Oral L-arginine improves endothelium-dependent dilation in hypercholesterolemic young adults.

P Clarkson, … , D S Celermajer, J E Deanfield

J Clin Invest. 1996;97(8):1989-1994. https://doi.org/10.1172/JCI118632.

Research Article

In hypercholesterolemic rabbits, oral L-arginine (the substrate for endothelium derived nitric oxide) attenuates endothelial dysfunction and atheroma formation, but the effect in hypercholesterolemic humans is unknown. Using high resolution external ultrasound, we studied arterial physiology in 27 hypercholesterolemic subjects aged 29+/-5 (19-40) years, with known endothelial dysfunction and LDL-cholesterol levels of 238+/-43 mg/dl. Each subject was studied before and after 4 wk of L-arginine (7 grams x 3/day) or placebo powder, with 4 wk washout, in a randomized double-blind crossover study. Brachial artery diameter was measured at rest, during increased flow (causing endothelium-dependent dilation, EDD) and after sublingual glyceryl trinitrate (causing endothelium-independent dilation). After oral L-arginine, plasma L-arginine levels rose from 115+/-103 to 231+/-125 micromol/liter (P<0.001), and EDD improved from 1.7+/-1.3 to 5.6+/-3.0% (P<0.001). In contrast there was no significant change in response to glyceryl trinitrate. After placebo there were no changes in endothelium-dependent or independent vascular responses. Lipid levels were unchanged after L-arginine and placebo. Dietary supplementation with L-arginine significantly improves EDD in hypercholesterolemic young adults, and this may impact favorably on the atherogenic process.

Oral L-Arginine Improves Endothelium-dependent Dilation in Hypercholesterolemic Young Adults

Peter Clarkson,* Mark R. Adams,‡ Amanda J. Powe,* Ann E. Donald,* Robin McCredie,‡ Jacqui Robinson,‡ Susan N. McCarthy,* Anthony Keech,‡ David S. Celermajer,‡ and John E. Deanfield*

**Cardiothoracic Unit, Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London WC1N 3JH, UK; and ‡Department of Cardiology and the Heart Research Institute, Royal Prince Alfred Hospital, Missenden Road, Camperdown, NSW 2050, Australia*

Abstract

In hypercholesterolemic rabbits, oral L-arginine (the substrate for endothelium derived nitric oxide) attenuates endothelial dysfunction and atheroma formation, but the effect in hypercholesterolemic humans is unknown. Using high resolution external ultrasound, we studied arterial physiology in 27 hypercholesterolemic subjects aged 29±5 (19–40) years, with known endothelial dysfunction and LDL-cholesterol levels of 238±43 mg/dl. Each subject was studied before and after 4 wk of L-arginine (7 grams × 3/day) or placebo powder, with 4 wk washout, in a randomized double-blind crossover study. Brachial artery diameter was measured at rest, during increased flow (causing endothelium-dependent dilation, EDD) and after sublingual glyceryl trinitrate (causing endothelium-independent dilation). After oral L-arginine, plasma L-arginine levels rose from 115±103 to 231±125 μmol/liter ($P < 0.001$), and EDD improved from 1.7±1.3 to 5.6±3.0% ($P < 0.001$). In contrast there was no significant change in response to glyceryl trinitrate. After placebo there were no changes in endothelium-dependent or independent vascular responses. Lipid levels were unchanged after L-arginine and placebo. Dietary supplementation with L-arginine significantly improves EDD in hypercholesterolemic young adults, and this may impact favorably on the atherogenic process. (*J. Clin. Invest.* 1996. 97:1989-1994.) Key words: nitric oxide • atherosclerosis • vascular ultrasound • cholesterol • nitrosoprotein

Introduction

Hypercholesterolemia is a major risk factor for atherosclerosis in animal models and in man (1) and may be associated with damage to the vascular endothelium which is involved in the initiation of the atherosclerotic process (2). Healthy vascular endothelium is known to produce nitric oxide (NO, or a related compound) (3) which is a potent vasodilator and also inhibits platelet aggregation and adhesion, leucocyte/vessel wall interactions and smooth muscle proliferation (4). Elevated serum cholesterol is associated with reduced endothelium-dependent vasodilation (5, 6) and decreased endothelial NO activity (7), well before any structural changes in the arterial wall are detectable.

L-arginine is the substrate for nitric oxide synthase (NOS), the enzyme which catalyses the production of NO in vascular endothelial cells (8). Experimental work in cholesterol fed rabbits has shown that dietary supplementation with L-arginine causes attenuation of endothelial dysfunction, with increased NO activity resulting in reduced platelet activation (9) and monocyte adhesion (10). In addition, it is associated with a marked reduction in aortic and coronary atherosclerosis (11, 12).

In hypercholesterolemic humans, acute intravenous administration of L-arginine may improve endothelial responses in resistance vessels in the peripheral and coronary circulation (13, 14), but the effects of chronic oral administration of L-arginine on large vessel physiology in humans are not known. This study has shown that dietary supplementation of L-arginine for a 4-wk-period improves endothelium-dependent dilation (EDD)[1] of the conduit arteries in young asymptomatic subjects with hypercholesterolemia.

Methods

Subjects. The study subjects, recruited from specialist lipid clinics in London and Sydney, had elevated LDL-cholesterol levels (> 162 mg/dl), were aged 18 to 40 yr, lifelong nonsmokers, and were not hypertensive or diabetic. None had regular exposure to environmental tobacco smoke, and all were of average fitness levels. Subjects with clinical evidence of atherosclerosis or who were on vasoactive medication were excluded. Those taking HMG CoA reductase inhibitors in a stable dose for > 6 mo who still met the entry criteria for cholesterol were recruited. Females using reliable contraception and not intending to become pregnant for the duration of the study were eligible. The study was approved by the local research ethics committees and each subject gave written informed consent. Consecutive subjects meeting the study entry criteria underwent a baseline study of vascular reactivity (see below) and those with impaired flow mediated dilation (defined as a flow mediated dilation below 1 standard deviation

Address correspondence to John E. Deanfield, Cardiothoracic Unit, Great Ormond Street Hospital for Children NHS Trust, Great Ormond Street, London WC1N 3JH. Phone: 171-813-8223; FAX: 171-831-4850.

Received for publication 22 September 1995 and accepted in revised form 9 February 1996.

J. Clin. Invest.

0021-9738/96/04/1989/06 $2.00
Volume 97, Number 8, April 1996, 1989–1994

1. *Abbreviations used in this paper:* EDD, endothelium-dependent dilation; NO, nitric oxide; L-NMMA, *N*-monomethyl L-arginine; GTN, glyceryl trinitrate.

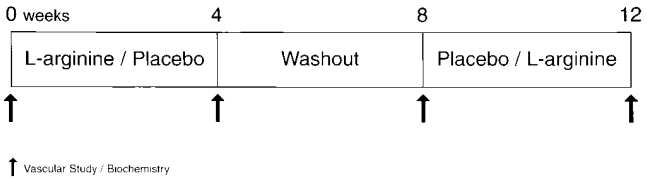

Figure 1. Study protocol. Arrows represent time points where vascular studies were performed and blood was sampled for biochemistry, L-arginine levels and lipid profile.

from the mean responses seen in 210 subjects without vascular risk factors, matched for vessel size) were enrolled in the trial. Thirty subjects were studied using a randomized, placebo controlled, double blind crossover design with two 4-wk treatment periods, separated by a 4-wk washout period (Fig. 1). The study size was based on power calculations derived from previous studies of variability in vascular reactivity measurements (15). L-arginine was given at a dose of seven grams three times a day for the 4-wk treatment period. Both the active drug preparation and placebo were prepared as identical powder formulations, which when mixed with water made a palatable drink (L-arginine and placebo were made up in pineapple flavoring and supplied by Scientific Hospital Supplies, Liverpool, UK). The amount of L-arginine supplementation was chosen after preliminary studies demonstrated that this dose was well tolerated and resulted in an approximately twofold increase in plasma arginine levels following each dose (similar to the dose given in the hypercholesterolemic rabbit model (11), calculated on a weight for weight basis).

The noninvasive assessment of endothelium-dependent dilation was performed before and at the end of each treatment period. Post-treatment studies were performed between 1 and 2 h after the last dose of L-arginine or placebo. Subjects fasted overnight before each assessment and supine blood pressure was measured at the time of each study.

Vascular reactivity study. Arterial endothelial and smooth muscle function were studied non-invasively by examining brachial artery responses to endothelium-dependent and independent stimuli as we have previously described (16). Arterial diameter was measured from B-mode ultrasound images at rest, in response to reactive hyperemia (with increased flow producing endothelium-dependent vasodilation), again at rest and after sublingual glyceryl trinitrate (GTN, an endothelium independent vasodilator), using a standard 7 mHz linear array transducer and Acuson 128XP/10 system (Acuson, Mountain View, California).

The subject lay at rest for at least 10 min before the first scan and remained supine throughout the study. The brachial artery was scanned in longitudinal section 2–10 cm above the elbow and the center of the artery identified when the clearest picture of the anterior and posterior wall layers was obtained. The transmit (focus) zone was set to a depth of the near wall, in view of the greater difficulty in evaluating the near compared with the far wall "m" line (the interface between the media and adventitia). Depth and gain settings were set to optimize images of the lumen/arterial wall interface, images were magnified using a resolution box function (leading to a line width of $\sim$ 0.065 mm) and machine operating parameters were not changed during the study.

When a satisfactory transducer position was found, the skin was marked and the arm remained in the same position throughout the study. A resting scan was then recorded. Increased flow was then induced by inflation of a pneumatic tourniquet placed around the forearm (distal to the segment of artery being scanned) to a pressure of 250–300 mmHg for 4.5 min, followed by rapid release. A second scan was taken 30 s before release of the cuff and continued for a further 90 s after cuff deflation. The brachial artery dilation to flow using this technique can be blocked by infusion of *N*-monomethyl L-arginine (L-NMMA) (17), a specific antagonist for NO synthase, and responses correlate with invasive tests of coronary endothelial function (18). Thereafter, 10–15 min was allowed for vessel recovery after which a further resting scan was taken. Sublingual GTN spray (400 μg) was then administered and 3–4 min later the last scan was performed. Doppler derived flow measurements (using a pulsed wave Doppler signal at a 70° angle to the vessel with the range gate (1.5 mm) in the center of the artery) were obtained during the first resting scan (baseline blood flow) and again during the first 15 s of reactive hyperemia (allowing the flow increase to be expressed as a percentage of the baseline flow). The electrocardiogram was monitored continuously throughout the study.

Data analysis. All scans were recorded on super VHS videotape for later analysis. Vessel diameters were measured by two blinded observers unaware of the clinical details and the stage of the experiment, at the center where the data was collected. Tapes were then transferred to the sister center where a further analysis was performed by a third independent observer. The mean of the three observations was then taken. We have shown previously that this method is accurate and reproducible for measurement of small changes in arterial diameter, with low inter-observer error for measurement of flow mediated dilation (15). The arterial diameter was measured at a fixed distance from an anatomical marker (such as a fascial plane or vein seen in cross section) using ultrasonic calipers. Measurements were taken from the anterior to the posterior "m-line" at end diastole. The mean diameter was calculated from four cardiac cycles incident with the R-wave on the electrocardiogram (ECG). For the reactive hyperemia scan, diameter measurements were taken 50 to 60 s after cuff deflation. Diameter changes were derived as percentage change relative to the mean of the two baseline scans (100%). Volume blood flow was calculated by multiplying the velocity time integral of the Doppler flow signal (corrected for angle) by the heart rate and the vessel cross-sectional area ($\pi \times r^2$) (16). The flow velocity used in our calculation is taken from the center of the artery and therefore absolute flow values may be overestimated, but relative values before and after cuff inflation are accurate. Reactive hyperemia was calculated as the maximum flow measured during the first 15 s after cuff deflation divided by the flow during the first resting (baseline) scan.

Biochemical studies. Venous blood samples were collected before the initiation of each treatment and after 1 and 4 weeks of treatment (between 1 and 2 h after the treatment dose). Measures of full blood count (Coulter Counter analyzer), urea, ammonia, creatinine, aspartate transaminase and alkaline phosphatase were performed in an automated analyzer (Kodak Systems, UK). In female subjects, blood samples were taken 2 h after the first dose of each powder for ammonia estimation in order to exclude a rapid rise in ammonia levels that may have signalled the presence of heterozygous arginase deficiency. This was not observed in any subject. Fasting lipid analyses were performed for total cholesterol, high density lipoprotein (HDL) cholesterol (after precipitation of apoprotein B containing lipoproteins) and triglycerides using Boehringer-Mannheim GmbH, diagnostica apparatus in London and a Hitachi 747 autoanalyzer in Sydney. Low density lipoprotein (LDL) cholesterol was calculated according to the Friedwald formula (19). Lipoprotein (a) was measured using an immuno-radiometric assay (Pharmacia, Milton Keynes, UK). Plasma was deproteinized with 2% sulphur salicylic acid and analyzed for free L-arginine with an automated amino acid analyzer (Pharmacia, Milton Keynes, UK) in London and high-performance liquid chromatography in Sydney.

Nitrosoprotein assay. We measured circulating nitrosoprotein levels as an indicator of the endogenous plasma levels of bioavailable NO (20) in a subgroup of 11 patients. Blood samples were collected into heparin on ice, immediately transported to the laboratory and centrifuged for 20 min at −40°C. The supernatant plasma was removed to a separate tube on ice and assayed immediately for protein-bound NO (21), after displacement with Hg^{2+}, by diazotization of sulphanilamide followed by coupling with N-(1-naphthl)-ethylene diamine, according to the method of Saville (22). Preliminary studies used nitrosylated bovine serum albumin to establish the half-life of

nitrosoproteins under conditions of blood sampling and assay. No detectable loss of nitrosylated bovine serum albumin was found during 2 h storage at room temperature. Standard curves were produced using sodium nitrite (linear, $r > 0.99$ in all cases). Control incubations in which Hg^{2+} was omitted indicated that plasma free nitrite levels were below the limits of detection of the assay. Triplicate determinations of nitrosoproteins on a batch of plasma, assayed on the same day, gave standard deviations of < 5%.

Statistics. Descriptive data are expressed as mean and standard deviation. The primary study endpoint was change in flow mediated dilation (posttreatment values minus pretreatment values) on L-arginine and on placebo. The change on L-arginine and placebo were compared by paired student *t* tests (23). Determinants of the degree of change in flow-mediated dilation (FMD), such as gender, current use of HMG CoA reductase inhibitors and biochemical measures, were explored by standard univariate and multiple regression analyses. Baseline values, before each powder was administered, and other outcome variables were compared by paired *t* tests corrected for multiple comparisons (24), except nitrosoprotein levels which were compared using a Wilcoxson signed rank test. The treatment "order effect" was explored using standard regression techniques in terms of changes in flow mediated dilation and in terms of changes from baseline for other outcome variables. For all values, comparisons of changes between treatment arms used intention-to-treat analyses (i.e., all patients were included irrespective of compliance to assigned treatment) unless otherwise stated. Statistical significance was inferred at two-sided $P < 0.05$.

Results

Subjects. Of the 56 hypercholesterolemic subjects screened by vascular ultrasound, 30 subjects fulfilled the selection criteria and were randomized. There were three withdrawals related to the subjects' time commitments and geographical difficulty in attending hospital, all within the first 4-wk treatment phase (1 received active medication, 2 placebo). The remaining 27 subjects (18 men, 9 women, mean age 29±5 [19 to 40] years) completed the study. Seventeen had familial hypercholesterolemia (defined as having a total plasma cholesterol > 268 mg/dl or a LDL-cholesterol > 196 mg/dl with tendon xanthomata in the patient or a relative) and 10 had "polygenic" hypercholesterolemia. Lipid profiles are shown in Table I. Total cholesterol was 294±50 mg/dl and LDL-cholesterol was 238±43 mg/dl. Fifteen subjects were receiving HMG CoA reductase inhibitors (Simvastatin or Pravastatin) at a stable dose throughout the study; LDL-cholesterol levels (217±35 vs. 224±45 mg/dl) were similar in those receiving and those not receiving HMG CoA reductase inhibitors ($P = 0.61$).

