Regression of Atherosclerosis
Role of Nitric Oxide and Apoptosis

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Background—We have recently found that administration of l-arginine to hypercholesterolemic rabbits induces regression of preexisting lesions. Others have previously shown that activation of the l-arginine/nitric oxide (NO) synthase pathway can induce apoptosis of vascular cells in vitro. Accordingly, the current study was designed to determine if dietary supplementation of l-arginine induces apoptosis of intimal lesions and if this effect is mediated through the NO synthase pathway.

Methods and Results—Male New Zealand White rabbits were fed a 0.5% cholesterol diet for 10 weeks and subsequently placed on 2.5% l-arginine HCl in the drinking water, and the cholesterol diet was continued for 2 weeks, at which time the aortas were harvested for histological studies. l-Arginine treatment increased the number of apoptotic cells (largely macrophages) in the intimal lesions by 3-fold (11.9±3.9 vs 3.9±1.4 apoptotic cells/mm², P<0.01). In subsequent studies, aortas were harvested for ex vivo studies. Aortic segments were incubated in cell culture medium for 4 to 24 hours with modulators of the NO synthase pathway. The tissues were then collected for histological studies and the conditioned medium collected for measurement of nitrogen oxides by chemiluminescence. Addition of sodium nitroprusside (10⁻⁵ mol/L) to the medium caused a time-dependent increase in apoptosis of vascular cells (largely macrophages) in the intimal lesion. l-Arginine (10⁻³ mol/L) had an identical effect on apoptosis, which was associated with an increase in nitrogen oxides released into the medium. These effects were not mimicked by d-arginine, and they were antagonized by the NO synthase inhibitor l-nitro-arginine (10⁻⁴ mol/L). The effect of l-arginine was not influenced by an antagonist of cGMP-dependent protein kinase, nor was the effect mimicked by the agonist of protein kinase G or 8-BR cGMP.

Conclusions—These results indicate that supplemental l-arginine induces apoptosis of macrophages in intimal lesions by its metabolism to NO, which acts through a cGMP-independent pathway. These studies are consistent with our previous observation that supplementation of dietary arginine induces regression of atheroma in this animal model. These studies provide a rationale for further investigation of the therapeutic potential of manipulating the NO synthase pathway in atherosclerosis. (Circulation. 1999;99:1236-1241.)

Key Words: apoptosis ▪ cells ▪ plaque ▪ nitric oxide ▪ angioplasty

Apoptosis is a form of actively regulated cell death. Apoptosis is initiated by specific stimuli that trigger signaling pathways leading to endonuclease activation and DNA fragmentation. Characteristic morphological features of apoptosis include nuclear condensation, cytoplasmic blebbing, and phagocytosis of the apoptotic bodies by the neighboring cells.1,2 Programmed cell death is required during embryogenesis for those structures fated to remodel or regress.3–5 Apoptosis continues to play a role in remodeling of tissues throughout life. It is therefore conceivable that apoptosis contributes to changes in vascular structure that occur in response to humoral factors or hemodynamic forces.6–8 Indeed, apoptosis of vascular cells has been observed in atherosclerotic plaque.2,9–13 The regulation of apoptosis in atherosclerosis has not been fully characterized. Although in vitro studies indicate that macrophages and vascular smooth muscle cells may undergo apoptosis through p53-dependent and p53-independent pathways,14,15 we hypothesize that vascular nitric oxide (NO) synthase may be another determinant of apoptosis in plaque cells. The enzyme is induced in vascular cells of atherosclerotic plaque and its activity results in high local concentrations of NO. Furthermore, NO is known to trigger apoptosis of vascular cells in vitro.15,16 We have recently shown that administration of l-arginine to hypercholesterolemic rabbits can increase vascular NO synthesis, which is associated with an apparent regression of
preexisting intimal lesions. Specifically, New Zealand White (NZW) rabbits were fed a 0.5% cholesterol diet for 10 weeks, at which time they exhibited intimal lesions occupying ~30% of the surface area of the thoracic aorta. Subsequently, animals received L-arginine (2.25%) or vehicle in their drinking water while the 0.5% cholesterol diet was continued. Thoracic aortas were harvested at subsequent weeks for vascular reactivity studies and histomorphometry. In those animals receiving vehicle, there was a progressive impairment of endothelium-dependent vasodilation and an increase in lesion surface area (to 60% of the thoracic aorta) over the course of weeks 10 to 23. By contrast, in the L-arginine–treated animal, there was an improvement in endothelium-dependent vasodilation and significant reduction in lesion surface area. Indeed, after 23 weeks, L-arginine–treated animals, with persistent improvement in endothelium-dependent NO-mediated vasodilation, exhibited a lesion surface area of only 5%. This previous study suggested to us that enhancement of vascular NO activity could induce regression of preexisting intimal lesions. We hypothesized that NO-induced regression may be mediated by apoptosis of cells in the intimal lesion. Accordingly, the present study was designed to determine if NO plays a significant role in apoptosis of vascular cells in vivo and to further determine if NO-induced apoptosis can be modulated.

