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EXPERIMENTAL STUDIES

Arginine Restores Nitric Oxide Activity and Inhibits Monocyte Accumulation After Vascular Injury in Hypercholesterolemic Rabbits

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Objectives. **This study sought to determine whether the alterations in vascular function and structure after balloon injury in hypercholesterolemic rabbits could be inhibited by dietary arginine.**

Background. **Administration of arginine (the nitric oxide [NO] precursor) restores vascular NO activity in hypercholesterolemic animals. We and other investigators have shown that enhancement of vascular NO activity can inhibit myointimal hyperplasia after vascular injury in normocholesterolemic animals.**

Methods. **Twenty-eight New Zealand White rabbits received either normal rabbit chow, 0.5% cholesterol diet or 0.5% cholesterol diet plus L-arginine hydrochloride (2.25% wt/vol) in the drinking water. After 6 weeks of dietary intervention, the left iliac artery of each animal was subjected to a balloon injury. Four weeks later, the iliac arteries were harvested for vascular reactivity studies and immunohistochemical analysis.**

Results. **Vascular injury induced intimal thickening that was largely composed of vascular smooth muscle cells and extracellular matrix. In the setting of hypercholesterolemia, vascular injury induced an exuberant myointimal lesion that was augmented by the accumulation of lipid-laden macrophages. Dietary arginine reduced intimal thickening in the injured vessels of hypercholesterolemic animals and substantially inhibited the accumulation of macrophages in the lesion (from 28% to 5% of the lesion area, $p < 0.001$).**

Conclusions. **We report that lesions induced by vascular injury in hypercholesterolemic animals are markedly reduced by oral administration of arginine. Moreover, we find that the nature of the lesion is altered, with a striking reduction in the percentage of macrophages comprising the lesion.**

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Coronary angioplasty is frequently complicated by restenosis, a vascular response to injury that is, in part, secondary to myointimal hyperplasia. Myointimal hyperplasia is a lesion that is largely composed of phenotypically altered vascular smooth muscle cells and matrix proteins secreted by these cells (1–3). The proliferation of these cells is under the influence of paracrine substances, such as fibroblast growth factor (4) and platelet-derived growth factor released by vascular smooth muscle cells and adherent platelets at the site of injury (5–7). These paracrine growth factors are normally counterbalanced by endothelial factors, such as nitric oxide (NO), which suppress vascular smooth muscle proliferation (8). This endothelial influence is transiently lost after a denuding injury. Even when the endothelium regenerates it remains dysfunctional, as manifested by an attenuation of endothelium-dependent relaxation (9,10). This deficit is more profound when the injury occurs in the setting of hyperlipidemia (11). The impairment in endothelium-dependent relaxation is thought to be secondary to a reduced elaboration or increased degradation of endothelium-derived NO (12,13).

In addition to its role as a vasodilator, NO suppresses vascular smooth muscle growth (8), inhibits platelet adherence and aggregation (14–16) and reduces monocyte adherence (17). Because these are key processes involved in the cellular response to injury in hypercholesterolemic states, we hypothesized that the loss of NO activity may promote lesion formation. Conversely, restoration of NO activity should limit intimal proliferation.

Vascular NO activity can be restored acutely in hypercholesterolemic animal models and humans by intravenous administration of the precursor L-arginine (12,18). Long-term oral administration of arginine induces a sustained improvement in vascular NO activity that is associated with a downregulation of endothelial adhesiveness for monocytes (19) and a striking inhibition of monocyte accumulation in the vessel wall (20,21).

In normocholesterolemic animal models, oral arginine enhances the vasodilator function of regenerating endothelium

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Abbreviations and Acronyms

cGMP = cyclic guanosine monophosphate
DCM = Stanford Department of Comparative Medicine
EC_{50} = median effective concentration
HDL = high density lipoprotein
I/M = intimal/medial ratio
NE = norepinephrine
NO = nitric oxide
PSS = physiologic saline solution

and inhibits myointimal hyperplasia after vascular injury (22-24). The effect of arginine is blocked by antagonism of NO synthase (22). However, it is not known whether dietary arginine will restore vascular function and inhibit lesion formation after vascular injury in the setting of hypercholesterolemia. The present investigation was designed to determine whether dietary L-arginine would reduce macrophage or vascular smooth muscle accumulation, or both, in the intimal lesion formed after balloon catheter injury in the hypercholesterolemic rabbit.

