Dose-related effect of intravenous L-arginine on muscular blood flow of the calf in patients with peripheral vascular disease: a $H_2^{15}O$ positron emission tomography study

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1. Endothelium-derived nitric oxide (NO) contributes to the regulation of vascular tone and blood pressure. Infusion of L-arginine produces systemic vasodilatation via stimulation of endogenous NO formation. Vasodilatation is accompanied by an increase in peripheral arterial blood flow. However, it is not known whether capillary nutritive blood flow increases as well. The time course and dose-response pattern of this effect remain to be elucidated.

2. Two groups of ten patients with peripheral vascular disease (PVD) received an intravenous infusion of 8 g or 30 g of L-arginine over a period of 40 min. Blood pressure and heart rate were monitored non-invasively. Muscular blood flow (MBF) of the calf was determined at 0, 20, 40, 60, 80 min by positron emission tomography with $H_2^{15}O$ as flow tracer. Plasma L-arginine and cyclic GMP (cGMP) levels were determined at the same time points.

3. L-arginine induced a dose-related decrease in blood pressure during the infusion period. MBF and plasma cGMP levels during and after the infusion of 8 g of L-arginine did not change significantly. In the patients receiving 30 g of L-arginine, MBF was enhanced significantly from $1.56 \pm 0.14$ to $2.09 \pm 0.21$ ml min$^{-1}$ 100 ml$^{-1}$ at 40 min and $2.23 \pm 0.15$ ml min$^{-1}$ 100 ml$^{-1}$ after 80 min ($+43.0\%$). The increase in MBF was paralleled by an increase in plasma cGMP from $4789.8 \pm 392.2$ nmol/l at baseline to $9223.2 \pm 1233.6$ nmol/l at 40 min.

4. We conclude that intravenous L-arginine enhances nutritive capillary MBF in patients with PVD via the NO–cGMP pathway in a dose-related manner. This effect might be therapeutically beneficial in patients with PVD.

INTRODUCTION

Endothelium-derived nitric oxide (NO), which is synthesized from the terminal guanidino nitrogen of the amino acid precursor L-arginine [1], has been shown to account for the biological activity of endothelium-derived relaxing factor [2]. These actions, mainly relaxation of vascular smooth muscle cells and inhibition of platelet aggregation and adhesion, are mediated by the intracellular second messenger cyclic GMP (cGMP) [3]. Basal secretion of NO in healthy blood vessels has been shown to contribute to the regulation of blood pressure by inducing an active vasodilatory tone [4]. In hypercholesterolaemic and atherosclerotic blood vessels, endothelium-dependent vascular relaxation is well known to be impaired [5–8]. Exogenous administration of L-arginine, the precursor of endogenous NO, has been shown to enhance endothelial function and to reduce intimal plaque area in animal models of hypercholesterolaemia [8–10].

In healthy human subjects, intravenous L-arginine induces peripheral vasodilatation, inhibits platelet aggregation and concomitantly increases urinary NO$^\cdot$ and cGMP excretion rates [11]. L-Arginine has also been shown to enhance acetylcholine-induced, endothelium-dependent vasodilatation in hypercholesterolaemic or atherosclerotic patients [12, 13]. Moreover, we have recently demonstrated in a double-blind, controlled study that a single intravenous infusion of L-arginine induces peripheral vasodilatation in patients with critical limb ischaemia [14]. These results have to be further clarified in two respects. Firstly, the question arises whether the observed increase of blood flow is due to an increase of nutritive tissue perfusion or merely to an opening of arterio-venous (AV) shunts. Second, further information about the time course

Key words: L-arginine, cyclic GMP, nitric oxide, positron emission tomography, skeletal muscle.

Abbreviations: ANOVA, analysis of variance; AV, arterio-venous; cGMP, cyclic guanosine monophosphate; MBF, muscular blood flow; NO, nitric oxide; OPA, o-phthalaldehyde; PET, positron emission tomography; PVD, peripheral vascular disease; VOP, venous occlusion plethysmography.

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of the drug effect and about the dose/effect relationship are needed.

