Reduced Endothelial Nitric Oxide Synthase Expression and Production in Human Atherosclerosis

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Background—NO regulates vascular tone and structure, platelets, and monocytes. NO is synthesized by endothelial NO synthase (eNOS). Endothelial dysfunction occurs in atherosclerosis.

Methods and Results—With a porphyrinic microsensor, NO release was measured in atherosclerotic human carotid arteries and normal mammary arteries obtained during surgery. eNOS protein expression was analyzed by immunohistochemistry. In normal arteries, the initial rate of NO release after stimulation with calcium ionophore A23187 (10 μmol/L) was 0.42±0.05 (μmol/L)/s (n=10). In contrast, the initial rate of NO release was markedly reduced in atherosclerotic segments, to 0.08±0.04 (μmol/L)/s (n=10, P<0.0001). NO peak concentration in normal arteries was 0.9±0.09 μmol/L (n=10) and in atherosclerotic segments, 0.1±0.03 μmol/L (n=10, P<0.0001). Reduced NO release in atherosclerotic segments was accompanied by marked reduction of immunoreactive eNOS in luminal endothelial cells, although specific endothelial cell markers (CD31) were present (n=13). Endothelial cells of vasa vorum of atherosclerotic segments, however, remained positive for eNOS, as was the endothelium of normal arteries.

Conclusions—In clinically relevant human atherosclerosis, eNOS protein expression and NO release are markedly reduced. This may be involved in the progression of atherosclerosis. (Circulation. 1998;97:2494-2498.)

Key Words: atherosclerosis ■ arteries ■ endothelium ■ nitric oxide ■ stroke

Atherosclerosis accounts for half of the morbidity and mortality in Western countries. Its pathogenesis, however, is not clear. Risk factors of atherosclerosis, ie, LDL levels, diabetes mellitus, hypertension, and smoking, are associated with vascular dysfunction, including monocyte adhesion and invasion, smooth muscle proliferation and migration, platelet activation, and extracellular matrix formation.12 In animal models, the endothelial NO pathway appears to be involved in atherosclerosis.3-6 However, little is known about this in human atherosclerosis.7

In endothelial cells, NO is formed from L-arginine8,9 by eNOS.10 eNOS is constitutively expressed and activated by cell surface receptors or mechanical forces such as shear stress and stretch.1111 NO relaxes vascular smooth muscle, inhibits platelet activation, and modulates migration and growth of vascular smooth muscle. Furthermore, NO regulates genes that lead to the expression of adhesion molecules for monocytes.12-14

Indirect evidence suggests that alterations in the NO pathway might be involved in endothelial dysfunction and atherosclerosis. In hypercholesterolemia and atherosclerosis, endothelium-dependent relaxation is reduced.3-5 Chronic administration of L-arginine to hypercholesterolemic animals improves endothelium-dependent relaxation.9 In patients with coronary artery disease, basal NO release seems to be impaired, as suggested by a blunted response to L-NMMA.7 However, in human atherosclerosis, no direct measurement of NO release or NOS protein has been reported.

This study presents the first direct evidence that NO release and eNOS expression are markedly reduced in clinically manifest human atherosclerosis.

Methods

Blood Vessels

Thirty-five arteries of patients undergoing carotid atherectomy or coronary bypass surgery were collected. For NO measurement, carotid arteries from 10 patients (mean age, 66 years; range, 53 to 78 years) and IMAs (normal segments) from 10 age-matched control subjects (mean age, 66 years; range, 54 to 75 years; P=NS) were used. The presence or absence of atherosclerosis was confirmed visually, microscopically, and by histology. The absence of atherosclerosis in IMAs is in line with previous studies.15 Immunohistochemistry for eNOS and CD31 was performed in 13 carotid arteries and 2 IMAs.

Porphyrinic NO Microsensor

Measurements of NO were carried out with a porphyrinic microsensor as described16-17 immediately after surgical removal of segments (<30 minutes). The amperometric signal was recorded with a chart recorder (Recom Electronic AG), and NO concentration was determined from the measured current by means of a calibration curve.
Experimental Protocols
Isolated vascular segments were cut longitudinally and pinned on the bottom of organ chambers filled with fresh HBSS buffer. The active tip of the L-shaped NO microsensor was placed on the endothelial surface of the IMA or carotid strips. A precision stereo zoom microscope (PZM) and a micromanipulator (M3301) were used for positioning. Ten microliters of a 10 μmol/L calcium ionophore A23187 solution was injected on the luminal side of the vascular strips with a pneumatic picoinjector (PV820) positioned with a micromanipulator (PZM, PV820, and M3301 are all from World Precision Instruments). In each tissue sample, NO release was measured at 3 different anatomic sites.

Immunohistochemistry
Serial paraformaldehyde-fixed paraffin sections were mounted onto silane-coated slides, dewaxed and rehydrated, washed in PBS, and incubated in 1 μg/mL anti-eNOS monoclonal antibodies (Transduction Laboratories; 2 hours at room temperature or overnight at 4°C). Adjacent sections were incubated in a 1:100 dilution of primary monoclonal antibodies for CD-31 (J7/7A; Dako) or preimmune control serum for 2 hours at room temperature, washed in PBS for 10 minutes, incubated in biotinylated secondary antibody for 30 minutes at room temperature, and visualized by use of the avidin-biotin-peroxidase labeling system (ABC-elite kit; Vector Laboratories). Sections were counterstained with hematoxylin and mounted with Kaiser’s solution (Merck).

Calculations and Statistical Analysis
For statistical analysis, the initial rate of NO release [slope of NO peak, (μmol/L/s)] and the maximal concentration of NO produced (NO peak; μmol/L) were measured. In each tissue sample, NO release was measured at 3 different anatomic sites, and the mean value was calculated for n=1. In each set of experiments, n is the number of blood vessels studied. Data are given as mean±SEM. Statistical analysis was performed with unpaired Student’s t test. Values of P<0.05 were considered statistically significant.

Results

NO Release
Representative amperometric curves of NO release are shown in Figure 1A. In normal segments, a rapid release of NO was observed after injection of 10 μmol/L calcium ionophore A23187 solution (Figure 1A, top). In contrast, the initial rate of NO release and NO peak concentration were significantly reduced in atherosclerotic carotid arteries (Figure 1A, bottom). The initial rate of NO release after calcium ionophore administration was 0.42±0.05 (μmol/L/s) in normal arteries but only 0.08±0.04 (μmol/L/s) in atherosclerotic carotid arteries (Figure 1B, left; n=10, P<0.0001). NO peak concentration was 0.9±0.09 μmol/L in normal arteries but was reduced to 0.1±0.03 μmol/L in atherosclerotic arteries (Figure 1B, right; n=10, P<0.0001).

Expression of eNOS
Labeling of normal arterial segments with monoclonal antibody for eNOS revealed high-level expression of eNOS in luminal endothelial cells (Figure 2A). In contrast, immunoreactive eNOS was undetectable in luminal endothelial cells of carotid segments with advanced atherosclerosis (Figure 3A). However, endothelial cells of the vasa vasorum inside the atherosclerotic plaques were strongly positive for eNOS staining (Figure 4A and 4C), indicating that the negative staining in luminal endothelial cells was not due to technical failure but rather reflects downregulation of the enzyme. Smooth muscle cells of normal segments and of atherosclerotic carotid arteries as well as foam cells and macrophages in the plaque were negative for eNOS.

Expression of Endothelial Cell Marker
Anti-CD31 staining, a specific marker for endothelial cells, was positive in all instances, indicating the presence of endothelial cells (Figures 2C, 3C, and 4D) and demonstrating that both eNOS-expressing cells in normal arterial segments and eNOS-negative cells in atherosclerotic carotid arteries were indeed endothelial cells. The negative staining with control serum demonstrated the specificity of both anti-eNOS and anti-CD31 antibodies (Figures 2B, 3B, and 4B).
Discussion

This study provides the first direct evidence that NOS protein expression and NO release are diminished in advanced human atherosclerosis. Initial and peak NO release as determined by direct in situ measurement with a porphyrinic sensor were markedly reduced in atherosclerotic carotid arteries. The impaired NO release was accompanied by a substantial reduction of immunoreactive eNOS protein in luminal endothelial cells of atherosclerotic plaques but not in vasa vasorum.

Endothelial NO plays an important role in vascular homeostasis, adhesion of white blood cells, and platelet function. There are at least 3 major mechanisms in the NO pathway that may lead to endothelial dysfunction and consequently atherogenesis: (1) functional abnormalities of NOS due to substrate or cofactor deficiency; (2) increased breakdown of NO; or (3) reduced expression of eNOS. Until now, even with invasive in vivo studies in humans, no clear distinction between these possibilities could be made. Moreover, studies of the NO pathway in rabbit models of...
Atherosclerosis gave unexpected results, ie, both eNOS protein and mRNA are increased in the atherosclerotic aorta of these animals, despite impaired endothelium-dependent vascular relaxation (most likely due to inactivation of NO by superoxide). In contrast, our results provide the first direct evidence that eNOS protein expression is markedly diminished in advanced human atherosclerosis and NO release is reduced. These findings are supported by studies in explanted vein grafts in which eNOS expression is reduced only in diseased segments. The differences in NO release observed in normal and atherosclerotic segments were mirrored by marked differences in NOS expression and are in line with previous work showing potent endothelium-dependent relaxations in normal mammary arteries and reduced responses in atherosclerotic human coronary arteries.

We confirmed the presence of endothelial cells in both normal and atherosclerotic segments by light microscopy and by endothelial cell marker (CD31). A heterogeneity in vascular beds is an unlikely explanation for the observed differences, because both mammary and carotid arteries are branches of the subclavian artery and brachiopheal trunk. Differences in NO production due to anatomic heterogeneity in receptor expression could be excluded by use of the receptor-independent agonist calcium ionophore. Moreover, the amount of NO released from normal arteries was comparable to that of other normal vessels. Finally, it should be noted that IMA samples were obtained from age-matched patients with documented atherosclerosis in the coronary circulation and angiographically normal mammary artery.

The discrepancies between our findings in human atherosclerosis and animal models might be explained by the duration of the atherosclerotic process, ie, months in Watanabe rabbits versus decades in patients. Furthermore, the severity of clinically relevant lesions and species differences may play a role. Indeed, vascular lesions of hypercholesterolemic rabbits mimic human plaques only in part. Nevertheless, it is conceivable that eNOS protein expression and NO release may be normal or increased in early human atherosclerosis, whereas at later stages NOS expression and NO release are reduced. Hence, abnormal endothelium-dependent responses during early atherosclerosis might be due to reduced substrate and/or cofactor concentrations or increased superoxide formation. This could explain why the administration of l-arginine or the cofactor tetrahydrobiopterin improves endothelial function only in early stages of atherosclerosis and in the microcirculation, where no plaques develop, but not in epicardial coronary arteries of patients with clinically relevant coronary disease. Thus, at later stages of the atherosclerotic process, when eNOS expression and NO production are decreased, these therapies are no longer effective.
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