Methods

Protocol I: Effect of Dietary L-Arginine on Apoptosis

Animals
Male NZW rabbits (n=14) were placed on a 0.5% cholesterol diet for 12 weeks. During weeks 10 to 12, the animals were randomized to 2.25% L-arginine HCl in the drinking water or water alone. Animals were killed at week 12 with an overdose of sodium pentobarbital (50 mg/kg), and the aortas were harvested and placed in 10% buffered formalin immediately. At this time, there is no significant difference in intimal area between the thoracic aortas of the vehicle-treated and L-arginine–treated animals (evidence of significant difference in intimal area between the thoracic aortas of the vehicle-treated and L-arginine–treated animals (evidence of regression is not seen until the fourth week of L-arginine supplementation)."

Histomorphometric Studies
The formalin-fixed vessels were embedded in paraffin, sectioned (5 μm thick), and stained with hematoxylin and eosin for light microscopy and histomorphometry. Assessments of intimal and medial cross-sectional areas were made with light microscopy at a magnification of ×40 and the cross-sectional areas digitized with an Image Analyst program (Automatrix)."

Quantitation of Apoptosis
The formalin-fixed sections were deparaffinized, hydrated through xylene and graded alcohol series, and stained for 20 minutes with Hoechst 33342 (Molecular Probes; 5 μg/mL). Immediately thereafter, the nuclear morphology was observed by fluorescence microscopy under ultraviolet light. We have developed rigorous criteria by using the Hoechst technique to quantitate apoptotic nuclei to determine an apoptotic index. A brightly staining, condensed nucleus with fragmentation into apoptotic bodies is a required criterion for a cell to be considered apoptotic. The apoptotic nuclei in the intimal lesions were counted in 5 high-power fields for each of 3 cross sections per vascular segment. These values were averaged and expressed as the number of apoptotic nuclei/mm2 intimal area. This technique for quantitation of apoptosis has been validated in vitro with time lapse videomicroscopy. The results with this technique are consistent with TUNEL staining and DNA fragmentation assessed by gel electrophoresis.

Protocol II: Development of an Ex Vivo Model of Apoptosis
The findings in protocol I indicated that dietary L-arginine increased apoptosis of cells in the intimal lesion in vivo. To elucidate the mechanisms of this phenomenon, the following protocol was performed to establish the utility and validity of an ex vivo model. Male NZW rabbits (n=5) received 0.5% cholesterol diet for 6 weeks. In the third week, balloon angioplasty of the abdominal aorta was performed. The rabbits were anesthetized with a mixture of ketamine (5 mg/kg) and xylazine (35 mg/kg) intravenously. The right carotid artery was exposed, and a coronary angioplasty catheter (Advanced Cardiovascular Systems) was passed over a steerable guide wire (0.014 in.) into the abdominal aorta. Beginning at the bifurcation of the abdominal aorta, the contrast-filled balloon was inflated to 10 atm and withdrawn to the infrarenal abdominal aorta. This procedure was performed 3 times. Subsequently, the animals were allowed to recover from anesthesia and the 0.5% cholesterol diet was continued for 4 more weeks, at which time the aortas were harvested.

The combination of hypercholesterolemia and vascular injury creates a lesion that is composed of macrophages as well as vascular smooth muscle cells, unlike in protocol I, in which the lesion is composed almost exclusively of macrophages. However, this model reproducibly produces an atheromatous lesion in the abdominal aorta that is characterized by an intense infiltration of macrophages, thereby facilitating ex vivo studies of lesional apoptosis.

Cytokine-induced activation of inducible NO synthase (iNOS) or NO donors is known to induce apoptosis in cultured vascular smooth muscle cells and macrophages. The following protocol was designed to determine if we could reproduce this phenomenon in an ex vivo model; to determine if we could reproduce the effects of supplemental arginine in an ex vivo model; and to determine the role of endogenous NO in apoptosis.