Methods

Animals. Male New Zealand White rabbits weighing 2.5 to 3.0 kg (n = 28) were entered into the study after a 1-week period of acclimation in the housing facilities of the Stanford Department of Comparative Medicine (DCM), during which time the animals were fed normal rabbit chow and received water ad libitum. All rabbits were inspected before the study by the DCM veterinarian and monitored daily by DCM technicians and the investigators. All experimental 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.

Animals received either normal rabbit chow (n = 9), 0.5% cholesterol diet (Dyets) (n = 9) or 0.5% cholesterol diet plus L-arginine hydrochloride (2.25% wt/vol) in the drinking water (n = 10). This dose represents a sixfold increase in the daily L-arginine intake, and in previous studies from this laboratory (20,21) was associated with a doubling of the plasma arginine concentration.

After 6 weeks of dietary intervention, the left iliac artery of each rabbit was subjected to balloon injury, as previously described (25). Rabbits were anesthetized using a mixture of ketamine (5 mg/kg body weight) and Rompun (35 mg/kg). The level of anesthesia was continuously monitored during the procedure, and additional anesthesia was given as needed. The left superficial femoral artery was exposed and isolated, and the distal portion was ligated with a small surgical clip. A 2F Fogarty arterial embolectomy catheter (Baxter Healthcare) was inserted into the artery and advanced proximally into the iliac artery, past the bifurcation into the aorta. The balloon was inflated with 0.5 ml of saline, then withdrawn distally into the iliac artery while inflated to a point just proximal to the insertion site, then deflated and readvanced. Three successive passes were made with the inflated balloon. The proximal portion of the superficial femoral artery was then ligated and hemostasis achieved.

Serum chemistry. Blood samples after 5 weeks of dietary intervention and at the end of the study were collected from rabbits in each group for determination of cholesterol, high density lipoprotein (HDL) and plasma free arginine levels. Arginine was measured using an automated amino acid analyzer, as described previously (20). Serum cholesterol and HDL were analyzed using a modification of the enzymatic method of Allain et al. (26).

Vascular reactivity. Twenty-eight days after the balloon injury, rabbits were killed with an overdose of intravenous pentobarbital. Both iliac arteries were removed and immediately placed into oxygenated physiologic saline solution (PSS) at 37°C composed of the following (mmol/liter): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 11.1 glucose. Adherent fat and connective tissue were removed, and the iliac arteries were cut into 3- to 4-mm wide rings. Two vascular rings from the uninjured iliac artery were mounted horizontally on stainless steel stirrups through the lumen and connected to force transducers. Vascular reactivity studies were technically difficult to perform on the balloon-injured iliac arteries; the intimal lesions significantly reduced the lumen diameter and made it difficult to mount the rings without damaging the endothelium. Therefore, the uninjured right iliac artery was used in each case in the bioassay to assess the effect of the dietary interventions on the activity of endothelium-derived NO.

The vascular rings were suspended in organ chambers filled with oxygenated PSS at 37°C. Over a period of 60 min, rings were progressively stretched to the optimal point of their length-tension relation (determined previously to be 2 g). Subsequently, the median effective concentration (EC_{50}) of norepinephrine (NE) was determined by exposing the tissues to increasing doses of NE (in half-log increments from 10^{-9} to 10^{-4} mol/liter). Once a maximal response was obtained, the rings were washed repeatedly with fresh PSS for 60 min until the tension returned to the previous baseline value. Response to vasodilators was studied after precontracting the rings with the EC_{50} of NE. After a stable contraction was obtained, the rings were exposed to increasing doses of the vasodilator.

Histologic studies. On completion of the vascular reactivity studies, the iliac rings were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with an elastic van Gieson stain for light microscopy and histomorphometric measurements. Measurements of intimal and medial cross-sectional areas (mm^2) were made by a skilled observer (B.W.) who had no knowledge of the treatment groups. An eyepiece grid with 100 counting points (line intersections), each 0.4 mm apart, was used (20,27,28). Total grid area (mm^2) was determined by measurement of grid dimensions with a stage micrometer at the same microscope magnification used for point counts. Total area of the intima (or media) was defined as the

