Protection by Shear Stress From Collar-Induced Intimal Thickening
Role of Nitric Oxide

Giuseppe Marano, Sergio Palazzesi, Alessandro Vergari, Alberto U. Ferrari

Abstract—Nitric oxide (NO) has potent relaxant and antiproliferative effects on vascular smooth muscle cells, which may represent an important antiatherosclerotic mechanism. Since one of the major stimuli for NO release is flow-related shear stress, we have investigated (1) the effect of increased shear stress on neointimal formation induced in the rabbit carotid artery by enclosing the vessel in a nonconstrictive silicone soft collar and (2) the role of NO in the antiproliferative effect of increased shear stress. Forty-three New Zealand White rabbits were used. High shear stress in the left common carotid artery (CCA) was induced by ligation of the contralateral right internal carotid artery; intimal thickening was produced by the positioning a nonconstrictive silicone soft collar around the left CCA. To evaluate the role of NO, N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) was orally administered at a subpressor dose. In all rabbits, arterial blood pressure, heart rate, arterial diameters, and blood flow velocities of both CCAs were determined at days 0, 3, 7, and 14. At the end of the study, all rabbits were euthanized, and histological analyses were performed on both CCAs of each animal. The presence of the collar was associated with a marked degree of intimal hyperplasia (intimal/medial area ratio 29±3.0% in collared arteries compared with 3 ±0.7% in sham control [noncollared] arteries, P<0.001). The increase in blood flow almost completely inhibited neointimal formation and induced an increase in arterial diameter of ≈30%. The effects of increased blood flow were reversed by the administration of L-NAME. In conclusion, we demonstrate that in collar-induced intimal thickening, a chronic increase in shear stress (1) almost completely inhibits intimal thickening, and (2) this protective effect is mediated by NO production. (Arterioscler Thromb Vasc Biol. 1999;19:2609-2614.)

Key Words: shear stress • intimal hyperplasia • collar model • nitric oxide • flow-dependent vasodilation • rabbits

Nitric oxide (NO) has potent relaxant and antiproliferative effects on vascular smooth muscle cells. These effects have been well documented in vivo and in vitro.\textsuperscript{1–5} It is believed that one of the major stimuli to NO synthesis and release from the arterial wall is the shear stress to which the vascular endothelial lining is subjected. Therefore, it is conceivable that a high blood flow/shear stress hemodynamic regimen may be associated not only with NO-dependent functional relaxation but also with inhibition of the intimal proliferative response to growth-promoting stimuli. For example, it was recently reported that high intra-arterial blood flow inhibits neointimal formation in graft- and balloon-induced intimal thickening models\textsuperscript{6–8}; ie, neointimal formation is inhibited after the native endothelium is lesioned by various mechanical traumas. Although it was suggested that regenerated endothelial cells play a key role in this effect,\textsuperscript{7} it is to be considered that in deendothelialized vessels NO synthesis and release may be taken over by the smooth muscle cells themselves, probably as a consequence of their exposure to shear stress,\textsuperscript{9} and that the neoendothelium is functionally different from the original cell layer; ie, it has defective production of NO and prostacyclin.\textsuperscript{10,11} Thus, it is not yet known whether and by which mechanisms high blood flow is capable of inhibiting the intimal proliferative response to growth-promoting stimuli when the native endothelium is present.

The above premises prompted us to verify whether high flow and NO release may modulate the vascular proliferative response in the collar model of neointimal formation, ie, in a setting in which growth develops under the original, noninterrupted, nonregenerated endothelium. The goals of the present study were (1) to evaluate whether an increased blood flow/shear stress can inhibit collar-induced intimal thickening, with the increase in flow being obtained in the collared common carotid artery (CCA) by placing a ligation on the contralateral internal carotid artery, and (2) to establish whether the NO system is involved in inhibiting collar-induced growth, ie, whether and to what extent the adminis-
treatment of the NO synthesis inhibitor N ω-nitro-L-arginine methyl ester (L-NAME) is able to reverse the protective effect of increased blood flow/shear stress on intimal thickening. Because of the controversial results previously reported, an additional goal of the present study was to further assess the changes in carotid artery diameter in response to large increases in blood flow.

To this aim, measurements of blood flow velocity and arterial diameter of experimental rabbits were obtained in both CCAs immediately before and after as well as at days 3, 7, and 14 after ligation of the right internal carotid artery, and histological studies were carried out after euthanizing the animals at day 14.

Methods

Experimental Groups and Drug Treatments

The experiments were conducted in 43 male New Zealand White rabbits (Charles River, Italy) weighing 2.5 to 3.0 kg and complied with the recommendations of the Council of European Community (86/609/EEC) in all phases.

Forty rabbits were randomly divided into 5 groups of 8 rabbits each. In group 1 (collared+natal shear stress), rabbits were subjected to a nonconstrictive silicone soft collar application (see details in the next paragraph) in the left CCA, whereas the contralateral internal carotid artery was isolated but not ligated. In group 2 (collared+high shear stress), a collar was positioned around the left CCA; contralaterally, the right internal carotid artery was isolated and ligated. In groups 3 (collared+high shear stress+placebo) and 4 (collared+high shear stress+L-NAME treatment), the same surgical procedures as in group 2 were performed, but the animals additionally received drug vehicle or L-NAME (160 μg/mL in drinking water), respectively. This dose of L-NAME was chosen because it has been demonstrated in rabbits to be effective on the vascular wall without affecting arterial blood pressure. Treatment was started 7 days before collar application to obtain effective NO synthase inhibition at the time of the surgical procedure, and the treatment was continued for 14 days. In group 5 (sham control), both CCAs were exposed, and the left CCA was surgically manipulated in an identical fashion and for the same time as the carotid artery enclosed by the collar. All animals in each group underwent histological analysis.

In another experimental series, 3 rabbits underwent ligation of the right internal carotid artery. A telemetry probe (TL1.1 mol/L2-CXT-P50, Data Sciences Inc) was inserted in the femoral artery 7 days before vessel ligation to evaluate whether the surgical procedure used to increase blood flow in the left CCA produced significant changes in hemodynamic conditions during the entire period of study.

Surgical Procedures

Anesthesia was induced intramuscularly with ketamine (10 mg/kg) and midazolam (0.1 mg/kg), and then orotracheal intubation was attempted immediately after the rabbits lost consciousness. A cuffed endotracheal tube (3.0-mm ID, D.A.R.) was inserted into the trachea and connected to a respiratory ventilator (model 7900, Ohmeda) set at a tidal volume of 10 mL/kg. The ventilatory rate was adjusted to 30 to 35 breaths per minute to keep end-tidal CO2 between 35 and 37 mm Hg. End-tidal anesthetic and carbon dioxide levels were continuously monitored (gas monitor, 5250 RGM, Ohmeda). Pancuronium bromide (0.1 mg/kg IV) was injected to induce muscle relaxation and facilitate mechanical ventilation. Anesthesia was maintained with end-tidal 1.8% isoflurane (1mmunium alveolar concentration) in a gaseous mixture of nitrous oxide (N2O) and oxygen (O2) (50/50% [vol/vol]). Body temperature and lead II of the ECG were monitored continuously. A 24-gauge catheter needle was inserted percutaneously into the marginal ear vein, and an intravenous flow control system (DIAL-A-FLO, Abbott) was attached to it. Lactated Ringer’s solution was infused at 4 mL/kg per hour throughout the study. A 22-gauge catheter needle was percutaneously inserted into the central ear artery in all animals at days 0, 3, 7, and 14 to determine arterial blood pressure and heart rate. