L-Arginine Attenuates Platelet Reactivity in Hypercholesterolemic Rabbits

Philip S. Tsao, Gregor Theilmeier, Alan H. Singer, Lawrence L.-K. Leung, John P. Cooke

Abstract Platelets are capable of producing nitric oxide (NO) through the l-arginine–NO synthase pathway. Acute exposure to supraphysiological concentrations of L-arginine in vitro increases the production of NO by platelets and is associated with an increase in platelet cyclic GMP (cGMP) levels and a reduction in platelet aggregation. The purpose of this study was to determine if chronic oral administration of L-arginine decreases platelet aggregation in hypercholesterolemic animals and to determine if this effect is mediated by the metabolism of L-arginine to NO. Male New Zealand White rabbits were fed normal chow (Con), a 1% cholesterol diet (Chol), or a 1% cholesterol diet supplemented with a sixfold enrichment of dietary L-arginine (Arg) or L-methionine (Met). After 10 weeks, cholesterol levels were equally increased in Chol and Arg animals, whereas plasma arginine levels were doubled in the Arg group. There was no difference in maximum aggregation initiated by ADP (100 μmol/L) between washed platelets from Con, Met, and Chol animals, but aggregation of platelets from Arg animals was significantly decreased (P<.05). In aggregating platelets from Arg animals, cGMP levels were significantly higher than the other groups (P<.05). When platelets were incubated ex vivo with the NO synthase inhibitor Nω-monomethyl-L-arginine, the effects of dietary L-arginine were reversed. Chronic dietary supplementation of L-arginine decreases platelet aggregation in hypercholesterolemic rabbits. This effect appears to be due to the metabolism of L-arginine to NO. (Arterioscler Thromb. 1994;14:1529-1533.)

Keywords • nitric oxide • atherosclerosis • endothelium • endothelium-derived relaxing factor • aggregometry

Atherosclerosis is thought to be initiated as a response to injury of the endothelium.1 This alteration of endothelial function favors platelet adherence and aggregation to the luminal surface, with the subsequent release of platelet-derived growth factors and vasoactive substances that contribute to the process of atherogenesis.

One of the earliest alterations of the endothelium that has been observed in hypercholesterolemic animals and humans is decreased activity of endothelium-derived relaxing factor (EDRF).2-4 EDRF is nitric oxide (NO) or a nitroso-containing compound derived from the metabolism of L-arginine by NO synthase.5-8 This nitrosodiuretoid also inhibits platelet adhesion9 and aggregation via the stimulation of intracellular guanylate cyclase to increase intracellular levels of cyclic GMP (cGMP).10,11 NO synthase and guanylate cyclase are also active in platelets, and platelet-derived NO auto-regulates aggregation.12,13 A reduction in the activity of NO synthase by hypercholesterolemia could therefore lead to increased platelet–vessel wall interaction and enhanced atherogenesis.

Recent studies in this laboratory have shown that chronic oral supplementation of the EDRF precursor L-arginine significantly improved NO-dependent vaso-dilation in hypercholesterolemic animals; this effect was associated with a striking reduction in intimal lesions.14 To determine if this antiatherogenic effect of L-arginine is associated with decreased platelet reactivity and enhancement of platelet-derived NO activity, we tested the hypothesis that (1) dietary L-arginine reduces platelet aggregation and (2) this effect is mediated by the metabolism of L-arginine to NO.

Methods

Animals

Male New Zealand White rabbits received one of the following dietary interventions for 10 weeks: normal rabbit chow (Con), rabbit chow enriched with 1% cholesterol (Chol) (ICN Biomedical), or a 1% cholesterol chow supplemented with L-arginine (Arg) added to the drinking water throughout the course of the study. Supplementation with 2.25% L-arginine HCl in the drinking water results in a sixfold enrichment of this amino acid (based on the daily average water intake of the animals) and induces a twofold increase in plasma arginine levels.14 To determine if any effects of L-arginine supplementation were mediated by changes in caloric or nitrogen balance, a fourth group received a 1% cholesterol diet supplemented with 0.9% L-methionine (Met) in the drinking water to induce a sixfold increase in the daily intake of this amino acid.

