Basal nitric oxide production is impaired in offspring of patients with essential hypertension

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ABSTRACT

There is considerable evidence that endothelium-dependent nitric oxide (NO)-mediated vasodilatation in response to acetylcholine is impaired in essential hypertension, whereas the endothelium-independent response to sodium nitroprusside is normal. More limited data have suggested that there is also reduced vasoconstriction in response to \( \text{N}^\text{G} \)-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of basal NO release. As it is not known whether endothelial dysfunction in hypertension, if indeed present, is a cause or consequence of the condition, we have studied the normotensive offspring of parents with essential hypertension. Both basal and stimulated vascular responses were examined in 12 normotensive offspring [mean age (± S.E.M.) 26.1 ± 1.4 years] of parents with essential hypertension and compared with those in 12 age-matched offspring (mean age 25.6 ± 1.1 years) of normotensive subjects. Forearm blood flow was measured simultaneously in both arms by venous occlusion plethysmography, both at baseline and during intra-arterial brachial infusion of increasing doses of acetylcholine, sodium nitroprusside, noradrenaline and L-NMMA. There were no significant differences between the groups in the responses to acetylcholine, sodium nitroprusside or noradrenaline. In contrast, the vasoconstrictor response to L-NMMA was significantly blunted in the offspring of hypertensive parents compared with that in the offspring of normotensive parents (\( P = 0.005 \)). Thus endothelial dysfunction, as demonstrated by impaired basal production of NO, is present in subjects at high risk of essential hypertension, and does not occur simply as a consequence of the condition.

INTRODUCTION

Furchgott and Zawadzki [1] first demonstrated in 1980 that vasodilatation induced by acetylcholine was mediated by the release of an endothelial-derived mediator (endothelium-derived relaxing factor), which was later identified as nitric oxide (NO). Since then, the role of the endothelium in normal vascular function and in cardiovascular disease has been the focus of increasing interest. NO is synthesized within the endothelium from L-arginine by the endothelial constitutive isoform of the enzyme NO synthase. Within the vascular smooth muscle, NO activates soluble guanylate cyclase to increase the levels of cytoplasmic cGMP, with a resultant decrease in calcium influx, thus causing vasodilatation. Sodium nitroprusside produces endothelial-independent vasodilatation by providing an inorganic source of NO. Basal release of NO has also been investigated by infusing the L-arginine analogue \( \text{N}^\text{G} \)-monomethyl-L-arginine (L-NMMA), which acts as a competitive inhibitor of endothelial constitutive NO synthase activity [2]. There is considerable evidence from both animal work

Key words: acetylcholine, endothelium, hypertension, nitric oxide, plethysmography.
Abbreviations: FBF, forearm blood flow; L-NMMA, \( \text{N}^\text{G} \)-monomethyl-L-arginine.
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[3–8] and studies of the coronary and forearm circulations in humans [9–22] that endothelial-dependent relaxation in response to acetylcholine is impaired in hypertension. However, these findings were challenged by Cockcroft et al. [23], who demonstrated that the vasodilator responses to sodium nitroprusside, carbachol and acetylcholine were similar in hypertensive and normotensive subjects. More limited evidence in humans has suggested that the decrease in forearm blood flow (FBF) induced by L-NMMA is smaller in hypertensive than in normotensive subjects [13,16,24,25]. Basal NO production was not examined in the study of Cockcroft et al. [23].

A fundamental question which remains unanswered is whether endothelial dysfunction in hypertension, if indeed present, is a cause or consequence of the condition. Whereas studies in hypercholesterolaemia have shown the reversal of endothelial dysfunction with effective treatment [26,27], this has not been consistently demonstrated in hypertension [11,12,28–31]. The study of individuals who, although clinically normal, are at increased genetic risk for hypertension offers an alternative approach which avoids any confounding effects of treatment. It is also conceivable that early stages of vascular disease may involve more subtle defects, perhaps in basal rather than stimulated formation of NO [32]. A recent study employing such a strategy examined only stimulated NO production, and showed a reduction in acetylcholine-induced vasodilation in offspring of hypertensive compared with normotensive parents [33].

In the present study we have examined both basal and stimulated NO production in a group of normotensive offspring of parents with essential hypertension, and compared these with results from a comparable number of offspring of normotensive control subjects.

METHODS

Subjects
A total of 24 healthy Caucasian volunteers were recruited from hospital staff or through family practices with which links have been established. Of these, 11 had either a maternal or a paternal history of hypertension, and for one subject both parents were hypertensive, as confirmed by hospital or general practice records. The remaining 12 subjects, for whom neither parent had any evidence of hypertension (defined as blood pressure < 140/90 mmHg and confirmed from general practice records or by an investigator), served as controls. One subject in each group had a parental history of ischaemic heart disease, and two in each group had a family history of type II (non-insulin-dependent) diabetes mellitus. One subject in each group smoked 10–15 cigarettes per day. The study was approved by the Research Ethical Committee of the Queen’s University of Belfast and was in accordance with the Declaration of Helsinki (1989) of the World Medical Association, and all subjects gave written informed consent.

None of the subjects had any significant past medical history; nor were they receiving medication before or during the study. The clinical characteristics of the two groups are summarized in Table 1. Biochemical results shown were determined from fasting serum samples. Systolic and diastolic blood pressures represent the average values over a 24 h period, assessed by ambulatory monitoring every 30 min (QuietTrak; Welch Allyn, Skaneateles Falls, NY, U.S.A.). There were no significant differences in any variable between the two groups.

Protocol
Studies were performed in the morning in an undisturbed temperature-controlled environment (22–24 °C, within ± 0.5 °C during each study). Subjects lay supine with both forearms supported above the level of the right atrium. Each individual fasted from 22.00 hours the night before, and abstained from ingestion of alcohol or caffeine-containing beverages during the same period. A 27 G unmounted needle (Cooper’s Needle Works, Birmingham, U.K.), sealed with dental wax to an 18 G epidural catheter (Portex, Hythe, Kent, U.K.), was inserted into the brachial artery of the non-dominant side (usually the left), under sterile conditions and after local anaesthesia with 1% (w/v) lignocaine hydrochloride (Antigen Pharmaceuticals Ltd., Roscrea, Ireland). The infusion of saline or drug solutions throughout the study was at a constant rate of 1 ml/min using syringe pumps (Ivac P2000; Ivac Corp., Welmed Ltd., Hants., U.K.).

FBF was measured in both arms simultaneously by venous occlusion plethysmography using mercury-in-silastic strain gauges placed at the widest part of the forearms. The strain gauges were electrically calibrated [34] to measure the percentage change in forearm volume. These were connected to a plethysmograph (Vascular SPG16; Medasonics, Mountain View, CA, U.S.A.), which in turn was connected to a computerized chart recording system (Maclab; AD Instruments, Castle Hill, NSW, Australia). The hands were excluded from the circulation by the inflation of wrist cuffs to 200 mmHg for 1 min before and during each measurement period. Upper arm cuffs were inflated to 40 mmHg by a rapid cuff inflator (model E-20; Hokanson Inc., Bellevue, WA, U.S.A.) to occlude venous outflow. Blood flow was recorded for 10 s out of every 15 s during the final 1.5 min of each infusion period.

Resting control FBF values were obtained at least 30 min after needle placement, to allow stabilization of blood flow. In the first part of the study, subjects then received four incremental doses of acetylcholine (Miochol; Iolab, Bracknell, Berks., U.K.) at 25, 50, 100 and 200 nmol/min (3.7, 7.4, 14.7 and 29.4 μg/min respect-
Table 1  Characteristics of study subjects
Values are means ± S.E.M. No statistical differences between groups were observed at P < 0.05. Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.

