that the amount of NO released is likely to decrease with time. In addition, in the present study, it was clear that the AHF and IOP reducing effects of the NO donors were concentration-dependent. Therefore, sufficient amount of the drug will be required at the ciliary body to elicit a similar reduction in the AHF and IOP *in vivo*. It would have been useful to see if the final drug concentration in the blood circulation or at the ciliary epithelium was adequate to elicit any effect in the above human study. However, the authors were unable to use higher doses because of the potential side effects of the drugs. In contrast, the advantage of the present *in vitro* setup was that it allowed desired concentration of drugs (up to 100 μM) be quickly and directly perfused to the ciliary body without being metabolised in the blood stream.

The isolated perfused pig eye setup has several other advantages: (1) this system is free from systemic effects in terms of absorption and metabolism of drugs; (2) the isolated whole eye is free from the influence of any nervous or hormonal effects; (3) it allows simultaneous measurements of the arterial flow rate, arterial pressure, AHF and IOP; (4) it allows the use of high drug concentration that would otherwise be impossible in live animals; (5) it also allows both baseline and control data to be obtained from the same eye; (6) the whole organ preparation involves no traumatic isolation of the ciliary body and therefore preserves the integrity of the CE preparation; (7) lastly, instead of killing live animals for experiments, pig eyes are readily available from local abattoirs. However, it also has a few drawbacks technically: (1) reperfusion of the eye may be hindered by blood clot in the ocular vasculature if the eyes are not fresh enough; (2) occasionally, fine air bubbles in the perfusion system can block small arteries and thereby increase the perfusion pressure beyond acceptable limit of 140 mmHg; (3) despite adequate precautions, some blood vessels are severed in the dissection process and perfusate may leak out from the eye. In our experience, these drawbacks were easily identified and did not cause significant problem in the study.

The mean baseline AHF rate in the isolated perfused pig eye, as determined by fluorescein dilution technique, was 2.7 ± 0.5 ($n = 100$) μl min^{-1} and the mean anterior chamber volume was 237.4 ± 15.5 ($n = 100$) μl. There are few published data on the AHF and IOP of *in vivo* pig eye for comparison with our *in vitro* results. In big eye such as that in the bovine, the anterior chamber volume was found to be 1.69 ml and the AHF rate was also found to be high at 12 μl min^{-1} (Wilson *et al*., 1993). In *in vivo* human eye, the anterior chamber volume was reported to be 247 μl (Toris *et al*., 1999) with an AHF rate of 2.56 μl min^{-1} (Kaplan *et al*., 1996). Given the comparable size between the pig and human eyes, the present data on both the AHF and anterior chamber volume of pig eye are in a similar order of magnitude as the human eye.

The mean baseline IOP of the *in vitro* pig eye was 11.8 ± 0.2, ($n = 100$) mmHg. In this model, we removed most of the extraocular muscles and cut short all the vortex veins to encourage venous drainage and facilitate perfusion of the eye. Thus, there would be little vortex, orbital or ophthalmic venous pressures. Although there may be a small pressure in the remaining episcleral veins, this is likely to be small as well. Therefore, without these opposing pressures, the IOP recorded in the isolated pig eye is expected to be lower than the *in vivo* data. In fact, in a similar setup, the isolated bovine eye gave a lower IOP value of 9.4 mmHg (Wilson *et al*., 1993) than that recorded in the calves, 16.5 mmHg (Woellfel *et al*., 1964).

There are growing evidences that NO is involved in the modulation of AHF in human and animals. For example, cNOS activity has been detected in the bovine ciliary processes (Geyer *et al*., 1997), and neuronal and inducible NOS have also been localised in the porcine CE (Meyer *et al*., 1999). L-NAME inhibitable NO production has been demonstrated in the isolated human and porcine ciliary processes (Haufschild *et al*., 2000). Cyclic AMP, forskolin and isoproterenol have been shown to stimulate NO production in the pig ciliary processes (Liu *et al*., 1999). The nitrite/NO production in pig ciliary processes induced by norepinephrine,

an adrenergic agonist, can be blocked by L-NAME, an NOS inhibitor (Liu *et al*., 1998).