L-arginine and placebo treatments were generally well tolerated with minor complaints of increased stool frequency and abdominal bloating in five subjects on L-arginine and two subjects on placebo powder. While on L-arginine, three subjects suffered diarrhoea, requiring that the dose be reduced to seven grams twice daily without unblinding. Symptoms quickly resolved on this modified regime.

Vascular reactivity studies. Baseline vessel diameter, blood flow and degree of reactive hyperemia after cuff release did not alter significantly over the course of the study (Table I). Baseline flow mediated dilation was similar in those subjects taking and those not receiving HMG CoA reductase inhibitors (2±1.5 vs. 1.8±1.4%, $P = 0.71$). There was a significant improvement in flow mediated dilation in subjects while taking L-arginine (1.7±1.3% to 5.6±3.0%) compared with placebo (2.3±1.9 to 2.3±2.4%, Change(Δ) on L-arginine was 3.9±3.0% versus Δ on placebo 0.1±2.2%, $P < 0.001$) (Table I, Fig. 2). In contrast, no difference in response to GTN was found during the two treatment phases. Of the 27 subjects, flow mediated dilation improved by > 2% in 18 subjects on L-arginine (67% of subjects).

In the 15 patients who received the active L-arginine treatment phase first, there was no difference in baseline vessel diameter (4.0±0.6 vs. 4.1±0.4 mm) the degree of reactive hyperemia (401±168 vs. 411±246%), or flow mediated dilation (2.2±1.4 vs. 2.1±2.0%) between the baseline scan and the scan following the washout phase ($P > 0.50$ for each comparison). Similarly, analysis of the results using multiple linear regression did not reveal any "order effect."

There were no significant changes in resting heart rate (64±10 bpm to 65±10 bpm on L-arginine and 61±10 bpm to 64±9 bpm on placebo) and supine blood pressure (119±10/70±8 mmHg to 119±7/76±7 mmHg on L-arginine and 117±8/77±8 mmHg to 118±8/76±7 mmHg on placebo) during active or placebo treatment phases, suggesting no direct hemodynamic effects of L-arginine therapy.

Biochemical measurements. Lipid profiles did not change significantly during L-arginine and placebo treatment phases (Table I). Full blood count, creatinine, alkaline phosphatase, aspartate transaminase and alanine transaminase also re-

Table I. Results of Biochemical and Vascular Studies Pre and Post L-Arginine and Placebo

		L-arginine		Placebo		Δ (change)		
		Pre	Post	Pre	Post	L-arginine	Placebo	*P*
Total cholesterol	(mg/dl)	294±50	302±46	317±50	313±50	5±36	3±39	NS
Triglyceride	(mg/dl)	121±76	112±23	132±92	133±80	19±100	34±153	NS
HDL-cholesterol	(mg/dl)	50±15	46±12	54±19	50±15	6±58	6±74	NS
LDL-cholesterol	(mg/dl)	238±43	232±50	236±46	236±46	3±39	2±32	NS
Lp(a)	(mg/l)	364±381	600±500	454±239	612±475	130±383	41±151	NS
L-arginine	(μmol/l)	115±103	231±125	149±1.29	98±60	113±109	60±101	< 0.02
Nitrosoproteins	(μmol/l) ($n = 11$)	0.60±1.47	2.72±3.03	0.85±1.09	0.23±0.61	2±3	1±1	NS
Baseline vessel diameter	(mm)	4.0±0.6	4.1±0.6	3.9±0.6	4.0±0.5	0.1±0.4	0.1±0.2	NS
Baseline blood flow	(l/min)	65±40	73±44	47±29	57±31	9±40	8±23	NS
Reactive hyperemia	(%)	461±255	409±174	470±239	420±196	68±266	50±259	NS
Flow mediated dilation	(%)	1.7±1.3	5.6±3.0	2.3±1.9	2.3±2.4	3.8±3.0	0.1±2.2	< 0.001
GTN mediated dilation	(%)	15.0±6.0	13.1±4.5	16.2±5.5	14.3±4.6	1.8±5.5	1.7±4.6	NS

