Flow-mediated dilatation following wrist and upper arm occlusion in humans: the contribution of nitric oxide

Sagar N. DOSHI*, Katerina K. NAKA†, Nicola PAYNE‡, Christopher J. H. JONES†, Moira ASHTON†, Malcolm J. LEWIS* and Jonathan GOODFELLOW†

*Department of Pharmacology, Cardiovascular Sciences Research Group, Wales Heart Research Institute, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, U.K., †Department of Cardiology, Cardiovascular Sciences Research Group, Wales Heart Research Institute, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, U.K., and ‡Department of Medical Computing and Statistics, University of Wales College of Medicine, Heath Park, Cardiff CF14 4XN, U.K.

ABSTRACT

Flow-mediated dilatation (FMD) of the brachial artery assessed by high-resolution ultrasound is widely used to measure endothelial function. However, the technique is not standardized, with different groups using occlusion of either the wrist or the upper arm to induce increased blood flow. The validity of the test as a marker of endothelial function rests on the assumption that the dilatation observed is endothelium-dependent and mediated by nitric oxide (NO). We sought to compare the NO component of brachial artery dilatation observed following wrist or upper arm occlusion. Dilatation was assessed before and during intra-arterial infusion of the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) following occlusion of (i) the wrist (distal to ultrasound probe) and (ii) the upper arm (proximal to ultrasound probe) for 5 min in ten healthy males. Dilatation was significantly greater after upper arm occlusion (upper arm, $11.62 \pm 3.17\%$; wrist, $7.25 \pm 2.49\%$; $P = 0.003$). During L-NMMA infusion, dilatation after wrist occlusion was abolished (from $7.25 \pm 2.49\%$ to $0.16 \pm 2.24\%$; $P < 0.001$), whereas dilatation after upper arm occlusion was only partially attenuated (from $11.62 \pm 3.17\%$ to $7.51 \pm 2.34\%$; $P = 0.006$). The peak flow stimulus was similar after wrist and upper arm occlusion. We conclude that dilatation following upper arm occlusion is greater than that observed after wrist occlusion, despite a similar peak flow stimulus. L-NMMA infusion revealed that FMD following wrist occlusion is mediated exclusively by NO, while dilatation following upper arm occlusion comprises a substantial component not mediated by NO, most probably related to tissue ischaemia around the brachial artery. FMD following wrist occlusion may be a more valid marker of endothelial function than dilatation following upper arm occlusion.

INTRODUCTION

The endothelium plays a key role in the development and progression of atherosclerosis [1]. Endothelial dysfunction in the coronary arteries of humans is found in the presence of coronary atherosclerosis and cardiovascular risk factors [2,3], and precedes the development of clinically detectable disease [4,5]. In recently published prospective studies, coronary endothelial dysfunction was found to be associated with an increased risk of coronary events independent of classical risk factors [6,7]. Clear prospective evidence of outcome benefit

Key words: endothelial function, flow-mediated dilatation, nitric oxide, ultrasound.

Abbreviations: EDD, end-diastolic diameter; FMD, flow-mediated dilatation; L-NMMA, N^G-monomethyl-L-arginine.

Correspondence: Dr J Goodfellow (e-mail GoodfellowJ@cardiff.ac.uk).

following improvement in endothelial function is not yet available, but considerable circumstantial evidence supports the strategy of improving endothelial function. For example, statins, angiotensin-converting enzyme inhibitors, spironolactone and fish oils improve cardiovascular outcome [8–11], and have also been shown in separate experimental studies to improve endothelial function [12–15].

Coronary endothelial function correlates closely with endothelial function in large peripheral arteries as measured by flow-mediated dilatation (FMD), indicating the systemic nature of the abnormality [16]. Furthermore, there appears to be a graded relationship between endothelial dysfunction and cardiovascular risk [7], and FMD of the brachial artery correlates inversely with the extent of coronary atherosclerosis [17,18].

FMD is a phenomenon whereby increased flow in an artery results in vasodilatation mediated by changes in shear stress, detected by endothelial cells. It is abolished by removal of or damage to the endothelium [19,20]. The phenomenon is mediated in larger arteries by endothelial nitric oxide (NO), and selective blockade of NO production with N^G-monomethyl-L-arginine (L-NMMA) abolishes the response [21].