Table 1. Biochemical Measurements

	5 Weeks			10 Weeks		
	Control (n = 9)	Chol (n = 9)	Arg (n = 10)	Control (n = 9)	Chol (n = 9)	Arg (n = 10)
Total cholesterol (mg/dl)	27.1 ± 4.4	683.3 ± 115*	708.5 ± 58.5*	59.7 ± 16.7	719.6 ± 115.2*	691.8 ± 75.5*
HDL (mg/dl)	22.9 ± 3.9	31.5 ± 3.4	41.6 ± 6.5	31.2 ± 9.2	43 ± 8.4	35 ± 7.4
Arginine (mmol/liter)	0.21 ± 0.01	0.20 ± 0.02	0.44 ± 0.08*	0.21 ± 0.02	0.18 ± 0.04	0.44 ± 0.08*

*Significantly different from control at the same time points. Data presented are mean value ± SEM. Arg = 0.5% cholesterol chow supplemented with L-arginine; Chol = 0.5% cholesterol chow; Control = normal rabbit chow; HDL = high density lipoprotein.

number of grid points overlying the intima (or media). Area of each was calculated by the formula 0.01 (P) × grid area, where P is the number of points counted over the intima (or media). At least six cross sections from each vascular segment were analyzed and the values averaged.

Immunohistochemical analysis. Immunohistochemical analysis was performed on tissue fixed in formaldehyde and embedded in paraffin, as previously described. Antibodies against rabbit macrophage (RAM 11, Dako Corp.) and alpha-actin (Sigma Chemical Co.) were used to identify macrophage and smooth muscle cells. Sections were incubated with the primary antibody for 1 h at room temperature, anti-rabbit immunoglobulin G (biotin conjugate) for 30 min and avidin peroxidase for 20 min. Peroxidase was visualized with chromagen (Zymed Laboratories Inc.).

Regions of the arterial cross sections stained for macrophages or actin were quantified using the method of Ameli et al. (29). Measurements were made from projected photographs of the immunostained tissue. The total area occupied by macrophages or by cells staining for actin was quantified by videomicroscopy (using computer-assisted planimetry) by an observer with no knowledge of the treatment groups. Area of the vessel wall was defined by the intimal plus medial areas and the percent of the vessel stained for macrophages or actin-positive cells as determined by the previous method.

Drugs. All solutions were prepared in distilled water, made fresh the day of the experiment, and stored on ice. Norepinephrine bitartrate, acetylcholine chloride and L-arginine hydrochloride were purchased from Sigma Chemical Co. and nitroglycerin from DuPont Chemicals.

Data analysis. Results are expressed as mean value ± SEM. Dose–response curves to norepinephrine are expressed as contractions in grams above the rest tension. The dose–response curves to acetylcholine or nitroglycerin are expressed as the percent of tension attained by precontraction with norepinephrine. To analyze dose–response curves, we determined the maximal response (expressed as percent relaxation of the contraction in response to norepinephrine) and the median effective dose (expressed as −log M) for each dose–response curve. These results are given as mean value ± SEM, and comparisons between the three experimental groups were made by analysis of variance. Significance was accepted at the 95% confidence interval.

Results

Serum chemistry. At the 5- and 10-week time points, the plasma cholesterol level was markedly elevated in each of the two groups receiving the cholesterol diet compared with that in the rabbits receiving normal chow (Table 1). Dietary L-arginine did not have a significant effect on the plasma cholesterol level. Plasma HDL levels were similar in all three treatment groups. Plasma arginine levels were increased twofold in the group receiving L-arginine supplementation.

Vascular reactivity. Vasoconstriction of the noninjured vessel in response to norepinephrine was not significantly different between the three treatment groups (data not shown). Vasodilation in response to nitroglycerin was not different between the three groups (Table 2). By contrast, vasodilation in response to the endothelium-dependent vasodilator acetylcholine was attenuated in rabbits given the cholesterol diet compared with that in the group given normal chow. Arterial rings from hypercholesterolemic rabbits supplemented with L-arginine tended to have improved endothelium-dependent relaxation, although this difference from the other hypercholesterolemic group failed to achieve statistical significance

Table 2. Endothelium-Dependent and Endothelium-Independent Vasodilator Responses of Noninjured Rabbit Iliac Arteries

	EC_{50} (−log M)			Maximal Relaxation (%)*		
	Cont (n = 9)	Chol (n = 9)	Arg (n = 10)	Cont (n = 9)	Chol (n = 9)	Arg (n = 10)
Acetylcholine	7.3 ± 0.1	7.2 ± 0.1	7.0 ± 0.2	72 ± 5	32 ± 6†	46 ± 3†
Nitroglycerin	6.4 ± 0.2	6.4 ± 0.3	6.3 ± 0.3	92 ± 2	96 ± 2	97 ± 1

*Percentage of initial tension in response to norepinephrine. †$p < 0.05$ versus control (Cont). Data presented are mean value ± SEM. Other abbreviations as in Table 1.