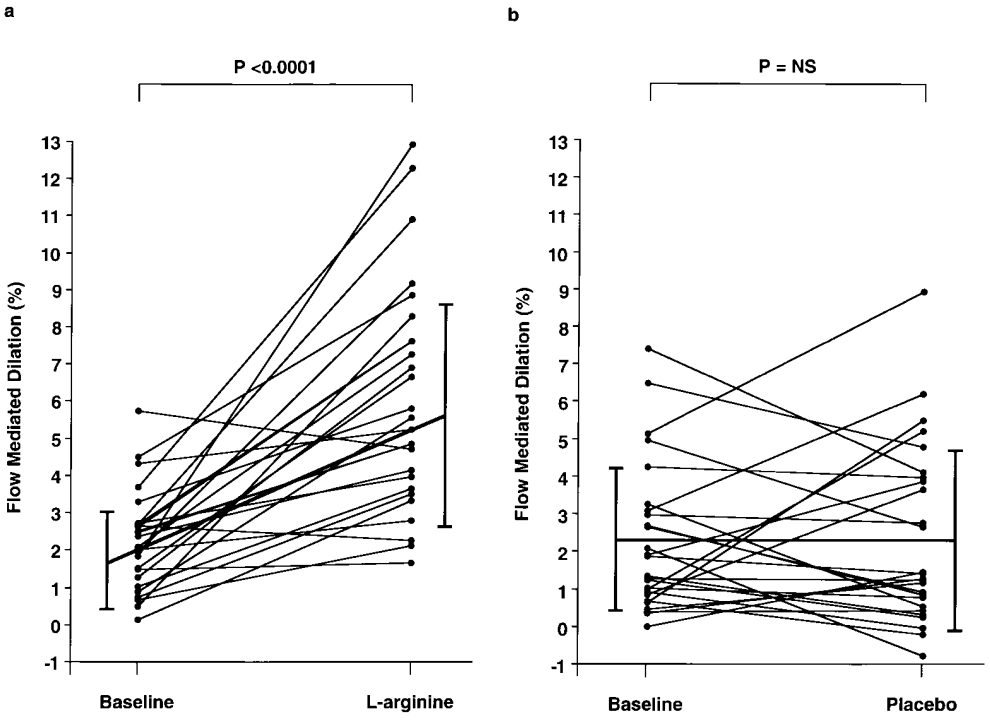

Figure 2. Flow mediated dilation before and after 4-wk dietary supplementation with L-arginine (*a*) or Placebo (*b*). Bold lines represent mean values±standard deviation.

mained constant throughout the study. There was, however, a small rise in blood urea during the L-arginine phase (from 4.62±1.46 mmol/liter to 6.15±1.82 mmol/liter on L-arginine and 5.27±1.66 mmol/liter to 4.81±2.41 mmol/liter on placebo, Δ on L-arginine 1.53±1.17 mmol/liter, Δ on placebo 0.36±2.38 mmol/liter, $P < 0.05$). Urea levels returned to baseline during the washout period in those who received L-arginine first. Serum L-arginine levels rose significantly during the active treatment phase (115±103 to 231±125 μmol/liter, $P = 0.002$) and there was also a modest rise in nitrosoprotein levels (0.6±1.4 to 2.7±3.0 μmol/liter, $\Delta P = 0.31$), although this was not statistically significant. No change in L-arginine levels or nitrosoprotein levels was detected on placebo treatment.

To investigate the effects of L-arginine administration on hormone levels, we measured plasma glucose, growth hormone (GH) and insulin-like growth factor-1 (IGF-1) in a subgroup of eight patients before and after 4 wk of L-arginine. No significant change in levels were found (glucose 4.8±0.5 to 4.8±0.7 mmol/liter, $P = 0.81$; GH 6.5±10.5 to 3.7±4.1 mU/liter, $P = 0.34$; IGF-1 337±54 to 364±118 ng/ml, $P = 0.42$). There were no significant correlations between any of these hormone levels and observed changes in vascular reactivity.

Determinants of change in endothelium-dependent dilation. The relationship between change in endothelium-dependent dilation on L-arginine powder and sex, HMG CoA reductase inhibitor therapy, LDL-cholesterol levels, lipoprotein (a) (Lp[a]) levels and plasma L-arginine levels was explored using univariate and multivariate analyses. There were no significant relationships detected. Thus, although there was a wide range of endothelium-dependent dilation (EDD) changes following L-arginine therapy (−1.0–11.1%) this could not be explained by any of the measured parameters, nor by the plasma L-arginine levels.

Discussion

We have shown that dietary supplementation with the amino acid L-arginine, results in significant improvement in endothelium-dependent dilation in hypercholesterolemic young adults, after just 4 wk. As oral L-arginine was not associated with adverse hemodynamic or clinical events, this agent may have therapeutic applications.

Previous experimental studies which have examined the effect of L-arginine in isolated tissue preparations and animal models of hypercholesterolemia have demonstrated acute improvement of microvascular and conduit artery endothelium-dependent dilation (25–27). Furthermore, in cholesterol fed rabbits, dietary supplementation with L-arginine has been shown to attenuate the development of endothelial dysfunction and early atheroma formation in the aorta (11). However, it is difficult to extrapolate these findings to humans as the time course and pathophysiology of atherogenesis are known to differ from animal models.

Clinical studies with L-arginine have been conducted previously in older individuals in whom atherosclerosis and endothelial dysfunction are likely to be well established. Parenteral L-arginine has been shown to improve microvascular endothelium-dependent responses in both the forearm (13, 28) and coronary circulation (14, 29) of hypercholesterolemic subjects, but not in those with normal cholesterol levels (14). Short term therapy with L-arginine had no effect on the endothelial physiology of the large conduit arteries in these studies. Our work differs from these reports since we have examined the effects of chronic oral administration at a much earlier stage in the disease process, when intuitively there is more likely to be a response to intervention. Indeed, we have demonstrated previously that endothelial dysfunction is already present in the

conduit arteries in hypercholesterolemic children as young as 7 yr of age (6). By studying subjects early in the natural history of their disease, we have been able to demonstrate, for the first time, that an oral agent can improve large artery physiology in presymptomatic subjects at risk of atherosclerosis. The improvement that we have observed in young adults contrasts with the lack of response in older patients and may be related to shorter duration of their disease or perhaps to the longer period of administration possible with oral therapy. However, despite receiving a regime that produced an approximately 2 fold increase in L-arginine levels, endothelium-dependent responses (5–7%) in these young hypercholesterolemic adults did not improve to the levels found in normocholesterolemic subjects (7–9%) (30). These findings are consistent with work in the cholesterol fed rabbit where a comparable dose of oral L-arginine resulted in only partial attenuation of endothelial dysfunction over a similar time course (11).