In 1992, Celermajer and colleagues [22] described a simple yet elegant method to study endothelial function in humans. They postulated that FMD, measured non-invasively using high-resolution ultrasound, could serve as an indication of the functional integrity of the endothelium [22]. FMD assessed by ultrasound measurement of the increase in the diameter of the brachial artery during reactive hyperaemia is now a widely used non-invasive research tool for the assessment of endothelial function in humans. However, the technique has not been standardized and important methodological differences exist, particularly with regard to the position of the occlusive cuff relative to the imaged section of the target vessel. The rationale for using FMD as a surrogate of NO-mediated endothelial function rests on the assumption that it is an endothelium-dependent, predominantly NO-mediated, process. We sought to compare the NO component of brachial artery dilatation following release of wrist and upper arm occlusion in healthy subjects.

METHODS

Subjects

For the main study comparing cuff positions, ten healthy males were recruited. All volunteers were free from medications and from factors associated with endothelial dysfunction, namely hyperlipidaemia (total cholesterol > 6.5 mmol/l), hypertension (blood pressure $> 140/80$ mmHg), diabetes mellitus, family history of premature coronary disease (age < 60 years), hyperhomocysteinaemia (> 15 μmol/l) and smoking. A further five male subjects with the same inclusion/exclusion criteria were recruited for an L-NMMA dose-ranging study. The investigations conformed to the principles outlined in the Declaration of Helsinki, and the Local Research Ethics Committee approved the study protocol. Written informed consent was obtained from each subject. Baseline characteristics of the volunteers are shown in Table 1.

Protocol for cuff position study

All volunteers were studied after an 8 h fast. Venous blood was collected into Vacutainers, and lipids, glucose and creatinine were analysed conventionally on the day of sampling. The sample for assay of total plasma homocysteine was centrifuged immediately [1630 *g* (3000 rev./min) for 10 min] and the plasma was stored at -70 °C until analysis by enzymic immunoassay (Abbot IMx; Abbot Diagnostics). A 27G needle was inserted into the brachial artery of the non-dominant arm under local anaesthesia. Normal saline was infused through the needle at a constant rate of 0.5 ml/min. The brachial artery was imaged 7–10 cm distal to the puncture site. After at least 10 min of stabilization, FMD was measured (see below) following release of a cuff at the wrist (distal to the ultrasound probe) that was inflated to 250 mmHg for 5 min (protocol 1). End-diastolic diameter (EDD) was recorded at 60 s intervals for 5 min, and then at 10 min, to examine the time course of diameter changes after cuff release. After return of the vessel diameter to a stable baseline, FMD was measured after release of a cuff placed on the upper arm (proximal to the ultrasound probe) that was inflated to 250 mmHg pressure for 5 min (protocol 2). EDD was recorded at 60 s intervals for 5 min and then at 10 min after cuff release. Following return of the vessel diameter to baseline, the NO synthase inhibitor L-NMMA (Clinalfa, Länfelfingen, Switzerland) was infused at 3 mg/min (0.5 ml/min) for 15 min in place of normal saline. FMD was then measured again using both protocols during continuous infusion of L-NMMA.

Blood pressure was measured continuously using photo-plethysmography (Finapres). Blood velocity was measured using an 8 MHz continuous-wave Doppler probe mounted in a stereotactic device at an angle of 60° to the vessel. Blood flow was calculated immediately after cuff release as the product of the Doppler time velocity integral, heart rate and brachial artery diameter measured by ultrasonic wall tracking as described below.

Protocol for L-NMMA dose-ranging study

To determine whether complete suppression of stimulated endothelial NO synthase activity was achieved with L-NMMA after release of the upper cuff, a dose-ranging study was performed. In five further male subjects an intra-arterial needle was inserted into the brachial artery

Table 1 Characteristics of study subjects

LDL, low-density lipoprotein. Data are expressed as means (S.D.).

Characteristic	Cuff positions study ($n = 10$)	L-NMMA dose-ranging study ($n = 5$)
Age (years)	34 (5)	33 (4)
Cholesterol (mmol/l)	5.1 (0.7)	5.8 (0.8)
Triacylglycerols (mmol/l)	0.88 (0.34)	1.16 (0.34)
LDL cholesterol (mmol/l)	3.31 (0.7)	3.92 (0.8)
Glucose (mmol/l)	5.0 (0.4)	4.8 (0.1)
Homocysteine (μmol/l)	10.2 (1.7)	9.5 (4.3)
Systolic pressure (mmHg)	133 (12)	126 (6)
Diastolic pressure (mmHg)	78 (6)	73 (3)
Heart rate (beats/min)	59 (9)	58 (11)

as described above. Dilatation was then measured following release of an upper arm cuff inflated for 5 min during infusion of: (i) normal saline (control), (ii) 3 mg/min L-NMMA and (iii) 12 mg/min L-NMMA, all at a constant rate of 0.5 ml/min.

