Systemic vascular endothelial cell dysfunction in normal pressure glaucoma

Christine Buckley, Patrick W F Hadoke, Emer Henry, Colm O’Brien

Aim: Vascular risk factors, and particularly vasospasm, are thought to play a part in the pathogenesis of normal pressure glaucoma (NPG). This study aimed to determine whether the function of systemic resistance arteries was altered in patients with NPG.

Methods: Contractile and relaxant function was assessed in arteries dissected from gluteal fat biopsies (11 NPG, 12 control) using small vessel myography.

Results: Responses to K+ and noradrenaline were similar in patients and controls and were unaffected by endothelial removal. In contrast, responses to 5-hydroxytryptamine (5-HT; pD2 7.29 (SD 0.16) vs 6.66 (0.19); p=0.03) and endothelin-1 (ET-1; pD2 9.12 (0.10) vs 8.72 (0.13); p=0.03) were enhanced in arteries from patients with NPG. Removal of the endothelium enhanced responses to 5-HT (pD2 6.66 (0.19) v 7.66 (0.08); p=0.003) and ET-1 (pD2 8.72 (0.13) v 9.66 (0.39); p=0.02) in control arteries but not in those from patients. ET-1 mediated contraction in control and patient arteries was reduced in the presence of (10−5 M) nifedipine. Endothelium dependent and independent relaxation was not impaired in arteries from patients.

Conclusions: This study has identified dysfunction of the systemic vascular endothelial cell in patients with normal pressure glaucoma. The vascular endothelium modulates contractile responses to 5-HT and ET-1 in human subcutaneous resistance arteries but this effect is lost in patients with NPG, indicating a selective defect in agonist mediated release of endothelium derived vasodilators. Selective antagonists of 5-HT and ET-1 may, therefore, help to prevent vasospasm in patients with NPG.
measurement of plasma cholesterol. The study protocol was approved by Lothian research ethics committee and conformed to the tenets of the Declaration of Helsinki. All subjects gave written informed consent before enrolment in the study.

Preparation of arteries
A biopsy of skin and subcutaneous fat (2 cm long × 1 cm × 1 cm) was taken from the gluteal region following subcutaneous injection of local anaesthetic (Xylocaine; 2% lidocaine hydrochloride with adrenaline, Astra Pharmaceuticals, Kings Langley, UK). Arteries were dissected from the biopsy and mounted as ring preparations (approximately 2 mm in length) on two 40 µm intraluminal stainless steel wires in a small vessel myograph (JP Trading, Aarhus, Denmark) for measurement of isometric tension. The vessels were bathed in physiological salt solution (PSS; composition in mM: NaCl 119, KCl 5.5, KH2PO4 1.17, MgSO4 1.18, D-glucose 5.5, K2 EDTA 0.026, NaHCO3 25, CaCl2 2.5) maintained at 37°C and gassed with 95% oxygen, 5% carbon dioxide. The length of each vessel segment was measured by light microscopy using a travelling micrometre eyepiece. Following a 30 minute equilibration period, the resting tension–internal circumference relation was determined for each artery by stepwise stretching of the vessel and application of the Laplace equation as previously described. From this, the internal circumference (L) that the artery would have in situ when relaxed under a transmural pressure of 100 mm Hg (13.3 kPa) was calculated. Each vessel was then set to an internal circumference of 0.9L at which these vessels develop maximum active tension.

The vessels were allowed to equilibrate at their optimum resting tension for 30 minutes before undergoing a standard start protocol in which the viability of each artery was assessed. This consisted of stimulating the vessels twice with NAK (a high potassium solution (125 mM; KPSS), prepared by equimolar substitution of KCl for NaCl in normal PSS, containing noradrenaline (NA; 5 × 10^-7 M)), once with NA (5 × 10^-7 M), and allowed to relax fully for at least 20 minutes before the next drug was tested.