After 6 weeks, the abdominal aortas were harvested, divided into segments (3 mm in length), and incubated for 2 to 24 hours at 37°C in DMEM-F12 medium plus (a) vehicle (control), (b) sodium nitroprusside (SNP, a nitric oxide donor, 10−3 mol/L), (c) L-nitroarginine (10−4 mol/L, an NOS antagonist), or (d) L-arginine (10−3 mol/L). The media were replaced with fresh media and drugs every 4 hours for 48 hours. After incubation, the vessel segments were fixed and stained as described above for assessment of apoptosis.

Protocol III: Is the Effect of L-Arginine Mediated by NOS?

The previous studies revealed that the ex vivo model reproduced the in vivo observation that supplemental L-arginine enhances apoptosis in the intimal lesion. The following study was performed to determine if the effect of L-arginine requires its metabolism by NOS and to determine if cGMP-dependent or cGMP-independent pathways were involved.

Six male NZW rabbits were exposed to the balloon injury and dietary intervention described above in protocol II and the aortas harvested for ex vivo studies. The aortas were divided into segments and incubated in DMEM-F12 medium plus (a) vehicle (control), (b) L-arginine (10−3 mol/L), (c) d-arginine (10−3 mol/L), or (d) L-arginine (10−3 mol/L) + L-nitroarginine (10−4 mol/L), (e) 8-Br-cAMP (10−3 mol/L) or 8-Br-cGMP (10−3 mol/L), (f) Rp8-pCPT-cGMP (200 μmol/L, an inhibitor of cGMP-dependent protein kinase Ia), or (g) Sp8-pCPT-cGMP (500 μmol/L; an agonist of cGMP-dependent protein kinase Ia). Every 4 to 6 hours the conditioned medium was collected for NO measurement and replaced with fresh media containing the appropriate agents. The aortic segments were collected after 24 hours of incubation and prepared for histological examination as described above.
Regression and Apoptosis

Immunohistochemistry
Immunohistochemical analysis was performed on tissue fixed in formalin and embedded in paraffin as described above. Mouse anti-rabbit antibodies against macrophages (RAM 11, Dako Corp) or α-actin (Sigma Chemical Co) were used to identify macrophages or smooth muscle cells, respectively. Sections were incubated with the primary antibody for 1 hour at room temperature, goat anti-mouse IgG (biotin conjugate) for 30 minutes, and avidin peroxidase for 20 minutes. Peroxidase was visualized with Chromagen. In some studies, formalin-fixed and paraffin-embedded tissue sections were first stained for macrophages and then stained with Hoechst 33342 (as described above) to observe the apoptotic bodies and macrophages in the same cross section.

Chemiluminescence
In some experiments, the aortic segments were prepared for ex vivo study as described above and incubated with 2 mL HBSS medium containing calcium ionophore (1 μmol/L; Sigma) and L-arginine (100 μmol/L; Sigma). At selected time points (0, 30, 60, and 120 minutes), samples of the medium (100 μL) were collected for measurement of nitrogen oxides (NO and 1-electron oxidation products of NO). Nitrogen oxides (NO2 and NO3) were measured with a commercially available chemiluminescence apparatus (model 2108, Dasibi) after reduction of the samples in boiling acidic vanadium (III) at 98°C. Boiling acidic vanadium quantitatively reduces NO2 to NO, which is quantified by the chemiluminescence detector after reaction with ozone. Signals from the detector were analyzed by a computerized integrator and recorded as areas under the curve. Standard curves for NO2 and NO3 were linear over the range of 100 pmol to 5 nmol.

Data Analysis
Data are expressed as mean±SEM. The comparisons were made by a multivariate ANOVA of independent groups to determine the overall difference, followed by a post hoc Fisher test to determine statistical significance between groups; comparisons between the 2 experimental groups were made by Student’s t test (2-tailed); statistical significance was accepted at the 95% significance level (P<0.05). 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 Accreditation of Laboratory Animal Care.