Non-invasive assessment of peripheral blood flow has mainly relied on two methods: venous occlusion plethysmography (VOP) and duplex ultrasound. Both methods are appropriate to estimate total blood flow to an extremity or to a segment of an extremity. However, neither method provides any information on nutritive capillary blood flow in a defined tissue compartment of an extremity. Moreover, lack of accuracy in each single recording requires repeated testing to generate one averaged value [15, 16].

Positron emission tomography (PET) is a well-established method to provide absolute values of nutritive capillary tissue flow in brain [17], heart [18] and tumors [19]. This is accomplished most often with the flow tracers NH3 or H2O. Recently, we have shown that H215O-PET is applicable to the assessment of skeletal muscle blood flow as well, particularly in peripheral vascular disease (PVD) [20]. The peculiarities of skeletal muscle perfusion, i.e. marked low flow at rest and great flow reserve, require precautions in designing protocols for data acquisition, data processing and image analysis. Provided the design requirements are met, accurate results in terms of absolute flow values can be obtained. Thus, in the present study we investigated whether L-arginine, given as a single intravenous infusion in two different dosages, increases nutritive muscular blood flow of the calf in patients with peripheral vascular disease.

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METHODS

Patients and study design

Twenty patients with PVD (Fontaine stages IIb–IV) confirmed by angiography were studied. The clinical characteristics of the patients are shown in Table 1. All patients gave written informed consent before participation in the study, which had been approved by the Hannover Medical School Ethics Committee.

Patients were placed in the supine position with the maximum calf diameter in the middle of the field of view of the PET camera. Attention was paid to ensure that the legs rested without compression by the patient's bed. For arterial blood sampling the radial artery was cannulated. An intravenous line for application of the infusions was placed into the cubital vein of the contralateral side. Blood pressure and heart rate were measured non-invasively throughout the study by an automatic device at intervals of 5 min. After 30 min of rest, a baseline PET scan was performed. Then the patients received a single intravenous infusion of L-arginine (Braun; L-arginine/HCl dissolved in 150 ml of 0.9% saline, pH 6.5) in a dose of 8 g or 30 g over a period of 40 min. Follow-up PET scans were performed at 20, 40, 60 and 80 min after the start of the infusion. At the time of each PET scan, blood samples were drawn for the determination of plasma L-arginine and cGMP concentrations.

PET

Data acquisition, data processing and image analysis are described elsewhere in detail [20]. Briefly, flow studies of the calf were performed with a Siemens/CTI ECAT 951/31 PET scanner. Thirty-one slices with a plane separation of 3.4 mm were obtained simultaneously. After intravenous bolus injection of 1.85 GBq of H215O a dynamic scan was acquired within 5 min (frame rate 12 × 5 s, 4 × 15 s, 2 × 30 s, 2 × 60 s). All image data were corrected for attenuation (10 min transmission) and radioactive decay. Arterial blood samples were taken at mid-frame times.

Mathematical modelling of the measured parameters (arterial input activity concentration and tissue activity concentration) is based on the assumption that water is freely diffusible. Thus, tracer kinetics can be described by the Kety–Schmidt equation [21]. We applied a linearized modification of this equation as described by van den Hoff et al. [22] to generate parametric flow maps on a pixel by pixel basis. This equation allows correction for delay and dispersion of the arterial input function as well as for fractional blood volume. The final value denotes capillary nutritive tissue blood flow in ml/min per 100 ml of tissue.

Quantitative image analysis was performed on the average of nine central slices. Definition of regions of interest was based on anatomical structures of the leg. MBF was calculated from a region of interest which included all muscles in the cross-section of the calf. Tibial and fibular bones were excluded as were the three main arteries (Fig. 1). Separate flow values were obtained for both legs at each scan.
L-Arginine enhances nutritive muscle blood flow

**Fig. 1.** Parametric flow image of the lower legs obtained by \( {^2H_2O-\text{PET}} \). The bones had been identified on the transmission scan. The arteries had been identified on images showing the early, i.e. the arterial phase, of tracer distribution. MUS indicates the region of interest from which muscle blood flow values were computed. TB = tibial bone; FB = fibular bone; ATA = anterior tibial artery; PTA = posterior tibial artery; PA = peroneal artery.