<table>
<thead>
<tr>
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<th>Offspring of hypertensive parents</th>
<th>Offspring of normotensive parents</th>
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<tbody>
<tr>
<td>Sex (male/female)</td>
<td>7/5</td>
<td>7/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>26.1 ± 1.4</td>
<td>25.6 ± 1.1</td>
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<tr>
<td>Body mass index</td>
<td>22.8 ± 0.8</td>
<td>23.7 ± 0.8</td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>123.5 ± 3.2</td>
<td>121.5 ± 3.1</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>69.9 ± 2.4</td>
<td>69.8 ± 2.1</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>4.3 ± 0.10</td>
<td>4.4 ± 0.14</td>
</tr>
<tr>
<td>Haemoglobin Hb (g/dl)</td>
<td>8.55 ± 0.14</td>
<td>8.75 ± 0.14</td>
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<tr>
<td>Fasting serum insulin (mU/ml)</td>
<td>13.2 ± 2.1</td>
<td>12.1 ± 2.0</td>
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<tr>
<td>Serum total cholesterol (mmol/l)</td>
<td>4.67 ± 0.15</td>
<td>4.54 ± 0.19</td>
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<tr>
<td>Serum LDL cholesterol (mmol/l)</td>
<td>2.84 ± 0.15</td>
<td>2.79 ± 0.15</td>
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<tr>
<td>Serum triacylglycerols (mmol/l)</td>
<td>1.09 ± 0.13</td>
<td>0.96 ± 0.11</td>
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<tr>
<td>von Willebrand factor (%)</td>
<td>84.7 ± 7.4</td>
<td>98.1 ± 11.7</td>
</tr>
<tr>
<td>Random urinary albumin/creatinine ratio</td>
<td>0.56 ± 0.12</td>
<td>0.40 ± 0.06</td>
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<tr>
<td>Timed overnight albumin excretion rate (mg/min)</td>
<td>3.82 ± 0.87</td>
<td>2.96 ± 0.47</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>81.0 ± 4.0</td>
<td>77.4 ± 3.0</td>
</tr>
<tr>
<td>Resting FBF (ml·min⁻¹·100 ml⁻¹)</td>
<td>2.93 ± 0.26</td>
<td>2.71 ± 0.29</td>
</tr>
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</table>

Effectively) for 4 min each, and four doses of sodium nitroprusside (Nipride; Roche, Welwyn Garden City, Herts., U.K.) at 2.5, 5, 10 and 20 nmol/min (0.75, 1.5, 3, and 6 µg/min respectively), also for 4 min each, to produce cumulative dose–response curves. The order of infusion of agents was randomized, and a 20–30 min rest period was given to allow FBF to return to baseline values between each infusion.

The second part of the study followed another 30 min rest period and further baseline FBF measurements. All but one subject received three doses of noradrenaline (Levophed 1:1000; Sanofi Winthrop, Guildford, Surrey, U.K.) at 100, 200 and 400 pmol/min (17, 34 and 68 ng/min respectively) for 5 min each. This was followed 20–30 min later, again after return to baseline, by infusion in all subjects of three doses of L-NMMA (Calbiochem-Novabiochem) at 2, 4 and 8 µmol/min (1, 2 and 4 mg/min respectively) for 5 min each.

Blood pressure and heart rate were recorded in the non-infused arm before each of the four infusion periods using an automatic oscillometric digital blood pressure monitor (HEM-705CP; Omron, Tokyo, Japan).

Statistical analysis
FBF is expressed as ml·min⁻¹·100 ml⁻¹ forearm volume, as established by Whitney [35]. The average of five recordings at each infusion step was calculated for both the infused and non-infused (control) arms. This allowed the calculation of the ratio of blood flow in the infused arm to that in the control arm (infused/control ratio), thus controlling for any effects of unavoidable external and systemic factors, which should affect blood flow in both arms similarly [36]. The FBF ratio (infused/control) measurement in response to drugs was expressed as a percentage of the ratio measured during the control period. These calculated values, when used to construct a dose–response curve for each drug in each subject, ensured that only the effects attributable to local administration of the drug were being assessed.

The overall response to each drug was measured as the area under the dose–response curve, calculated using the trapezium rule [37]. Results are expressed as means ± S.E.M., and were compared using Student’s t-test for paired or unpaired observations as appropriate.

RESULTS

Effects on FBF of acetylcholine, sodium nitroprusside and noradrenaline
Acetylcholine (25–200 nmol/min) and sodium nitroprusside (2.5–20 nmol/min) each produced dose-dependent augmentation of the basal FBF ratio (infused arm/control arm) in both groups of subjects. Dose–response curves were established for each drug in all subjects, and the area under the curve was calculated as outlined above. There were no statistically significant differences in the responses between the two groups (Figure 1).