After 10 weeks of dietary intervention, animals were lightly sedated with 3 mg subcutaneous injection of acepromazine maleate solution (Ayerst Laboratories), and the central ear artery was cannulated for measurement of intra-arterial blood pressure, followed by collection of blood samples. Whole blood samples were collected in one-tenth the volume of 3.8% sodium citrate at a pH of 6.3. Serum cholesterol measurements were performed on an automated analyzer (Monarch Chemistry System, Instrumentation Laboratory, Inc). These protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Platelet Isolation and Aggregation

Citrated blood was centrifuged at 200g for 15 minutes at room temperature. The platelet-rich plasma was removed and
centrifuged at 1500g for 15 minutes at room temperature. The platelet pellet was resuspended in a solution of 0.05 mL of 2% EDTA and 10 mL of Ca²⁺-free Krebs-Henseleit buffer consisting of the following (in mmol/L): NaCl, 120; NaHCO3, 12.5; dextrose, 10; KCl, 4.7; KHPO₄, 1.2; and MgSO₄ · 7H₂O, 1.2, at pH 7.4. The resuspended platelets were then spun at 1500g for 10 minutes at room temperature, and the resulting button was then resuspended in 10 mL of a 0.025% albumin solution consisting of the following (in mmol/L): NaCl, 136.9; tris(hydroxymethyl)aminomethane, 7.68; dextrose, 5.55; KCl, 2.6; MgCl₂ · H₂O, 1.04; CaCl₂ · 2 H₂O, 1.3. The platelet suspension was centrifuged at 1000g for 10 minutes at room temperature. The washed platelet button was then resuspended in Tyrode’s albumin buffer so that the platelet count was adjusted to 300,000/μL. Platelets were then warmed to 37°C for at least 10 minutes before aggregation studies.

Platelet aggregation was monitored with a dual-channel aggregometer (Chrono-Log Corp) at 37°C with continuous stirring at 800 rpm. Changes in light transmission occurring during platelet aggregation were recorded continuously, and results are presented in aggregation units as the extent of increase in light transmittance with Tyrode’s albumin buffer as reference. Aggregation was induced by ADP to give a final concentration of 0.1 to 100 μmol/L. In some experiments, the effect of 10⁻⁴-monomethyl-L-arginine (NMA) (Calbiochem) on platelet aggregation and cGMP accumulation was studied. NMA, dissolved in distilled water, was added directly to the cuvettes 15 minutes before aggregation to give a final concentration of 10 μmol/L.

cGMP Determinations

For cGMP studies, 0.4-mL platelet suspensions were incubated with 1 mmol/L 3-isobutyl-1-methylxanthine at 37°C for 15 minutes. Platelet aggregation was then initiated by ADP (100 μmol/L). After 5 minutes, the reaction was terminated by addition of 1 mL of trichloroacetic acid (6% wt/vol). The samples were immediately frozen in liquid nitrogen and stored at −70°C until cGMP levels were measured by radioimmunoassay (Amersham).

Intracellular Calcium Measurements

Changes in intracellular calcium concentration were measured by aequorin chemiluminescence using previously described methodology. Washed platelets were resuspended in calcium-free Krebs-Henseleit buffer (90 μL containing EGTA (5 mmol/L). Aequorin (1 mg) was dissolved in deionized water (333 μL), containing EGTA (7 mmol/L), and an aliquot (10 μL) of this solution was added to the platelets. To enhance aequorin loading, DMSO (1 μL) was added at 90-second intervals over a period of 7.5 minutes to reach a final concentration of 6%. Platelets were incubated in this solution for an additional 2.5 minutes at room temperature, washed twice, and resuspended in Tyrode’s albumin buffer. Platelet number was adjusted to 300,000/μL, and the suspension was placed in cuvettes for chemiluminescence and aggregometry. Increases in chemiluminescence in response to calcium ionophore (A23187, 10⁻⁶ mol/L) were detected using a dual-channel lumiaaggregometer (Chronolog Corp). Signals are reported as arbitrary chemiluminescence units, which reflect proportional increases in intracellular calcium. Light transmission aggregometry was performed on the same sample in parallel. All drugs, unless otherwise stated, were obtained from Sigma Chemical Co.

Data Analysis

All values in the text are mean±SEM of n independent experiments. Differences between specific means were tested by analysis of variance with post hoc analysis using Fisher’s protected least significant difference test. A value of P<.05 was accepted as statistically significant.

Results

Biochemical Measurements

Serum cholesterol levels were significantly increased in all of the animals receiving the 1% cholesterol diet when compared with animals receiving normal chow (40.5±9.5 versus 643±33 versus 687±50 versus 596±19; Con versus Chol versus Arg versus Met, respectively, P<.001). Serum cholesterol levels from hypercholesterolemic animals supplemented with either L-arginine or L-methionine were not different from those animals receiving 1% cholesterol diet alone.

Platelet Aggregation

In Con rabbits, ADP induced aggregation in a dose-dependent manner (Fig 1). After 10 weeks of a 1% cholesterol diet or a 1% cholesterol diet plus methionine supplementation, the extent of platelet aggregation to ADP was unchanged (Fig 1). By contrast, platelets isolated from hypercholesterolemic animals receiving arginine supplementation displayed markedly reduced aggregation to nearly all concentrations of the agonist (Fig 1). The NO synthase antagonist NMA reversed the reduction in aggregation observed in platelets from the Arg animals (from 47.6±12.5 to 83.8±11.2 aggregation units; n=3; Fig 2).

cGMP Determinations

None of the groups differed in basal production of cGMP in platelets (Con, 1.3±0.5 pmol/mg protein; Chol, 1.3±0.2 pmol/mg protein; Met, 1.7±0.4 pmol/mg protein; Arg, 2.1±0.5 pmol/mg protein). However, chronic oral supplementation with L-arginine significantly increased the ADP-stimulated production of cGMP in comparison to the other groups (Con, 2.1±0.7 pmol/mg protein; Chol, 1.5±0.2 pmol/mg protein; Met, 2.2±0.7 pmol/mg protein; Arg, 4.6±1.1 pmol/mg protein; P<.05; Fig 3). This enhancement of cGMP levels...
in platelets from the Arg animals was inhibited by preincubation with NMA (2.6±1.1; n=3).

**FIG 2.** Tracings of representative aggregation curves showing the reversal by N³-monomethyl-L-arginine (NMA) of the effect of dietary L-arginine on platelet aggregation. A, ADP (100 μmol/L) induced maximal aggregation in platelets from hypercholesterolemic animals. B, Chronic oral supplementation with L-arginine caused a decrease in platelet reactivity. This inhibitory effect is mediated in part by platelet-derived nitric oxide (NO), since the in vitro exposure of platelets to the NO synthase inhibitor NMA reversed the effect of dietary L-arginine (C).

**Intracellular Calcium Measurements**

Calcium ionophore (A23187; 10⁻⁶ mol/L) induced rapid elevations in intracellular calcium and platelet aggregation. Platelets from hypercholesterolemic rabbits exhibited an exaggerated response to calcium ionophore, which was blunted by dietary arginine treatment (Fig 4).

Specifically, calcium ionophore induced elevations in intracellular calcium that were 22.8±16 versus 17.2±4.5 versus 57.6±8.6 chemiluminescence units, respectively, for Con, Arg, and Choi groups (n=3 in each group; P<.05, Arg versus Choi).

The effects of dietary arginine to diminish cholesterol-induced platelet hyperreactivity was antagonized ex vivo by L-nitro-arginine (Fig 4). Exposure to L-nitro-arginine (10⁻⁴ mol/L) increased calcium ionophore-induced chemiluminescence by 67% (to 27.9±4.1 chemiluminescence units; n=3) in platelets from the Arg group. Similarly, exposure to this NO synthase antagonist enhanced aggregation of Arg platelets by 80% (to 20.7±1.7 aggregation units; n=3).

**Discussion**

This is the first demonstration that platelet aggregation in hypercholesterolemic animals is inhibited by dietary arginine. Our data also indicate that the antiplatelet effects of dietary arginine are mediated by its conversion to NO. In this study, the effect of L-arginine to inhibit platelet aggregation was associated with increases in platelet cGMP and inhibition of agonist-induced elevations of intracellular calcium. These effects of dietary arginine were reversed by in vitro exposure of the platelets to the inhibitor of NO synthase, NMA.

Because dietary supplements of L-methionine did not alter platelet reactivity, it is unlikely that the effects of L-arginine were mediated by changes in caloric or nitrogen balance. It is more likely that the antiplatelet effect of dietary L-arginine was due to its conversion to NO. NO and agents that release NO inhibit platelet aggregation by 67% (to 27.9±4.1 chemiluminescence units; n=3) in platelets from the Arg group. Similarly, exposure to this NO synthase antagonist enhanced aggregation of Arg platelets by 80% (to 20.7±1.7 aggregation units; n=3).