Drugs
All salts were obtained from BDH (Poole, Dorset, UK). Noradrenaline bitartrate, 5-hydroxytryptamine creatinine sulphate complex, acetylcholine chloride, bradykinin acetate, and nifedipine were from Sigma (Poole, Dorset, UK). Endothelin-1 and 3-morpholinosydnonimine hydrochloride were obtained from Alexis Biochemicals (Beeston, Nottingham, UK). All drugs were dissolved in deionised water except for ET-1 which was dissolved in 50% methanol and nifedipine which was dissolved in 50% ethanol before subsequent dilution in deionised water. Final bath concentrations of methanol and ethanol did not exceed 1.5% and 0.5% v/v, respectively.

Statistical analysis
Contractile responses are expressed as active force (mN/mm) and as a percentage of the maximum response to NAK obtained during the standard start (% max NAK). Relaxations are expressed as a percentage of the induced precontraction to NA. For each concentration–response curve the molar concentration required to produce 50% of the maximum contraction (EC50) or relaxation (IC50) was calculated by fitting the Hill equation using the curve fitting program Fig P (Biosoft, UK). The sensitivity of the vessels to each agonist was expressed as the negative logarithm of the EC50 (pD2) for constrictor agonists or –log IC50 for dilator agonists. All results are given as the mean (SEM) for n experiments, where n represents the number of subjects. Maximum responses and EC50 values were compared using Student’s unpaired t test and differences considered significant when p≤0.05.

RESULTS
There were no significant differences in the demographics between patient and control groups (Table 1). The internal diameters of resistance arteries isolated from gluteal biopsies used in this study were similar (p=0.53) in patients with glaucoma (258 (SEM 17) µm) and control subjects (238 (26) µm), and the contractile responses evoked by NAK were not significantly different between the two groups (patients 3.06 (0.22) mN/mm; controls 2.66 (0.29) mN/mm, p=0.26).

Responses to dilator agonists
ACh, BK, and SIN-1 all evoked concentration dependent relaxations in arteries precontracted with NA from both patients and controls. The relaxation in response to ACh and BK was significantly reduced following removal of the endothelium in both subject groups whereas the response to SIN-1 was unaffected (Table 2). Arteries from patients with

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Table 1  Characteristics of control subjects and patients with NPG

<table>
<thead>
<tr>
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<th>Control (n=12)</th>
<th>NPG (n=11)</th>
<th>p Value</th>
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</thead>
<tbody>
<tr>
<td>Sex (M:F)</td>
<td>7:5</td>
<td>6:5</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>57 (2)</td>
<td>59 (2)</td>
<td>0.68</td>
</tr>
<tr>
<td>IOP (mm Hg)</td>
<td>15.5 (0.9)</td>
<td>15.8 (0.8)</td>
<td>0.82</td>
</tr>
<tr>
<td>Mean arterial blood pressure (mm Hg)</td>
<td>83 (2)</td>
<td>87 (4)</td>
<td>0.41</td>
</tr>
<tr>
<td>Heart rate (BPM)</td>
<td>70 (3)</td>
<td>63 (3)</td>
<td>0.13</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>62.4 (3.1)</td>
<td>72.1 (4.7)</td>
<td>0.13</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.1 (0.4) (n=8)</td>
<td>4.9 (0.6) (n=8)</td>
<td>0.74</td>
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Values are expressed as mean (SEM)
endothelium from control vessels caused a significant increase in maximum contractile response (p = 0.045) and sensitivity (p = 0.003) to 5-HT (Table 3; Fig 2B) whereas endothelial removal from vessels from patients had no effect on either maximum response (p=0.66) or sensitivity (p=0.22; Table 3; Fig 2C).

ET-1 evoked contractions were of similar magnitude (p = 0.61) in arteries from patients with glaucoma and control subjects; the arteries from patients were, however, significantly more sensitive (p = 0.03) to ET-1 than control arteries (Table 3; Fig 3A). Comparison of individual concentration-response curves indicated that responses to ET-1 were similar in arteries from patients previously receiving betaxolol HCl and those from untreated patients. Endothelial removal from control vessels caused a 10-fold increase in sensitivity (p = 0.02) to ET-1 (Table 3; Fig 3B); denudation of vessels from patients, however, had no effect on sensitivity (p = 0.43) to this agonist (Table 3; Fig 3C). In endothelium intact arteries the maximum contraction in response to ET-1 was reduced following incubation with nifedipine (10⁻⁷ M) in both patients (Eₐₗ reduced from 3.45 (0.39) to 1.79 (0.32) mN/mm; n = 7; p=0.008) and controls (Eₐₗ reduced from 3.13 (0.49) to 1.65 (0.31) mN/mm; n = 6; p=0.04).