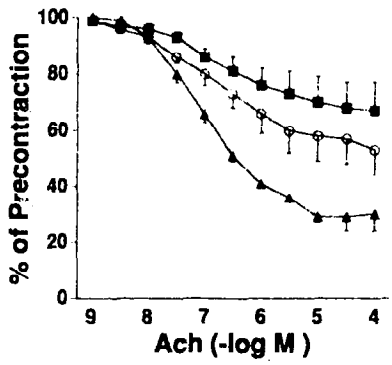

Figure 1. Endothelium-dependent relaxation of noninjured iliac artery segments. Endothelium-dependent relaxation in response to acetylcholine (Ach) is reduced in cholesterol-fed rabbits **(squares)** compared with that in rabbits fed normal chow **(triangles).** Endothelium-dependent relaxation is partially restored in the hypercholesterolemic rabbits receiving L-arginine supplementation **(circles).**

(Fig. 1). Although the results are directionally similar to our previous work, the present study may have lacked the power to detect a significant difference.

Histomorphometric analysis. There was only minor intimal thickening of the noninjured iliac arteries from rabbits given the cholesterol diet or the cholesterol diet plus L-arginine (intimal/medial [I/M] ratio 0.014 ± 0.009 vs. 0.015 ± 0.011, respectively, p = NS). Four weeks after balloon injury, the normocholesterolemic rabbits developed typical lesions of myointimal hyperplasia. In contrast, injury in the hypercholesterolemic animals produced a lesion that was quantitatively and qualitatively different. The mean intimal area after balloon injury in the hypercholesterolemic rabbits was 0.69 mm^2 versus 0.28 mm^2 for those given the normal diet ($p < 0.01$) (Table 3). The intimal thickness of the injured iliac arteries from the cholesterol-fed group was markedly increased compared with that in the injured iliac arteries from the normocholesterolemic group (I/M ratios 1.67 ± 0.36 vs. 0.90 ± 0.24, $p < 0.01$).

In the 10 hypercholesterolemic rabbits supplemented with L-arginine, intimal lesion formation was significantly reduced compared with that in the 9 hypercholesterolemic animals given vehicle, (0.34 vs. 0.69 mm^2, $p < 0.01$) and was not significantly different from that of injured vessels from the normocholesterolemic rabbits (Table 3). The mean I/M ratio of injured arteries from the group given arginine supplementation was also reduced compared with that in the vehicle-treated group (0.92 ± 0.25 vs. 1.67 ± 0.36, respectively; $p < 0.01$) (Fig. 1).

Table 3. Histomorphometric Measurements of Injured Iliac Arteries

Diet	Intima (mm^2)	Media (mm^2)	I/M Ratio
Control (n = 9)	0.28 ± 0.09	0.32 ± 0.05	0.90 ± 0.24
Chol (n = 9)	0.69 ± 0.12*	0.46 ± 0.04*	1.67 ± 0.36*
Arg (n = 10)	0.34 ± 0.08†	0.42 ± 0.03*	0.92 ± 0.26†

*Significantly different from Control group. †Significantly different from Chol group. Data presented are mean value ± SEM. I/M = intimal/medial; other abbreviations as in Table 1.

Immunohistochemical analysis. The injured iliac arteries from normocholesterolemic animals manifested intimal lesions that were composed primarily of smooth muscle cells as demonstrated by staining for alpha-actin, with only rare staining for macrophages.

Noninjured iliac arteries from rabbits given the 0.5% cholesterol diet displayed minimal thickening largely due to intimal macrophage infiltration. In the contralateral iliac arteries that had undergone injury, a marked increase in intimal thickening was noted. These lesions were composed of vascular smooth muscle cells and macrophages. There was also intense macrophage infiltration of both the media and adventitia (Fig. 2, top). An analysis of the cross-sectional area of macrophage staining in relation to total vessel area revealed that 28% of the vessel wall stained for macrophage versus only 2.9% in the lesions of injured vessels from normocholesterolemic rabbits (Fig. 3).