The current findings of improved endothelial function after oral L-arginine are only applicable to hypercholesterolemic subjects whose baseline endothelial physiology is abnormal. Whether larger oral doses of L-arginine, or more prolonged therapy, would have produced further improvement, or whether this represents maximal achievable benefit with L-arginine in such patients, is unknown. Similarly it remains to be tested whether an equivalent response could have been achieved with lower total doses or less frequent dosing. The beneficial effects on the endothelium were short lived and were no longer apparent 4 wk after cessation of treatment. It may be necessary, therefore, to continue long term therapy to maintain the improvement in vascular function. It will also be important, in future studies, to assess whether chronic oral therapy can improve endothelial function in normocholesterolemic subjects, or in those with high cholesterol levels but in whom flow mediated dilation is within the normal range.

Our simple noninvasive technique enables accurate and reproducible assessment of vascular responses in young, asymptomatic subjects (15). The method involves comparing vascular dilation in response to increased flow with dilation in response to glyceryl trinitrate. Flow mediated dilation depends on the ability of healthy endothelium to release NO in response to shear stress (31, 32), via the L-arginine/NO pathway and consequently can be inhibited by intra-arterial infusion of L-NMMA proximal to the measuring site (17). A close correlation has been demonstrated between endothelial function in the brachial artery, assessed using our method, and endothelial function in the coronary arteries assessed invasively using acetylcholine (18).

The mechanism whereby L-arginine may improve endothelial function remains uncertain. Experimental work on the vascular wall suggests that an important balance exists between the production of NO by the endothelium and its subsequent inactivation (33). In normal subjects there may already be sufficient NO activity, explaining the failure of L-arginine supplementation to increase EDD in such subjects (14, 34), although in these studies, L-arginine was administered for much shorter time periods. In hypercholesterolemia, the impaired vasodilation response to flow (6) and other endothelium-dependent stimuli (5) may result from increased NO inactivation by oxygen free radicals generated by the presence of oxidized LDL-cholesterol and/or Lp(a) particles in the subendothelial space (35) or, in some circumstances, by substrate limited NO production. In vitro work initially demonstrated that extracellular L-arginine levels were adequate for maintaining or increasing intracellular NO production (36) even in the presence of hypercholesterolemia. Recent work by Harrison and colleagues has, however, suggested that, in the presence of physiological concentrations of L-glutamine, the Km (substrate concentration at which the reaction velocity is half-maximal) of NOS for L-arginine may be much higher (37).

In this study, we were unable to measure directly whether L-arginine supplementation increased NO production. There was a non-significant increase in nitrosoprotein levels in some subjects; this may reflect inadequate statistical power, as other investigators have shown that L-arginine therapy is associated with an increase in a number of other surrogate markers of NO production, such as nitrite, nitrate and exhaled NO (10, 38). In our study, we did not find any changes in endocrine parameters with L-arginine therapy, which might have altered arterial physiology. Other investigators, however, have demonstrated that oral L-arginine may enhance insulin and prolactin secretion (39–41), and may influence the immune system, altering plasma interleukin and T lymphocyte responses (42, 43). It is uncertain if any or all of these changes might contribute to the beneficial effects of oral L-arginine on vascular physiology in hypercholesterolemic subjects. In our subjects, there was a wide range of changes observed in endothelium-dependent dilation after L-arginine; this suggests that other factors we were unable to measure might modulate an individual's response to oral L-arginine therapy. For example, inhibition of LDL-oxidation (44) may be an alternative or additive explanation for the observed effects of L-arginine on vascular physiology.

Nitric oxide is not only a potent vasodilator but reduces platelet aggregation and adhesion, monocyte–vessel wall interaction and smooth muscle cell proliferation — all important events in early atherosclerosis (4). Tsao et al. have demonstrated that dietary L-arginine supplementation decreases platelet activation (9) and inhibits monocyte adhesion to the endothelial cell surface (10) in hypercholesterolemic rabbits. In both experiments, the inhibition of these effects by L-NMMA suggests the mechanism is related to increased synthesis of NO from L-arginine. Preliminary studies suggest that L-arginine may have similar effects in human subjects (34).

A relationship is emerging between vascular risk factors, endothelial dysfunction and the subsequent development of atherosclerosis and its complications (2). Strategies for intervention in this sequence of vascular damage have traditionally concentrated on risk factor modification, such as cholesterol lowering or smoking cessation. By effecting a favorable alteration in the balance between NO production and catabolism, therapy with L-arginine might provide an additional approach for vascular protection. We have shown that oral L-arginine supplementation improves endothelial dysfunction in the conduit arteries of young asymptomatic adults with hypercholesterolemia. It may also be beneficial in individuals with other cardiovascular risk factors, such as cigarette smoking and diabetes mellitus, which may interact with hypercholesterolemia to cause vascular damage (30, 45). As in the hypercholesterolemic animal model, dietary L-arginine supplementation in humans may thus impact favorably on the atherogenic process.

Acknowledgments

The authors are very grateful to Dr. D.J. Betteridge, University College London Hospitals (Middlesex), London, UK; Dr. M. Seed, Char-

ing Cross Hospital; Dr. G.R. Thompson of the MRC Lipoprotein Team, Hammersmith Hospital, London, UK; and Dr. D. Sullivan, Clinical Biochemistry Department, Royal Prince Alfred Hospital, Sydney, Australia for allowing us to study their patients. We would also like to thank Dr. A. Nakagomi, Department of Cardiology, Royal Prince Alfred Hospital, Sydney, Australia for performing the nitrosoprotein assays and Teresa Bull for her help in the preparation of this manuscript.