### Plasma L-arginine and cGMP concentrations

Plasma L-arginine concentrations were determined by HPLC using pre-column derivatization with o-phthalaldehyde (OPA) as described previously [22a]. Briefly, plasma samples were extracted on CBA solid phase extraction cartridges (Varian), dried over nitrogen and dissolved in doubly distilled water for HPLC analysis. HPLC was carried out on a Gynkotek liquid chromatography system consisting of two HPLC pumps with a gradient controller (model M 480 HDG), a spectral fluorescence detector (RF 1002), and an automatic injector (model GINA 160). Samples and standards were incubated for exactly 30 s with the OPA reagent (5.4 mg/ml OPA in borate buffer, pH 8.5, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC system. Chromatographic separation was performed on a C6H5 column (Macherey and Nagel) with the fluorescence monitor set at \( \lambda \) excitation = 340 nm and \( \lambda \) emission = 455 nm. Samples were eluted from the column with 0.96% citric acid/methanol 2:1, pH 6.8, at a flow rate of 1 ml/min. The coefficient of variation of the method had previously been determined as 5.2% within-assay and 5.5% between-assay; the detection limit of the assay was 0.1 \( \mu \)mol/l.

For the determination of cGMP, plasma samples were thawed and centrifuged at 2500 \( \times \)g (4°C; 10 min). Supernatants were diluted 1:4 in PBC and acetylated by a mixture of acetic acid anhydride/triethylamine (1:2, v/v). cGMP content was measured by RIA using \(^{125}\)I-labelled cGMP as a tracer and globulin precipitation. The detection limit of the assay was 160 nmol/l.

### Calculations and statistical analysis

All values are given as means±SEM. Statistical comparison of paired observations was done by the Wilcoxon signed rank test. Unpaired observations were compared by the Mann–Whitney U-test. For comparison of plasma L-arginine levels, plasma cGMP concentrations and muscular blood flow values between the two treatment groups factorial analysis of variance (ANOVA) was performed. In all tests statistical significance was assumed for \( P < 0.05 \).

### RESULTS

#### Clinical observations

Intravenous infusion of L-arginine was tolerated well in both dosages. Only one patient in the 30 g group complained of discomfort of the mouth, the tongue and the hands in the first minutes of infusion. However, no physical signs could be observed. The infusion was continued and the sensations subsided. In both treatment groups two patients asked to interrupt the study after the third PET scan due to lower back pain or restlessness of the legs.

#### Plasma L-arginine and cGMP concentrations

The mean baseline plasma L-arginine concentration was 101.3 ± 14.1 \( \mu \)mol/l. Infusion of 30 g of L-arginine increased plasma L-arginine concentration to 8963.5 ± 1400.4 \( \mu \)mol/l at the end of the 40 min infusion period. At the end of the infusion of 8 g of L-arginine, L-arginine plasma concentration was increased to 1367.7 ± 136.3 \( \mu \)mol/l. The plasma concentrations differed significantly between the two groups at 20, 40, 60 and 80 min. After the end of the infusions, plasma L-arginine concentrations began to decrease; however, baseline plasma levels were not reached within the 40 min post-infusion period (Fig. 2).
Plasma cGMP concentration was 4789.8 ± 392.2 nmol/l at baseline. During the infusion of 30 g of L-arginine it increased to 9223.2 ± 1233.6 nmol/l at 40 min (+83.8%; P < 0.05 compared with baseline). After the end of the infusion, plasma cGMP decreased but did not reach baseline levels within the 40 min post-infusion period. During and after the infusion of 8 g of L-arginine, plasma cGMP only slightly increased by 12–18% (Fig. 3).

MBF

MBF is given as the mean value of the right and the left leg. No significant differences were observed between the legs at any time in any treatment group. Baseline values averaged 1.53 ± 0.16 ml/min per 100 ml in the 8 g group and 1.56 ± 0.14 ml/min per 100 ml of tissue in the 30 g group. In the 8 g group, MBF slightly increased during infusion of L-arginine, with a maximum of 1.67 ± 0.22 ml/min per 100 ml of tissue at 80 min (+9.0%). However, this increase was not statistically significant. In the 30 g group, the maximum value at 80 min was 2.23 ± 0.15 ml/min per 100 ml of tissue, denoting a relative flow increase of 43.0%. In this group, absolute flow values at 20, 40, 60 and 80 min differed statistically significantly from the baseline value, as did the corresponding 80 min values between the two treatment groups (Fig. 4).