Noradrenaline (100–400 pmol/min) resulted in a reduced blood flow ratio in all but one subject, in whom
there was modest vasodilatation. In one other subject there was an unexplained increase in blood flow with noradrenaline at 100 pmol/min, followed by diminished flow at higher doses. There were no differences in the response curves between the groups (Figure 2).

Effect on FBF of L-NMMA
L-NMMA (2–8 μmol/min) consistently produced a dose-dependent decrease in the FBF ratio (infused arm/control arm) in both groups of subjects (Figure 2). This response was significantly blunted in the offspring of hypertensive subjects compared with controls; the mean difference in area under the dose–response curve was 34.63 units (95% confidence intervals 11.77, 57.50; \( P = 0.005 \)). Incremental doses of L-NMMA (2, 4 and 8 μmol/min) reduced the blood flow ratio by 22.3 ± 3.3, 33.5 ± 4.2 and 45.3 ± 4.5% respectively of the baseline value in the offspring of hypertensive parents, as compared with 33.2 ± 2.7, 48.4 ± 2.8 and 63.0 ± 2.7% respectively in the offspring of normotensive control subjects.

DISCUSSION
There is continuing discussion concerning the presence of endothelial dysfunction in hypertension. The substantial body of evidence from animal data and isolated tissue preparations has been criticized, because such data are frequently limited to large conduit arteries. Most studies of endothelial function in human essential hypertension have examined endothelium-dependent NO production by observing its effects at the resistance vessel level, using venous occlusion plethysmography [35]. In addition, responses to the intra-arterial infusion of endogenous nitrovasodilators, such as acetylcholine, have been compared with those produced by exogenous nitrovasodilators, such as sodium nitroprusside, which provides an inorganic source of NO. The majority of these studies have demonstrated significantly impaired endothelium-dependent vasodilation in patients with essential hypertension compared with normotensive controls [9–17].

By contrast, Cockcroft et al. [23] showed similar vasodilator responses to acetylcholine, carbachol and sodium nitroprusside in the forearm resistance vessels of
hypertensive patients and normotensive controls, using these same techniques. These results seem difficult to reconcile with previous studies. One explanation is that endothelial dysfunction may not affect all patients with hypertension to the same degree [32]. Patient groups have also differed widely in the known duration and severity of hypertension, while the precise duration cannot be determined in most patients. The duration of withdrawal of anti-hypertensive treatment (including diuretics, beta-blockers, calcium antagonists, inhibitors of angiotensin-converting enzyme and centrally acting drugs) prior to study has varied from not at all to 4 weeks. In addition, the impact of diet, physical fitness and these variables in the assessment of endothelial function is difficult to determine.

It is also possible that early defects may involve the basal production of NO rather than its acetylcholine-stimulated release [32]. Identification of the methylated l-arginine analogue L-NMMA, which acts as a competitive inhibitor of NO synthase, has allowed an assessment of the contribution of basal NO release to the maintenance of vascular tone. Infusion of L-NMMA causes vasoconstriction, purely on an endothelium-dependent basis, demonstrating that there is a continuous physiological release of NO from the endothelium which contributes to the regulation of blood flow and blood pressure [2]. Several reports in hypertension have used infusions of L-NMMA to examine basal NO production [13,16,24,25], and in two of these studies the response to the non-specific endothelium-independent vasoconstrictor noradrenaline was also examined [24,25]. All but one [25] of these studies have demonstrated a significantly greater decrease in blood flow in normotensive controls than in hypertensive subjects, indicating impaired basal NO synthesis in the latter group. In the one exception [25], there was nonetheless a significant correlation in all subjects between the response to L-NMMA and blood pressure.