This work is supported by grants from the British Heart Foundation, the Coronary Artery Disease Research Association (CORDA), the National Heart Foundation of Australia and the National Health and Medical Research Council of Australia.

References

1. Anderson, K.M., W.P. Castelli, and D. Levy. 1987. Cholesterol and mortality. 30 years of follow-up from the Framingham study. *J. Am. Med. Assoc.* 257:2176–2180.

2. Ross, R. 1993. The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature (Lond.).* 362:801–809.

3. Palmer, R.M., A.G. Ferrige, and S. Moncada. 1987. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature (Lond.).* 327:524–526.

4. Cooke, J.P., and P.S. Tsao. 1994. Is NO an anti-atherogenic molecule? *Arterioscler. Thromb.* 14:653–655.

5. Creager, M.A., J.P. Cooke, M.E. Mendelsohn, S.J. Gallagher, S.M. Coleman, J. Loscalzo, and V.J. Dzau. 1990. Impaired vasodilation of forearm resistance vessels in hypercholesterolemic humans. *J. Clin. Invest.* 86:228–234.

6. Sorensen, K.E., D.S. Celermajer, D. Georgakopoulous, G. Hatcher, D.J. Betteridge, and J.E. Deanfield. 1994. Impairment of endothelium-dependent dilation is an early event in children with familial hypercholesterolemia and is related to the lipoprotein(a) level. *J. Clin. Invest.* 93:50–55.

7. Casino, P.R., C.M. Kilcoyne, A.A. Quyyumi, J.M. Hoeg, and J.A. Panza. 1993. The role of nitric oxide in endothelium-dependent vasodilation of hypercholesterolemic patients. *Circulation.* 88:2541–2547.

8. Palmer, R.M., D.S. Ashton, and S. Moncada. 1988. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature (Lond.).* 333:664–666.

9. Tsao, P.S., G. Theilmeier, A.H. Singer, L.L. Leung, and J.P. Cooke. 1994. L-arginine attenuates platelet reactivity in hypercholesterolemic rabbits. *Arterioscler. Thromb.* 14:1529–1533.

10. Tsao, P.S., L.M. McEvoy, H. Drexler, E.C. Butcher, and J.P. Cooke. 1994. Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circulation.* 89:2176–2182.

11. Cooke, J.P., A.H. Singer, P. Tsao, P. Zera, R.A. Rowan, and M.E. Billingham. 1992. Anti-atherogenic effects of L-arginine in the hypercholesterolemic rabbit. *J. Clin. Invest.* 90:1168–1172.

12. Wang, B.Y., A.H. Singer, P.S. Tsao, H. Drexler, J. Kosek, and J.P. Cooke. 1994. Dietary arginine prevents atherogenesis in the coronary artery of the hypercholesterolemic rabbit. *J. Am. Coll. Cardiol.* 23:452–458.

13. Creager, M.A., S.J. Gallagher, X.J. Girerd, S.M. Coleman, V.J. Dzau, and J.P. Cooke. 1992. L-arginine improves endothelium-dependent vasodilation in hypercholesterolemic humans. *J. Clin. Invest.* 90:1248–1253.

14. Drexler, H., A.M. Zeiher, K. Meinzer, and H. Just. 1991. Correction of endothelial dysfunction in coronary microcirculation of hypercholesterolaemic patients by L-arginine. *Lancet.* 338:1546–1550.

15. Sorensen, K.E., D.S. Celermajer, D.J. Spiegelhalter, J. Robinson, and J.E. Deanfield. 1995. Non-invasive measurement of endothelium-dependent arterial responses in man: accuracy and reproducibility. *Br. Heart J.* 74:347–253.

16. Celermajer, D.S., K.E. Sorensen, V.M. Gooch, D.J. Spiegelhalter, O.I. Miller, I.D. Sullivan, J.K. Lloyd, and J.E. Deanfield. 1992. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet.* 340:1111–1115.

17. Joannides, R., W.E. Haefeli, L. Linder, V. Richard, E.H. Bakkali, C. Thuillez, and T.F. Luscher. 1995. Nitric oxide is responsible for flow-dependent dilatation of human peripheral conduit arteries in vivo. *Circulation.* 91:1314–1319.

18. Anderson, T.J., A. Uehata, M.D. Gerhard, I.T. Meredith, S. Knab, D. Delagrange, E.H. Lieberman, P. Ganz, M.A. Creager, A.C. Yeung, and A.P. Selwyn. 1995. Close relationship of endothelial function in the human coronary and peripheral circulations. *J. Am. Coll. Cardiol.* 26(4):1235–1241.

19. Friedwald, W.T., R.I. Levi, and D.S. Fredrickson. 1958. Estimation of the concentration of low density lipoprotein cholesterol in the plasma without the use of the preparative ultracentrifuge. *Clin. Chem.* 18:499–502.

20. Stamler, J.S., O. Jaraki, and J. Osborne. 1992. Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc. Natl. Acad. Sci. USA.* 89:7674–7677.

21. Wennmalm, A., G. Benthin, and A. Edlund. 1993. Metabolism and excretion of nitric oxide in humans. *Circ. Res.* 73:11121–11127.

22. Saville, B. 1958. A scheme for the colorimetric determination of the microgram amounts of thiols. *Analyst.* 83:670–672.