The present data in the pig eye indicates that NO reduces the AHF and IOP via the NO-sGC-cGMP pathway. Exactly how cGMP modulates the ultimate effector (ion transporter, channel or other transport molecules) in the AHF of pig ciliary body remains to be elucidated. Na-K-ATPase is known to be important in AHF (Riley & Kishida, 1986) and it has been identified in the CE of rabbit (Flugel & Lutjen-Drecoll, 1988), ox (Ghosh *et al*., 1990), rat and mouse (Wetzel & Sweadner, 2001). NO and carbachol have been shown to inhibit the Na-K-ATPase activity in the bovine ciliary processes. This inhibition was correlated with an increase in the production of cGMP and was blocked by L-NAME and ODQ (Ellis *et al*., 2001). NO, NO donors or cGMP have also been reported to inhibit the bicarbonate and chloride transport in thick ascending limb of kidney by inhibiting Na-K-2Cl cotransporter (Ortiz *et al*., 2001). Na-K-2Cl cotransporter is a secondary active transport system which is thought to play an important role in the AHF in many species, including human (Hochgesand *et al*., 2001), rabbit (Crook *et al*., 2000) and ox (Do & To, 2000). Whether NO modulates the AHF *via* the Na-K-ATPase or Na-K-Cl or other transport proteins awaits further experimentation.

Recently, it has been reported that stimulation of NO/cGMP pathway induced membrane potential depolarisation in the porcine ciliary processes, which was predicted to lead to an increase in AHF (Fleischhauer *et al*., 2000). However, in the rabbit CE, cGMP produced an increase in the short circuit current when it was applied to the stromal side of the ciliary body, but decreased it when it was applied to the aqueous side (Carre & Civan, 1995). Therefore, it is difficult to predict the effect of cGMP on the AHF because of this 'sidedness' effect of cGMP. We have attempted to directly study the effect of cGMP on the fluid formation in an *in vitro* eye. Although the exact molecular events in terms of ion transport are still unknown, the present data has shown that cGMP decreased the AHF as well as IOP *via* sGC pathway. It would be very interesting to correlate the electrophysiological data with the fluid formation rate in future studies.

In the present investigation, we found no effect on ocular vasculature with any of the drugs used. The lack of vasodilating effect of AZ, SNP, L-arginine, or 8-pCPT-cGMP on ocular vasculature is not entirely unexpected. Apparently, these data are in contrast with some published reports, particularly reports involving anaesthetised pigs (Jacot *et al*., 1998) and in isolated bovine posterior ciliary artery sections (Delaey & Van de Voorde, 1998). However, we observed similar results previously in arterially perfused bovine whole eye preparation where atriopeptin, which also acts through cGMP, reduced AHF without any effect on the ocular vasculature (Millar *et al*., 1997). Furthermore, vascular relaxation by AZ can readily be detected in arterially perfused eye when precontracted by norepinephrine (Millar *et al*., 2001).

A probable explanation for the lack of vascular effects of NO donors might be that the uveal vessels of the perfused eye, particularly the small diameter vessels, were already maximally dilated due to the mechanical shear stress of the fluid flow. In fact, in the normal pig the *in vivo* mean arterial pressure has been reported to be 95 ± 18 mmHg (Gelzer *et al*., 2004). The present study has recorded a lower mean arterial pressure (53.52 ± 2.26 mmHg, $n = 100$). The fact that L-NAME failed to produce any effect on these dilated vessels argues against the notion that vasodilatation was due to NO. In addition, the perfusate (Krebs' solution) did not contain L-arginine, and there would not be sufficient L-arginine for the NOS enzyme to produce adequate NO for vasodilatation in the system. This suggestion is further supported by the fact that L-NAME was effective in reversing the L-arginine induced AHF reduction only in the presence of L-arginine. However, neither L-arginine nor L-NAME nor their combination has any significant effect on the vasculature of the present system. Thus, dilatation of ocular vessels in our system was unlikely to be due to NO. It has been shown that in the isolated perfused eye, the vasodilatation was mediated by endothelium-derived hyperpolarising factors (EDHF) (McNeish *et al*., 2001). Release of EDHF by vascular endothelium due to shear stress has been documented (Macedo & Lautt, 1998). The influence of endothelial mediators increases with decreasing diameter of porcine ocular vessels (Haefliger *et al*., 1993). It was also shown that most of the resistance in the perfused porcine eye is offered by the small arterioles with diameters of 100 μm or less (Meyer *et al*., 1993). Small arteries may be more prone to shear stress due to their thinner vessel wall. Thus, it may not be surprising that the maximally dilated vessels demonstrated no further dilatation with NO donors.