The purpose of the present study was to determine whether endothelial dysfunction pre-dates the development of hypertension in a high-risk group. Conceivably, the endothelium could have an integral role in the development of increased blood pressure, thereby contributing to the vascular risks associated with hypertension. Animal data suggest that endothelial dysfunction is a consequence, rather than a cause, of hypertension [5,38]. Studies in rats, including both spontaneous and induced hypertension, have shown improved endothelium-dependent relaxation after normalization of blood pressure. This has been observed in the abdominal aorta, carotid arteries or mesenteric vessels after treatment with hydralazine or reversal of the hypertensive stimulus. However, most of the human studies in which endothelial function has been assessed before and after normalization of hypertension with conventional therapy have not shown an improvement in the endothelial-dependent responses [12,28,29,31]. It seems, therefore, that endothelial dysfunction in human hypertension, once established, is more difficult to reverse than in the rat. This appears to contrast with the situation in hypercholesterolaemia, in which endothelial dysfunction is reversed by treatment [26,27].

Examination of subjects with normal blood pressure but at an increased risk of hypertension allows the detection of early abnormalities, while minimizing any possible secondary effects of hypertension and eliminating the confounding effects of treatment. Our results show a reduced response to L-NMMA in the offspring of parents with essential hypertension compared with offspring of normotensive control subjects, indicating an abnormality in basal endothelial-dependent NO function. Assessment of basal NO production has not previously been undertaken in such a group of patients at subsequent high risk for the development of hypertension. Although one study [33] in a similar population demonstrated impaired endothelium-dependent vasodilatation, as assessed by the response to acetylcholine, we were unable to confirm this. The reasons for the discrepancy between the previous study and ours are not apparent.

The reduced response to L-NMMA found in the present study may be indicative of diminished basal NO synthesis in the offspring of hypertensive subjects, thereby resulting in less vasoconstriction after inhibition of NO synthesis by L-NMMA. The findings are unlikely to be explained on the basis of clinical differences between the study groups, since subjects were closely matched for age, sex, body mass index, cholesterol and all other biochemical parameters, while none had any clinical evidence of vascular disease. Average blood pressure, measured by ambulatory monitoring over a 24 h period, was similar in the hypertension risk group (123.5 ± 3.2/69.8 ± 2.4 mmHg) and the control group (121.5 ± 3.1/69.8 ± 2.1 mmHg). Although an association between microalbuminuria and endothelial dysfunction has been reported in some, but not all, studies [39–41], and elevated levels of von Willebrand factor have also been described [39,42], in the present study there were no significant differences between groups in either of these measurements. Neither was there a significant difference in mean fasting insulin levels between the groups (offspring of hypertensives, 6.29 ± 0.60 m-units/l; controls, 4.83 ± 0.71 m-units/l).

Our findings not only provide further important independent support for an association between endothelial dysfunction and hypertension, but also suggest that endothelial dysfunction is a phenomenon that pre-dates the development of essential hypertension. Basal NO synthesis may be the first detectable evidence of endothelial dysfunction. However, our results do not exclude the possibility that hypertension subsequently exacerbates this dysfunction, perhaps irreversibly. The
lack of a difference in the responses to acetylcholine and sodium nitroprusside may have reflected insufficient patient numbers, given the large variability in responses. Our post-hoc power calculations revealed that, with reference to the acetylcholine response (Figure 1), 12 subjects would have allowed the detection of a 9-unit difference in the area under the curve for acetylcholine with 80% power. This would have corresponded to a difference in the percentage increase in the FBF ratio (infused limb/contralateral limb) between groups of 0% at dose 0, rising to 130% at 25 nmol/l, to 200% at 50 nmol/l, to 330% at 100 nmol/l and to 480% at 200 nmol/l. In addition to these power considerations, it should be noted that Angus and Lew [43] have suggested previously that this test may be influenced by many factors, not necessarily related to endothelial dysfunction.

In conclusion, our results show that endothelial dysfunction is present in healthy normotensive subjects at high risk for the development of essential hypertension, and thus does not simply occur as a consequence of the condition. They also demonstrate that reduced basal NO production may be among the earliest abnormalities of endothelial dysfunction. Careful follow-up of these subjects should offer further insights into disease pathogenesis. A major challenge for the future is to establish whether the progression of endothelial dysfunction can be retarded, particularly in subjects at increased risk for vascular disease.

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