Blood pressure and heart rate

Pre-infusion measurements of systolic blood pressure averaged 139.0 ± 6.6 mmHg in the 8 g group and 140.1 ± 4.2 mmHg in the 30 g group. Diastolic blood pressure values averaged 73.3 ± 2.4 and 71.1 ± 2.8 mmHg respectively. During the infusion period, systolic as well as diastolic blood pressure decreased in the 30 g group but not in the 8 g group (Fig. 5). In the 30 g group, the mean of all systolic measurements during infusion was 134.4 mmHg (−5.7 mmHg; P < 0.05) and the diastolic value was 65.5 mmHg (−5.6 mmHg; P < 0.05). In the 8 g group no significant changes could be detected. After the end of infusion, blood pressure increased rapidly in the 30 g group causing a significant overshoot. Neither of the doses of L-arginine had a significant effect on heart rate.
DISCUSSION

Our present study suggests that: (1) intravenous L-arginine increases nutritive MBF of the calf in patients with PVD via stimulation of the NO–cGMP pathway; (2) this effect of L-arginine on tissue perfusion is related to the dose; and (3) the increase in nutritive capillary flow persists when the blood pressure response to L-arginine has returned to baseline values.

Several studies have investigated the direct effects of L-arginine on peripheral blood flow in humans. Using VOP, Imaizumi et al. [23] demonstrated a dose-dependent increase in forearm blood flow in healthy humans by intra-arterial L-arginine in a dose range of 50–290 μmol/min. In this study D-arginine, in the same dose range, had no effect. Calver et al. [24] demonstrated by VOP that in a dose range from 10 to 40 μmol/min, neither L-arginine nor D-arginine had any vasodilatory effect in healthy humans. At a substantially higher dose (160 μmol/min), both L- and D-arginine induced vasodilatation in the present study. Panza et al. [25] also found no direct effect of intra-arterial L-arginine at an infusion rate of 40 μmol/min on forearm blood flow in hypercholesterolaemic patients, using the same method to assess forearm blood flow. However, they found an enhanced vasodilatory response to intra-arterial acetylcholine after L-arginine infusion.

Using intravenous infusions of L-arginine, Creager et al. [12] found no effect on forearm blood flow determined by VOP in patients with hypercholesterolaemia, nor in healthy human subjects, at an infusion rate of 3.3 mmol/min. However, we recently demonstrated by Duplex sonography that a single intravenous dose of L-arginine (4.75 mmol/min) increased femoral artery blood flow by 42.8% in patients with manifest atherosclerosis [14]. The conflicting results from these studies may be explained by the dose/effect relationships of L-arginine, with low doses having no direct vasodilatory effect on peripheral blood flow but enhancing acetylcholine-induced NO-dependent vasodilatation, medium doses inducing direct, NO-dependent vasodilatation, and with ultra-high doses of either L- or D-arginine inducing vasodilatation via unspecific mechanisms.

Up to now, no data has been available to demonstrate whether the blood flow response to L-arginine is due to an increased nutritive capillary blood flow or to an opening of AV shunts. Both reactions would result in increased blood flow velocity in the femoral artery, so that duplex ultrasound is inadequate to discriminate between these reactions. The same holds true for measurements by means of VOP. Furthermore, neither ultrasound nor VOP allow discrimination between cutaneous and muscular blood flow. In our present study, we applied H215O-PET to further investigate this issue. The reproducibility and accuracy of this method in measuring tissue blood flow have been proven for the heart by using microspheres in a dog model [26]. The blood flow values in that study covered a range between 20 and 400 ml/min per 100 ml. As shown previously, this method is also applicable to skeletal muscle blood flow measurements within a flow range between 1 and 60 ml/min per 100 ml [20]. In thigh muscles, H215O-PET has been used for the characterization of blood flow changes caused by hyperinsulinaemia [27]. In our present study, resting blood flow of patients with PVD did not differ from that previously measured in healthy subjects [28]. Intravenous infusion of L-arginine was followed by a marked increase in muscular blood flow after the 30 g dose (i.e. 4.75 mmol/min) whereas the 8 g dose (i.e. 1.27 mmol/min) induced virtually no reaction. The 43% flow increase in the 30 g group corresponds well to the flow increases determined by duplex ultrasound in healthy subjects (+44%) and in PVD patients (+43%) in our previous studies [11, 14]. This suggests that L-arginine obviously does not open AV shunts.