23. Sonn, S. 1993. Cross over trials in clinical research. John Wiley & Sons Ltd. Chichester, UK. 56pp.

24. Hochberg, Y. 1988. A sharper Bonferroni procedure for multiple tests of significance. *Biometrika.* 75:800–802.

25. Girerd, X.J., A.T. Hirsch, J.P. Cooke, V.J. Dzau, and M.A. Creager. 1990. L-arginine augments endothelium-dependent vasodilation in cholesterol-fed rabbits. *Circ. Res.* 67:1301–1308.

26. Cooke, J.P., N.A. Andon, X.J. Girerd, A.T. Hirsch, and M.A. Creager. 1991. Arginine restores cholinergic relaxation of hypercholesterolemic rabbit thoracic aorta [see comments]. *Circulation.* 83:1057–1062.

27. Rossitch, E., Jr., E. Alexander, P.M. Black, and J.P. Cooke. 1991. L-arginine normalizes endothelial function in cerebral vessels from hypercholesterolemic rabbits. *J. Clin. Invest.* 87:1295–1299.

28. Hirooka, Y., K. Egashira, T. Imaizumi, T. Tagawa, H. Kai, M. Sugimachi, and A. Takeshita. 1994. Effect of L-arginine on acetylcholine-induced endothelium-dependent vasodilation differs between the coronary and forearm vasculatures in humans. *J. Am. Coll. Cardiol.* 24:948–955.

29. Kuo, L., M.J. Davis, M.S. Cannon, and W.M. Chilian. 1992. Pathophysiological consequences of atherosclerosis extend into the coronary microcirculation. Restoration of endothelium-dependent responses by L-arginine. *Circ. Res.* 70:465–476.

30. Celermajer, D.S., K.E. Sorensen, D. Georgakopoulos, C. Bull, O. Thomas, J. Robinson, and J.E. Deanfield. 1993. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation.* 88:2149–2155.

31. Rubanyi, G.M., J.C. Romero, and P.M. Vanhoutte. 1986. Flow-induced release of endothelium-derived relaxing factor. *Am. J. Physiol.* 250:H1145–1149.

32. Ohno, M., G.H. Gibbons, V.J. Dzau, and J.P. Cooke. 1993. Shear stress elevates endothelial cGMP. Role of a potassium channel and G protein coupling. *Circulation.* 88:193–197.

33. Ohara, Y., T.E. Peterson, and D.G. Harrison. 1993. Hypercholesterolemia increases endothelial superoxide anion production. *J. Clin. Invest.* 91: 2546–2551.

34. Adams, M.R., C.J. Forsyth, W. Jessup, J. Robinson, and D.S. Celermajer. 1995. Oral L-arginine inhibits platelet aggregation but does not enhance endothelium-dependent dilation in healthy young adults. *J. Am. Coll. Cardiol.* 26(4):1054–1061.

35. Galle, J., J. Bengen, P. Schollmeyer, and C. Wanner. 1995. Impairment of endothelium-dependent dilation in rabbit renal arteries by oxidized lipoprotein (a). *Circulation.* 96:1582–1589.

36. Hecker, M., W.C. Sessa, H.J. Harris, E.E. Anggard, and J.R. Vane. 1990. The metabolism of L-arginine and its significance for the biosynthesis of endothelium-derived relaxing factor: cultured endothelial cells recycle L-citrulline to L-arginine. *Proc. Natl. Acad. Sci. USA.* 87:8612–8616.

37. Arnal, J.F., T. Munzel, R.C. Venema, N.L. James, C.L. Bai, W.E. Mitch, and D.G. Harrison. 1995. Interactions between L-arginine and L-glutamine change endothelial NO production. *J. Clin. Invest.* 95:2565–2572.

38. Kharitonov, A., G. Lubec, B. Lubec, M. Hjelm, and P.J. Barnes. 1995. L-arginine increases exhaled nitric oxide in normal human subjects. *Clin. Sci.* 88:135–139.

39. Besset, A., A. Bonardet, G. Rondouin, B. Descomps, and P. Passouant. 1982. Increase in sleep related GH and Prl secretion after chronic arginine aspartate administration in man. *Acta Endocrinol. (Copenh.).* 99 (1):18–23.

40. Elsair, J., K. Khelfat, A. Ghouini, and F. Ikhlef. 1985. Effects of arginine, administered orally, on the endogenous secretion of the STH-somatomedin complex in young human volunteers. *CR Seances Soc. Biol. Fil.* 179 (5):608–614.

41. Isidori, A., A. Lo Monaco, and M. Cappa. 1981. A study of growth hormone release in man after oral administration of amino acids. *Curr. Med. Res. Opin.* 7(7):475–481.

42. Hayde, M., H. Vierhapper, B. Lubec, C. Popow, M. Weninger, Z. Xi, and G. Lubec. 1994. Low-dose dietary L-arginine increases plasma interleukin 1 alpha but not interleukin 1 beta in patients with diabetes mellitus. *Cytokine.* Jan;6(1):79–82.

43. Daly, J.M., J. Reynolds, A. Thom, L. Kinsley, M. Dietrick-Gallagher, J. Shou, and B. Ruggieri. 1988. Immune and metabolic effects of Arginine in the surgical patient. *Ann. Surg.* 208:512–523.

44. Philis-Tsimakis, A., and J.L. Witztum. 1995. L-arginine may inhibit atherosclerosis through inhibition of LDL oxidation (abstract). *Circulation.* 92 (Suppl 1):I-422–423.

45. Poston, L., and P. Taylor. 1995. Impairment of endothelium-dependent vascular reactivity in insulin dependent diabetes mellitus. *Clin. Sci.* 88:245–255.