**DISCUSSION**

A number of studies have suggested that alterations in the systemic vasculature contribute to the aetiology and progression of NPG. Indeed, treatment with drugs that increase systemic blood flow can also improve visual field in some patients with NPG. This investigation, the first to describe vascular function in arteries isolated from patients with NPG, aimed to determine whether the functional characteristics of isolated resistance arteries were altered in patients with this condition.

The demonstration that endothelium dependent relaxation was maintained (indeed sensitivity to ACh was enhanced) indicates that the ability of the endothelium to release relaxing factors is not impaired in arteries from patients with NPG. The cause of the selective increase in sensitivity to ACh

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**Table 2** Maximum relaxation and sensitivity (−log IC₅₀) values for dilator agonists in endothelium intact and denuded arteries isolated from control subjects and patients with NPG

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>NPG</th>
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<tbody>
<tr>
<td></td>
<td>Intact (n=8–11)</td>
<td>Denuded (n=5)</td>
</tr>
<tr>
<td></td>
<td>% Relax</td>
<td>-log IC₅₀</td>
</tr>
<tr>
<td>ACh</td>
<td>95.87 (1.41)</td>
<td>4.00 (3.23)*</td>
</tr>
<tr>
<td>BK</td>
<td>95.72 (1.84)</td>
<td>15.54 (6.45)*</td>
</tr>
<tr>
<td>SIN-1</td>
<td>99.85 (0.83)</td>
<td>100.69 (1.49)</td>
</tr>
</tbody>
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Values are expressed as mean [SEM] *p<0.05 compared with intact vessels in the same subject group; †p<0.05 compared with endothelium intact control vessels.

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**Table 3** Maximum contractile responses (Eₐₗ) and sensitivity (pD₂) values for constrictor agonists in endothelium intact and denuded arteries isolated from control subjects and patients with NPG

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control</th>
<th>NPG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intact (n=8–11)</td>
<td>Denuded (n=5–6)</td>
</tr>
<tr>
<td></td>
<td>Eₐₗ (mN/mm)</td>
<td>pD₂</td>
</tr>
<tr>
<td>NA</td>
<td>2.48 (0.46)</td>
<td>1.75 (0.27)</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.67 (0.18)</td>
<td>2.56 (0.66)*</td>
</tr>
<tr>
<td>ET-1</td>
<td>3.13 (0.49)</td>
<td>3.25 (0.63)</td>
</tr>
<tr>
<td>K⁺</td>
<td>2.81 (0.52)</td>
<td>2.44 (0.77)</td>
</tr>
</tbody>
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Values are expressed as mean [SEM]. *p<0.05 compared with intact vessels in the same subject group; †p<0.05 compared with endothelium intact control vessels.

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**Figure 1** Cumulative concentration-response curves to acetylcholine in endothelium intact arteries isolated from control subjects (n = 11) and patients with NPG (n = 11). Each point represents the mean with SEM shown by vertical bars.
vascular function in hypertension, lar disparities reported between in vitro and in vivo studies of the present in vitro investigation are unclear but reflect simi-
The reasons for the differences between the in vivo study and those obtained using forearm plethysmography. Finally, dis-
parities between studies using forearm blood flow and isolated resistance arteries may reflect physiological variations be-
tween vessels of differing anatomical origin. Blood flow in the forearm occurs mainly through muscular arteries (with only a small contribution through cutaneous or subcutaneous vessels), whereas gluteal biopsies provide purely subcutaneous arteries. In the latter, ACh stimulates relaxation mainly via the action of endothelium derived hyperpolarising factor (EDHF) with a relatively small component mediated by NO.25 ACh also acts via endothelium derived NO in the forearm but the contribution of EDHF has not been determined in this territory.26 While the relative contributions of NO and EDHF to relaxation of arteries from control subjects and patients with NPG require further investigation, it is possible that impaired NO mediated relaxation in NPG (demonstrated in the forearm23) is masked in gluteal subcutaneous resistance arteries by enhanced EDHF activity.