**Fig 4.** A, Tracings of representative recordings of calcium ionophore-induced aggregation of platelets from hypercholesterolemic rabbits (Choi) and hypercholesterolemic rabbits receiving dietary arginine (Arg). In some aggregations, platelets from Arg animals were exposed to L-nitro-arginine (L-NA; 10⁻⁴ mol/L) for 10 minutes before aggregation. Platelets from hypercholesterolemic rabbits exhibit an exaggerated response to calcium ionophore, which is blunted by dietary arginine treatment. The effect of dietary arginine is antagonized by the nitric oxide (NO) synthase inhibitor L-NA. B, Tracings of continuously recorded chemiluminescence signals reflecting increases in intracellular calcium levels in platelets stimulated by calcium ionophore (simultaneously determined during the corresponding platelet aggregations in A). Again, platelets from hypercholesterolemic rabbits exhibit an exaggerated response to calcium ionophore, which is blunted by dietary arginine treatment, and the effect of dietary arginine is antagonized by the NO synthase inhibitor L-NA.
bition of platelet aggregation by dietary arginine is mediated by its metabolism to NO.

At least two sources of endogenous NO may modulate platelet reactivity. Pohl and Busse22 perfused isolated rabbit hearts with solutions of human platelets and collected platelets from the coronary sinus effluent for measurement of cGMP. When acetylcholine was added to the perfusate, platelet cGMP became significantly elevated. This was judged to be due to stimulated release of endothelium-derived NO, since isolated platelets did not respond to acetylcholine.23 This effect was blocked by antagonists of NO synthesis. The antiplatelet effect of endothelium-derived NO may be mediated by more stable S-nitrosothiol intermediates released from the endothelium into the systemic circulation.24-26

In addition to regulation by endothelium-derived NO, platelet reactivity may be modulated in an autocrine fashion by NO. Aggregating platelets have been shown to elaborate NO.13 Moreover, in vitro exposure of platelets to high concentrations of exogenous L-arginine increases the production of platelet-derived NO, elevates intracellular cGMP, and inhibits platelet aggregation.27

The effect of dietary arginine in the present study may be largely mediated by NO derived from the platelets rather than the endothelium. Basal cGMP levels tended to be elevated in unstimulated platelets harvested from arginine-treated animals, although not significantly so. More important, there was a significantly greater increase in cGMP in platelets from arginine-treated animals after aggregation; this latter effect must be due to platelet-derived NO, since the aggregation was performed ex vivo in the absence of endothelial influence. These findings are consistent with the recent observations of Yao et al.28 Intravenous infusion of L-arginine in dogs produced a significant decrement in ex vivo platelet aggregation when compared with saline infusion. The effects of L-arginine were thought to be mediated by NO, since infusion of NMA enhanced ex vivo platelet aggregation.

The antiplatelet effect of dietary L-arginine may contribute to the antiatherogenic properties of this amino acid. We have previously shown that intravenous infusion of L-arginine normalized NO-dependent vasodilation in hypercholesterolemic animals and humans.29-32 This effect of L-arginine to normalize NO-dependent vasodilation in vessels exposed to elevated levels of cholesterol has now been confirmed by other workers.33,34 More recently, we have demonstrated that chronic exposure to dietary L-arginine can also improve NO-dependent vasodilation in hypercholesterolemic animals.14 This enhancement of NO release is associated with a striking reduction in the extent of atherosclerotic lesions in the thoracic aorta and a reduction in intimal thickness.14 It is possible that this antiatherogenic effect of L-arginine is in part mediated by the antiplatelet action of endothelial and/or platelet-derived NO. Platelet–vessel wall interactions and release of platelet-derived growth factors may contribute to atherogenesis in this hypercholesterolemic animal model. For example, the effect of fish oil supplements to reduce atherogenesis in hypercholesterolemic rabbits has been attributed to the antiplatelet effects of eicosapentaenoic fatty acids in marine lipids.35,36 The resistance to atherogenesis manifested by swine with von Willebrand's disease is also likely due to the reduced platelet–vessel wall interactions observed in this animal model.37

In humans with type IIa hypercholesterolemia and in hypercholesterolemic animal models, there is an increased sensitivity to agonists of platelet aggregation.38-42 This hyperreactivity is associated with increased production by platelets of thromboxane A2.43,44 The effect of hypercholesterolemia may be mediated by direct effects of cholesterol on platelet membrane fluidity and arachidonic acid metabolism.45 In concordance with previous studies, we observed that hypercholesterolemia induced an enhancement of platelet aggregation in response to calcium ionophore. There was also a tendency for the lowest dose of adenosine diphosphate to elicit a greater aggregation response, consistent with previous reports.

To summarize, we have demonstrated for the first time that supplementation of dietary L-arginine inhibits platelet aggregation in hypercholesterolemic animals. This effect appears to be mediated by the metabolism of L-arginine to NO, a known antagonist of platelet reactivity. The antiplatelet effect of dietary arginine may explain in part the antiatherogenic properties of this amino acid.

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