We have previously shown that nitrate, the oxidative metabolite of NO, and cGMP are useful markers to assess NO formation in vivo [11, 29, 30]. However, as plasma nitrate concentrations show large intra- and inter-individual variability, and urine sampling was not possible in the setting of PET scanning, we used plasma cGMP levels as a biochemical indicator of NO–cGMP pathway activation in the present study. There is broad evidence from studies in vitro that extracellular, i.e. plasma, cGMP levels represent the extent of intracellular NO induced signalling [31–33]. The haemodynamic changes induced by two different doses of intravenous L-arginine correlated well with the difference in plasma cGMP concentrations in both groups. These observations, together with the about 6-fold higher increase in plasma L-arginine concentration in the 30 g group, strongly suggest that the increase in nutritive muscular tissue blood flow was due to activation of the NO–cGMP pathway.

In addition to its impact on local tissue blood flow, intravenous L-arginine had an effect on systemic arterial blood pressure. Decreased systolic and diastolic blood pressure in response to intravenous L-arginine was first reported by Nakaki et al. [34]. Subsequently, this finding was confirmed, and evidence grew that it is NO-dependent [11, 35], although some authors could not reproduce this finding [36]. Our results also indicate that the effect of intravenous L-arginine on systemic arterial blood pressure is dose related.

In the present study, the time course of blood pressure was markedly different from that of tissue blood flow. Paralleled by the plasma cGMP levels, the increase in nutritive muscle blood flow outlasted the infusion period of L-arginine by at least 40 min (Figs. 3 and 4). A similar difference between the responses of (systemic) arterial pressure and (local) tissue blood flow has been noted in our previous study in patients with critical limb ischaemia [14]. In that study, femoral artery blood flow, mea-
sured by duplex ultrasound, continued to increase after the 60 min infusion period of 30 g of intravenous L-arginine and still remained elevated even 90 min after the infusion had been stopped. This observation would imply that L-arginine/NO acts in a different way on arteriolar resistance vessels (> 25 μm in diameter), which contribute most to the regulation of peripheral vascular resistance, and on pre-capillary sphincter vessels (< 25 μm in diameter), which control local tissue blood flow [37, 38]. The present PET study does not provide more detailed information to support this idea. However, our findings fit well with experimental data reported by Ekelund and Mellander [39] who demonstrated that the response of vascular resistance to intra-arterial administration of NO-monomethyl-L-arginine followed by L-arginine was different between sections of ‘large bore’ resistance vessels and ‘small-arteriolar’ vessels in cat skeletal muscle.

In addition to the biological implications of our results, the study of nutritive capillary flow is significant in a clinical perspective. Patients with PVD might benefit from substances causing enhanced tissue blood flow in the leg muscles [40]. This is the mechanism by which substances like the prostaglandins are believed to act [41]. With its two main biological actions in man, increase of peripheral blood flow and inhibition of platelet aggregation [11], L-arginine has a pharmacological profile comparable with those compounds which are currently being used in the therapy of PVD. The fact that L-arginine not only increases peripheral blood flow in general but enhances nutritive capillary tissue blood flow provides a reason to set up clinical trials to study the possible therapeutic effects of L-arginine in PVD.

In conclusion, results from our present study indicate that the increase in arterial blood flow of the leg caused by intravenous L-arginine is due to increased nutritive capillary blood flow in skeletal muscle. This effect on local blood flow is most probably mediated by the NO–cGMP pathway. The increase in nutritive muscle blood flow by a well-tolerated intravenous dose of L-arginine might be beneficial in patients with PVD.

REFERENCES