The addition of L-arginine to the cholesterol diet significantly reduced macrophage accumulation in the injured iliac arteries. In hypercholesterolemic rabbits given arginine, there was a sixfold reduction in the percentage of the lesion that was composed of macrophages (5% vs. 28%, $p < 0.001$) (Fig. 3). The macrophages were largely confined to the intima, in contrast to the generalized involvement of the vessel wall observed in lesions from injured arteries of hypercholesterolemic animals given vehicle (Fig. 2, bottom).

Discussion

Salient findings. The salient findings of this investigation were as follows:

1. Vascular injury in the setting of hypercholesterolemia produces a lesion that is quantitatively and qualitatively different from that observed in normocholesterolemic rabbits. These lesions are more exuberant and are composed of intimal vascular smooth muscle cells as well as macrophages infiltrating the entire thickness of the vessel wall;

2. To our knowledge, we report for the first time that the lesions induced by *vascular injury in hypercholesterolemic rabbits* are markedly reduced by oral administration of arginine. Moreover, we find that the nature of the lesion is altered, with a striking reduction in the percentage of macrophages comprising the lesion.

3. We speculate that this effect of arginine is mediated by its metabolism to NO. We and other investigators (19–21) have shown that arginine administration enhances vascular NO activity, reduces endothelial adhesiveness for monocytes and inhibits intimal lesion formation in hypercholesterolemic rabbits. We suspect that a similar mechanism is operative in the present study, although we did not measure NO activity in the injured vessels.

Vascular injury: effects on structure and function. The response to balloon injury in both normocholesterolemic and hypercholesterolemic states has been previously described

Figure 2. Photomicrographs of representative cross sections of iliac arteries obtained 4 weeks after balloon injury. Sections were stained for macrophage with a monoclonal antibody to rabbit macrophage (RAM-11). **Top,** cholesterol-fed rabbit. Both intimal and medial thickening are observed, with foam cell infiltration. There is diffuse staining for macrophage. **Bottom,** cholesterol-fed rabbit supplemented with L-arginine. There is a reduction in intimal lesion, with a marked reduction of staining against macrophage.

(9–11). In the absence of mechanical injury, long-term administration of a hypercholesterolemic diet produces an arterial lesion that is characterized by lipid-laden macrophages, or "foam cells." By contrast, the lesion produced by vascular injury in normocholesterolemic rabbits is composed primarily of smooth muscle cells and a connective tissue matrix. In these vessels, the denuded endothelial layer eventually regenerates, but the endothelial cells may be irregularly sized, polygonal and not aligned with flow. When vascular injury occurs in the setting of hypercholesterolemia, a quantitatively and qualitatively different lesion is observed. This combination of vascular insults produces an exuberant myointimal lesion that is characterized by an intense infiltration of mononuclear cells, largely macrophages.

These alterations in vascular structure are associated with changes in vascular function. Even after the endothelial monolayer has regenerated weeks after a vascular injury, endothelium-dependent relaxation is attenuated (9,10). This reduction in endothelium-derived NO activity that occurs after vascular injury is exacerbated by hyperlipidemia (11).

These endothelium-dependent relaxations are largely due to NO, which is formed during the metabolic conversion of L-arginine to L-citrulline by NO synthase (30,31). The activity of NO is reduced in hypercholesterolemic animals and humans and is manifested by impaired endothelium-dependent vasodilation (32–36). This endothelial vasodilator dysfunction is rapidly reversed by intravenous administration of L-arginine (but not D-arginine, which cannot be utilized by NO synthase) (12,18). The reversal of endothelial vasodilator dysfunction by exogenous arginine is paradoxic in that the Michaelis constant (Km) of NO synthase is in the micromolar range, whereas plasma arginine levels are in the 0.1 mmol/liter range (and intracellular levels are even higher). An explanation for this paradox may have been provided by recent findings (37) that