remains unclear but may reflect an alteration at the level of the muscarinic receptor itself or in muscarinic receptor mediated release of endothelium derived relaxing factors. Certainly, the ability of the smooth muscle to relax in response to nitric oxide (NO) is not altered as responses to the NO donor, SIN-1, were unchanged in arteries from patients with NPG. These results contrast with a study of forearm blood flow in vivo performed by our own group18 which reported impaired ACh mediated relaxation (due to an abnormality in endothelial cell function) in patients with NPG. This is particularly relevant as some of the patients in whom forearm blood flow was assessed also provided biopsies for the present investigation. The reasons for the differences between the in vivo study and the present in vitro investigation are unclear but reflect similar disparities reported between in vitro and in vivo studies of vascular function in hypertension.11–12 insulin dependent diabetes mellitus (IDDM),13–14 and non-insulin dependent diabetes mellitus (NIDDM).15–16 As small vessel myography isolates resistance arteries from the humoral environment, it is possible that impaired ACh mediated relaxation in vivo is caused by enhanced degradation of endothelium derived NO by bloodborne factors (for example, free radicals). Alternatively, functional responses obtained using arteries suspended on intraluminal wires in vitro may not reflect the behaviour of those in vivo. However, even when pressure myography (which places the isolated arteries under conditions similar to those experienced in vivo) was used to assess vascular function in patients with NIDDM,14 the results did not correspond with those obtained using forearm plethysmography.18 Finally, disparities between studies using forearm blood flow and isolated resistance arteries may reflect physiological variations between vessels of differing anatomical origin. Blood flow in the forearm occurs mainly through muscular arteries (with only a small contribution through cutaneous or subcutaneous vessels), whereas gluteal biopsies provide purely subcutaneous arteries. In the latter, ACh stimulates relaxation mainly via the action of endothelium derived hyperpolarising factor (EDHF) with a relatively small component mediated by NO.25 ACh also acts via endothelium derived NO in the forearm but the contribution of EDHF has not been determined in this territory.26 While the relative contributions of NO and EDHF to relaxation of arteries from control subjects and patients with NPG require further investigation, it is possible that impaired NO mediated relaxation in NPG (demonstrated in the forearm23) is masked in gluteal subcutaneous resistance arteries by enhanced EDHF activity.
The endothelium plays an important part in the control of local vascular tone and blood flow and many vasoconstrictors modulate their own actions by receptor-mediated release of endothelium-derived relaxing factors. Removal of the endothelium attenuates this modulatory effect, resulting in augmented agonist-mediated contraction. The present study used a receptor-independent vasoconstrictor, KCl, which is unaffected by either basal or stimulated release of NO, as well as 5-HT, ET-1, and NA, which cause contraction by stimulating receptors (5-HT₁, ET₁, and α₁ adrenoceptors, respectively) on the vascular smooth muscle. In some arteries, contractile responses to 5-HT, ET-1, and NA are modulated in part by endothelium-derived relaxing factors. Removal of the endothelium did not reduce these responses. Since only the responses observed in patients with NPG were only found in intact arteries from patients with NPG. These findings may also have implications for the periphery as well. While neither study confirmed that the systemic vascular defects identified are responsible for optic nerve damage, both support the hypothesis that NPG is one manifestation of more widespread vascular abnormalities. Enhanced contractile responses in the periphery may contribute to vascular complications in these patients and further studies are required to assess whether 5-HT and ET-1 receptor antagonists may be useful in the prevention of vasospasm.

**ACKNOWLEDGMENTS**

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**REFERENCES**