Although the exact reason for the lack of vascular response of the isolated pig eye to SNP or AZ is yet to be understood, the fact that there was no vascular response by the drugs has facilitated the interpretation that the action of NO was exerted primarily on the CE. In other words, the effects of NO donors on the AHF and IOP reductions can be attributed to the CE but not the ciliary blood flow. Furthermore, we have shown earlier that the effect of nitrovasodilator on the IOP of the isolated perfused bovine eye was not *via* vasodilatation, but by direct action at the CE (Millar *et al*., 2001). Recently, using the same *in vitro* pig eye system, we have observed that significant variation of the arterial perfusion flow or pressure did not affect the AHF rate even when the arterial flow is increased by 100% or decreased by more than 75% (unpublished data). Similar results have been found by Kiel and Reitsamer in anesthetised rabbits (Reitsamer & Kiel, 2003).

In the present investigation, reduction of AHF was found to be about double the reduction of IOP in terms of percentage. For example, while10 μM AZ produced about 52% reduction in AHF, it produced only 24% reduction in IOP (Table 1 vs Table 2). Similar trends were found with all effective drugs at all concentrations used in this study (Table 1 vs Table 2). There are three plausible interpretations for this fall in IOP: it may due to (i) a reduction in AHF with unchanged outflow facility at the trabecular meshwork; (ii) a combined effect of reduction in AHF and increase in trabecular outflow facility or (iii) a combined effect of increases in both AHF and outflow facility. Both the (i) and (ii) are possible explanations to the observation. However, condition (iii) where both the AHF and outflow facility increase is unlikely. Assuming the AHF remains unchanged, increase in the outflow facility will improve the ease of aqueous outflow and decrease both the IOP and volume of the anterior chamber. Although the AHF rate is unchanged, such decrease in anterior chamber volume will lead to an increase in the fluorescein dilution rate, which is always translated into as an increase, but not a decrease, in AHF. However in the above experimental observation, the rate of fall in absorbance was not accelerated but slowed

down. It showed that the AHF was reduced and the IOP was decreased by a significant amount. In fact, the setup is more likely to underestimate the decrease of AHF if the outflow facility is increased. Similarly, this logic also argues against the possibility of any increase in the AHF with NO donors in the present experiment. However, the exact contribution of AHF rate and outflow facility to the reduction in IOP is unclear and awaits further experimentation. Although a number of reports have shown that NO donors reduced IOP by increasing trabecular outflow facility, we cannot confirm if NO has a dual effect of decreasing AHF and increasing outflow facility in the present study. We may reasonably conclude that NO donors reduce IOP at least in part by reducing AHF.

Nipradilol, a novel antiglaucoma drug, has been shown to improve retinal blood flow (Kida *et al*., 2001) and increased the optic nerve head circulation in human (Mizuno *et al*., 2002). Nipradilol has also been shown to offer neuroprotection in rats mainly through its NO releasing property (Mizuno *et al*., 2001; Nakazawa *et al*., 2002). The present results have shown that NO donors reduce the AHF and demonstrated ocular hypotensive action. Considering its effects of reducing AHF, increasing AH outflow and improving retinal circulation (Haefliger *et al*., 1994), NO and its donors have the potential to become the next generation of pharmacological treatment for glaucoma patients.

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