Results
Effect of Dietary L-Arginine on Apoptosis
Histological studies revealed intimal lesions in the thoracic aortas from hypercholesterolemic rabbits that were largely caused by accumulation of macrophages. Fluorescent microscopic examination after Hoechst staining revealed apoptotic nuclei within the lesion (3.9±1.4 apoptotic nuclei/mm2). In the hypercholesterolemic rabbits that received L-arginine supplementation for 2 weeks, there was a 3-fold increase in the number of apoptotic nuclei observed in the intima (11.9±2.1 apoptotic nuclei/mm2; P<0.01 compared with thoracic aortas from vehicle-treated hypercholesterolemic animals).

Ex Vivo Model of NO-Induced Apoptosis
Segments of abdominal aorta from hypercholesterolemic rabbits that had undergone aortic angioplasty (see “Methods”) were placed into organ culture. Segments were prepared for fluorescent microscopy and quantitation of apoptosis 0 to 48 hours after organ culture. At time 0 (immediately after death), a small percentage of the cells were undergoing apoptosis, as demonstrated by Hoechst staining. Over a period of 48 hours in tissue culture, there was a slight increase in the number of apoptotic nuclei/mm2.

Is the Effect of Arginine Mediated by NOS?
In a subsequent study, the vascular segments were treated in organ culture with vehicle; L-arginine (10⁻³ mol/L); D-arginine (10⁻³ mol/L); or L-arginine (10⁻³ mol/L) + L-nitro-arginine (10⁻⁴ mol/L) for 24 hours. The vessels were prepared for histological studies, and the conditioned medium in proliferating cells in the intimal lesions (Figure 1). Addition of SNP (10⁻⁵ mol/L) to the medium markedly increased the number of apoptotic cells in the intimal lesions. L-Arginine (10⁻³ mol/L) had an identical effect (Figure 1). Addition of d-arginine (10⁻³ mol/L) to the medium had no effect on the basal rate of apoptosis.

Figure 1. Quantitation of apoptotic nuclei in intimal lesions of vascular segments incubated in medium containing modulators of the NOS pathway. Under control (NC) conditions, there is a time-dependent increase in the number of apoptotic nuclei observed in the intimal lesion. Compared with control conditions, in the presence of SNP (10⁻⁵ mol/L), there was a striking increase in the number of apoptotic nuclei. This effect was mimicked by L-arginine (ARG, 1 mmol/L). By contrast, after treatment with L-nitro-arginine (NNA, 10⁻⁴ mol/L), the number of apoptotic nuclei was not different from control conditions.
was collected for measurement of NOx at 4-hour intervals. The addition of L-arginine to the medium enhanced the generation of NOx. This effect of L-arginine to enhance NO synthesis was not mimicked by D-arginine and was blocked by L-nitro-arginine (Figure 2). Fluorescent microscopy of intimal sections from the vascular segments revealed that the number of apoptotic cells in the intimal lesions was increased by the addition of L-arginine to the medium. This effect was not mimicked by D-arginine and was blocked by L-nitro-arginine (Figure 3). In parallel studies vascular segments were exposed to 8-Br-cGMP (the stable analogue of cGMP); 8-Br-cAMP (the stable analogue of cAMP); Rp8-pCPT-cGMP (the antagonist of cGMP-dependent protein kinase Ia); or Sp8-pCPT-cGMP (activator of cGMP-dependent protein kinase Ia). These agents had no effect on the basal rate of apoptosis in the intimal lesion (Figure 3), suggesting that the effect of NO to induce apoptosis may be cGMP independent. (See Table).

**Discussion**

The salient findings of the study are (1) Supplementation of L-arginine increases apoptosis of vascular cells in the intimal lesions of hypercholesterolemic rabbits. (2) The effect of L-arginine to enhance NO synthesis was not mimicked by D-arginine and was blocked by L-nitro-arginine (Figure 2). (3) Exogenous NO donors or endogenous NO largely modulates apoptosis of macrophages, with little observable effect on vascular smooth muscle cells in the intimal lesion. These studies shed light on our previous observation that dietary L-arginine induces regression of atherosclerosis in an animal model of atherosclerosis. Notably, the current study provides in vivo evidence that apoptosis can be manipulated to reverse atherogenesis.