Non-invasive measurement of arterial diameter

FMD was measured using high-resolution ultrasound and wall tracking, as described previously by us [15]. Using a specially adapted duplex colour flow echo machine (Toshiba Ultrasound) with a 7.5 MHz linear phased-array transducer, the brachial artery was imaged above the antecubital crease, 5–10 cm distal to the brachial artery puncture site. When a clear B-mode image of the brachial artery anterior and posterior walls was obtained, the transducer was fixed by means of a stereotactic clamp and the position was held constant for the duration of the study. The radio-frequency signals from the corresponding M-mode image were relayed to the wall tracking system (Vadirec, Oosterbeck, The Netherlands). Following a 10 s acquisition period, the radio-frequency signal is displayed as a waveform, allowing manual placement of cursors on the anterior and posterior brachial artery walls. Vessel wall movements are then tracked automatically using the acquired data. A displacement waveform is generated, enabling accurate measurement of EDD for a series of beats (theoretical spatial resolution 3 μm) [23]. FMD was reported as the greatest absolute increase in EDD from baseline (average of three recordings) during the first 3 min after cuff release, and is expressed as a percentage of the basal vessel diameter. The coefficient of variation for the measurement of FMD in our laboratory is 5.6%.

Statistical analysis

Results are expressed as means ± S.E.M. unless otherwise stated. Dilatation and blood flow data were analysed by one-way ANOVA, followed when significant by a post-hoc test (Tukey). The time courses of vessel diameter changes after cuff release were analysed by multivariate ANOVA. A P value of < 0.05 was considered statistically significant.

RESULTS

Dilatation after wrist and upper arm occlusion under baseline conditions

Dilatation following upper arm occlusion was significantly greater than that observed after wrist occlusion (11.62 ± 3.17 and 7.25 ± 2.49% respectively; $P < 0.05$), despite there being no difference in the peak flow stimulus following cuff release at the two positions (160.9 ± 41 and 155.1 ± 46 ml/min respectively) (Figure 1, Table 2). The difference between the diameter changes with the two protocols persisted throughout the initial 5 min, with both returning to baseline by 10 min (Figures 2 and 3).

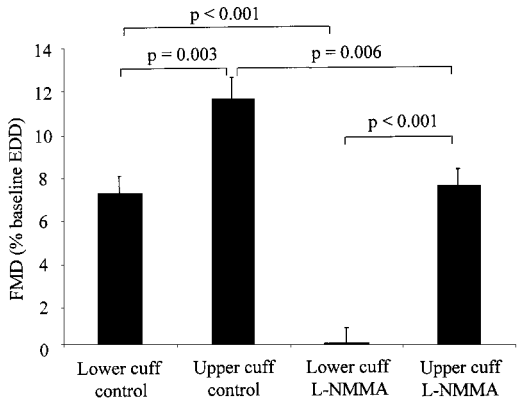

Figure 1 FMD following wrist (lower cuff) and upper arm (upper cuff) occlusion during control and L-NMMA infusion (3 mg/min)

The control infusion was of normal saline.

Table 2 Baseline and peak flow data during control and L-NMMA infusion in the cuff positions study

The control infusion consisted of normal saline (0.9% NaCl).

	Control			L-NMMA			
Cuff	Baseline flow (ml/min)	Peak flow (ml/min)	*P* value	Baseline flow (ml/min)	Peak flow (ml/min)	*P* value	*P* value*
Lower	25.3 (10)	155.1 (46)	< 0.001	15.4 (6)	140.2 (37)	< 0.001	0.85
Upper	23.6 (8)	160.9 (41)	< 0.001	14.1 (5)	157.7 (39)	< 0.001	0.99

* Comparison of peak hyperaemic flow after cuff release during control compared with L-NMMA infusion.

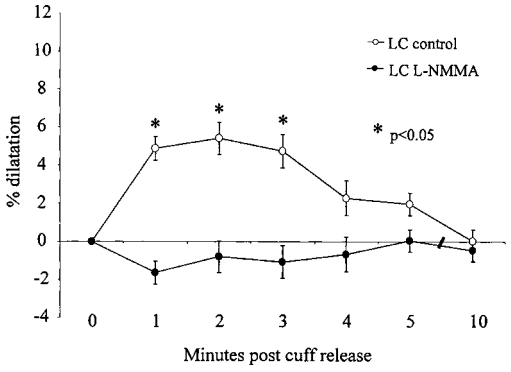

Figure 2 Time course of vessel diameter changes following lower (wrist) cuff release during control and L-NMMA infusion (3 mg/min)

LC, lower cuff. The change in vessel diameter is expressed as a percentage of baseline diameter. The control infusion was of normal saline.