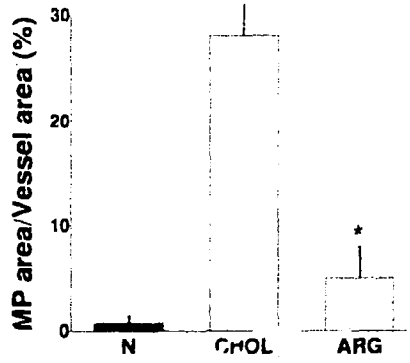

Figure 3. Macrophage (MP) content in iliac arteries 4 weeks after balloon injury. Data are expressed as a percent of the vessel (intimal plus medial areas) that contains macrophages (mean area ± SEM). Balloon injury in hypercholesterolemic rabbits (CHOL) results in a marked increase in arterial macrophage content compared with that in injured iliac arteries from rabbits fed normal chow (N). Macrophage content in iliac arteries from hypercholesterolemic rabbits receiving L-arginine (ARG) is significantly reduced compared with that in the CHOL group. *$p < 0.01$, ARG group versus N group.

the plasma level of the endogenous inhibitor of NO synthase, asymmetric dimethyl arginine, is elevated in hypercholesterolemic rabbits.

In addition to its vasodilator properties, NO is known to inhibit a variety of processes that are involved in atherogenesis, including platelet adherence and aggregation, vascular smooth muscle proliferation and monocyte adherence (13–17). Evidence from our laboratory and others (38–40) indicates that NO downregulates the endothelial expression of monocyte chemotactic protein (MCP-1) and vascular cell adhesion molecule (VCAM), which play critical roles in monocyte–endothelial cell interaction. Nitric oxide may exert these effects in part by inhibiting the activation of oxidant-responsive transcriptional pathways that mediate the expression of adhesion molecules and chemokines (38–42). Therefore, it seems plausible that the impairment of vascular NO activity observed in hypercholesterolemia could promote atherogenesis through a mechanism in which monocyte–endothelial cell interactions are increased. Restoration of NO activity would then be predicted to inhibit atherogenesis.

Arginine restores NO activity. Indeed, in hypercholesterolemic rabbits, arginine administration enhances NO elaboration (as documented by bioassay and chemiluminescence), reduces endothelial adhesiveness for monocytes and inhibits accumulation of monocytes in the vessel wall (19–21). By contrast, inhibition of endothelial NO synthase augments endothelial adhesiveness for monocytes and accelerates atherogenesis (19,43).

The results of the present study are concordant with this hypothesis. Indeed, administration of L-arginine reduced macrophage accumulation in the injured vessel wall by 80% in hypercholesterolemic rabbits. It is possible that the salutary effects of arginine in this study were in part mediated by NO synthase expressed by vascular cells in the media. Normally, NO synthase is not expressed by vascular smooth muscle cells. However, after balloon injury in animal models, NO synthase is induced locally in the vascular smooth muscle, as measured by bioassay and reverse transcription-polymerase chain reaction (44,45). The production of NO by vascular smooth muscle not only affects its contractility, but appears to suppress its proliferation. Earlier observations by Garg and Hassid (18) revealed that NO donors suppressed the proliferation of vascular smooth muscle cells in culture. This effect of NO donors may be due in part to elevations in intracellular cyclic guanosine monophosphate (cGMP) because analogues of cGMP, as well as other agonists of this pathway (i.e., atrial natriuretic peptide) have similar effects.

Dietary arginine suppresses neointimal formation in the balloon-injured iliac artery of *normocholesterolemic* New Zealand White rabbits (22–24). It is not known whether this effect is due to an enhancement of the NO synthase pathway in the regenerating endothelium or vascular smooth muscle, or both. Recently, we used a more direct method to determine whether activation of the NO synthase pathway in vascular smooth muscle cells could inhibit their proliferation in vivo after balloon injury (46). The common carotid artery of rats was subjected to balloon injury, then transfected with a control vector or a plasmid construct containing the complementary DNA for the constitutive isoform of NO synthase. Carotid arteries transfected with the NO synthase construct expressed significantly more NO synthase and elaborated nearly normal amounts of NO. The increased expression and activity of NO synthase in the vessel wall was associated with a 75% reduction in intimal thickening 2 weeks after the injury.

Conclusions. We demonstrated that in the setting of hypercholesterolemia, vascular injury produces an exuberant lesion composed of macrophages (which infiltrate the entire thickness of the vessel wall), as well as vascular smooth muscle cells. Dietary supplementation with L-arginine quantitatively and qualitatively alters the lesion, with a marked reduction in infiltrating macrophages. The effect of L-arginine is most likely mediated by its metabolism to nitric oxide, which is an antagonist of vascular smooth muscle cell proliferation and a potent inhibitor of adhesive interactions of circulating blood elements with the vessel wall.

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