Apoptosis has been reported to occur in vascular cells of human atherosclerotic plaque. Factors involved in the initiation and regulation of apoptosis in atherosclerosis have not been fully elucidated, but immunohistochemical studies provide evidence for several proteins known to participate in apoptosis, including p53 and interleukin-1β–converting enzyme. Among the myriad pathways that may be involved, there is accumulating evidence to implicate L-arginine/NOS. Cytokine-mediated activation of iNOS induces apoptosis of macrophages and vascular smooth muscle cells in vitro. The effect of iNOS activation in vitro is augmented by additional L-arginine and attenuated by antagonists of NOS. In the present study, L-arginine–enhanced apoptosis was associated with increases in the level of NO released into the medium. The involvement of the NOS pathway was also indicated by the observation that the effect of L-arginine was blocked by L-nitro-arginine. In this study, it is likely that iNOS expressed by cells within the lesion is responsible for the effect of L-arginine. Indeed, previous immunohistochemical studies have detected iNOS in the intimal macrophages and vascular smooth muscle cells of human atherosclerotic plaque. Under these conditions, vascular cells also produce superoxide anion. In this milieu, the product of iNOS is quickly transformed into peroxynitrite anion, a highly reactive free radical.

![Figure 2](image-url) Measurement by chemiluminescence of NOx in conditioned medium. There was a time-dependent increase in the accumulation of NOx in conditioned medium from vascular segments. Elaboration of NOx was increased by L-arginine (L-Arg, 1 mmol/L). This effect was not mimicked by D-arginine (D-Arg, 1 mmol/L).

![Figure 3](image-url) Histogram illustrating effects of NOS modulation on apoptosis. Vascular segments were incubated with L-arginine (L-Arg, 1 mmol/L), D-arginine (D-Arg, 1 mmol/L), or L-nitro-arginine (10^(-4) mol/L) plus L-arginine (1 mmol/L); NNA plus L-Arg; 8-Br cGMP; 8-Br cAMP; or vehicle (C) for 24 hours and then segments prepared for quantitation of apoptotic nuclei. Cyclic nucleotides had no effect on the number of apoptotic nuclei. Effect of L-arginine to enhance apoptosis was not mimicked by D-arginine and was blocked by L-nitro-arginine.
Peroxynitrite anion is cytotoxic and may induce apoptosis initially by causing DNA strand fragmentation. Peroxynitrite anion can also affect cell function by nitrosating tyrosine residues that are involved in the signal transduction of transmembrane receptors. Using monoclonal antibodies directed against nitrotyrosine, evidence of peroxynitrite formation has been observed in human atherosclerotic plaque. This is relevant to the present study because peroxynitrite anion may be the NO species mediating the effects that we observed.

Previous studies have suggested that apoptosis of macrophages induced by iNOS activity is independent of cGMP. Consistent with this observation is our finding that the various manipulations of the cGMP pathway did not influence apoptosis in the lesions of this animal model. By contrast, in cultured vascular smooth muscle cells, NO donors induce apoptosis by a cGMP-dependent mechanism. The activation of iNOS may have complex effects on the evolution of atherosclerotic plaque. By inducing cell death, iNOS activation may contribute to the development of the "necrotic core" of complex lesions. One might also speculate that iNOS may be involved in the characteristic atrophy of the media beneath atheroma or the dissolution of the fibrous cap by activated macrophages. NO or peroxynitrite anion produced by these activated macrophages could induce apoptosis of vascular smooth muscle. Furthermore, NO or peroxynitrite anion may reduce collagen formation by vascular cells and activate metalloproteinases, which degrade extracellular matrix. These actions of peroxynitrite anion would contribute to plaque instability and have led some to explore antagonism of iNOS as a potential therapeutic avenue. However, it is likely that such a strategy would have unintended consequences. Antagonism of iNOS activity could promote platelet aggregation, leukocyte adherence, vasoconstriction, and proliferation of vascular smooth muscle cells and macrophages. It should be considered that iNOS may be a factor in countervailing forces in the accretion of atherosclerotic plaque. Furthermore, by reducing proliferation and by promoting apoptosis of macrophages in the lesion, iNOS activation may lead to plaque stabilization and even regression, as suggested by the present study. It is worthy of emphasis that both macrophages and vascular smooth muscle cells contribute to the intimal lesion in the balloon-injured hypercholesterolemic rabbit, but in this study apoptosis was largely observed in the central, macrophage-rich area of the lesions.

To conclude, the present study provides evidence that supplementation of dietary l-arginine promotes apoptosis of cells in the atheromatous lesion. This effect of l-arginine is mediated by NOS in a cGMP-independent fashion. The effect of l-arginine to increase apoptosis of intimal cells may explain our previous observation that dietary supplementation of l-arginine induces regression of atheroma.

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