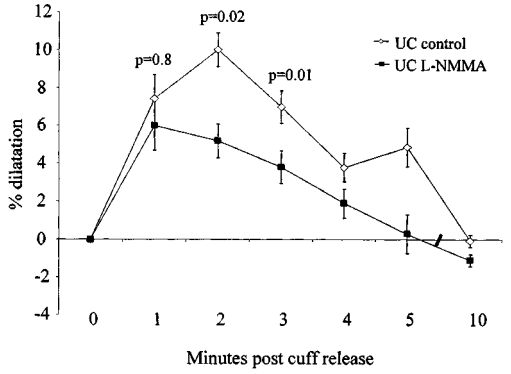

Figure 3 Time course of vessel diameter changes following upper (upper arm) cuff release during control and L-NMMA infusion (3 mg/min)

UC, upper cuff. The change in vessel diameter is expressed as a percentage of baseline diameter. The control infusion was of normal saline.

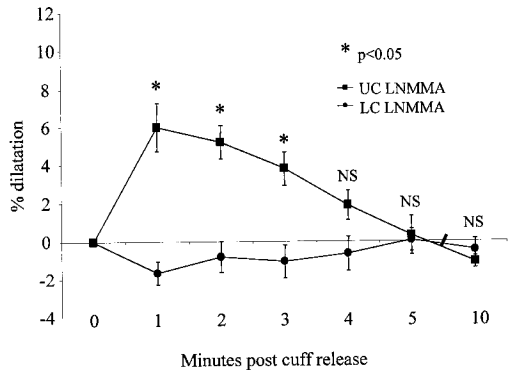

Figure 4 Time course of vessel diameter changes following upper (upper arm) and lower (wrist) cuff release during L-NMMA infusion (3 mg/min)

UC, upper cuff; LC, lower cuff; NS, not significant. The change in vessel diameter is expressed as a percentage of baseline diameter.

Dilatation after wrist and upper arm occlusion during L-NMMA infusion

During L-NMMA infusion (3 mg/min), dilatation after wrist occlusion was abolished, while the response following upper arm occlusion was only partially attenuated. As a result, dilatation differed significantly when comparing the two cuff positions during L-NMMA infusion (Figure 1). L-NMMA changed dilatation to a small overall constriction after release of wrist occlusion (Figure 2), but only partially decreased the dilatation following upper arm occlusion (Figure 3). No significant difference in peak hyperaemic blood flow was observed between the cuff positions during control or L-NMMA infusion, indicating no difference in the flow stimulus to dilatation (Table 2). Comparison of the time courses for changes in vessel diameter during L-NMMA infusion after cuff release showed significantly greater dilatation following upper arm compared with wrist occlusion for the first 3 min (Figure 4). Heart rate and blood pressure were unchanged during both study protocols.

L-NMMA dose-ranging study

In the five additional volunteers, dilatation following upper arm cuff release was $11.88 \pm 0.91\%$ in the control situation. L-NMMA infusion at 3 mg/min reduced dilatation to $8.31 \pm 1.96\%$ ($P = 0.014$), with no further

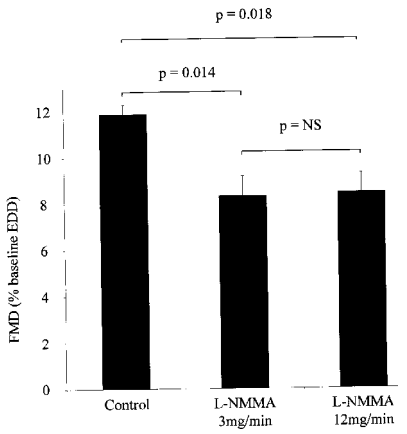

Figure 5 Dilatation following upper cuff release during infusion with normal saline (control), or L-NMMA at 3 or 12 mg/min

NS, not significant.

change during infusion with the higher dose of L-NMMA (12 mg/min; 8.46 ± 1.93 % dilatation) (Figure 5). No differences in blood pressure or heart rate were observed during the study. Basal brachial artery diameter did not differ significantly when comparing control with L-NMMA (3 mg/min) or L-NMMA (12 mg/min) infusion (3.85, 3.81 and 3.80 mm respectively).

DISCUSSION

This study has revealed two major findings. First, FMD following release of upper arm occlusion is significantly greater than that seen after release of wrist occlusion in healthy males. This finding is consistent with previous observations comparing upper arm with forearm occlusion of the same duration [24,25]. Secondly, our data demonstrate that dilatation after wrist occlusion is exclusively NO-mediated, while dilatation following upper arm occlusion is only partially mediated by NO, a finding not reported previously.

Infusion of the NO synthase inhibitor, L-NMMA, significantly reduced dilatation with both cuff positions (Figure 1). However, the response following wrist occlusion was abolished, while that following upper arm occlusion was only partially reduced, with significant differences between the two cuff positions during the 3 min following cuff release (Figure 4). A possible explanation is that the dose of L-NMMA used (3 mg/min) was inadequate to block stimulated NO production following upper arm cuff release. However, the dose-ranging study found no further suppression of dilatation using a 4-fold greater dose of L-NMMA, indicating that the dose chosen was adequate and likely to be at the top of the dose–response curve.

The greater dilatation observed with upper arm occlusion has been attributed, in part, to a difference in the flow stimulus observed between cuff positions [25]. However, we found no significant increase in peak flow after upper arm occlusion, and suggest that a different flow stimulus does not explain the observed difference in diameter change. However, other factors are more likely to explain the observed difference. Upper arm occlusion is more painful than wrist occlusion [24], presumably because it causes greater tissue ischaemia. The resulting ischaemia in the region of the imaged section of the brachial artery is associated with the release of vasodilatory metabolites (e.g. potassium, adenosine, ATP) and changes in pH, which could explain the non-NO-mediated dilatation observed after upper arm cuff release.

A further possibility is that loss of myogenic tone may account for greater dilatation after upper arm cuff release. Brachial artery pressure falls to zero during upper arm occlusion, but will remain normal during wrist occlusion. Myogenic influences on diameter are well recognized in larger conduit vessels [26] such as the brachial artery. Although hyperaemic flow will rapidly wash out ischaemic vasodilatory metabolites, restoration of myogenic tone is unlikely to be completed within the first 5 min after cuff release. The profound difference in intra-arterial pressures during cuff inflation between the two protocols will contribute to and possibly account entirely for the difference in diameter change after cuff release.

The finding that the wrist cuff protocol results in a 'purer' assessment of NO activity may not in itself justify the exclusive use of this technique. Endothelial dysfunction assessed by wrist cuff occlusion results in small changes in diameter (dilatation usually $< 3\,\%$) [15] that are difficult to measure. A Type-2 error might be the simple explanation for the inability to identify endothelial dysfunction in a group of subjects with cardiovascular risk factors using FMD with forearm occlusion in one recent study [25]. However, the vast majority of published studies have shown that endothelial dysfunction can be identified by careful measurement of FMD after forearm/wrist occlusion, allowing small improvements following interventions to be detected [15,22,27–31].

The present study had certain limitations. The study design resulted in measurement of lower cuff and then upper cuff responses, raising the possibility of an order effect. However, the results from the L-NMMA dose-ranging study, where only the upper cuff protocol was followed (12 mg dose), argue against this possibility, as no significant differences in the responses following upper cuff release at baseline were observed between experiments. Another limitation was the fact that we measured peak brachial artery flow, but not duration of peak flow or duration of the hyperaemic response. In contrast, others have demonstrated a higher peak flow and longer duration of hyperaemia with upper cuff

occlusion, and have suggested this as a possible explanation for the greater dilatation seen [32]. The degree of dilatation produced following cuff occlusion depends on peak hyperaemic flow and duration of hyperaemia [33]. L-NMMA does not affect the peak flow observed following post-ischaemic dilatation, as this is driven by ischaemic metabolites. L-NMMA will affect the late phase of the hyperaemic response, which is in part NO mediated. Therefore it is unlikely that L-NMMA would have differential effects on the duration of peak flow and thus influence the FMD observed after upper and lower cuff occlusion.

In conclusion, the present study has shown that FMD after release of wrist occlusion represents the activity of NO. In contrast, dilatation after upper arm occlusion is greater, with a significant component not mediated by NO and perhaps not by flow. These differences are not attributable to differences in the peak flow stimulus, but are more likely to be the result of ischaemia in the surrounding tissue or pressure changes in the brachial artery itself, induced by the occluding cuff. This may suggest that studies with L-NMMA should be performed before and after interventions that affect endothelial function, assessed with an upper arm cuff, as improved dilatation may not necessarily be due to improved NO activity.

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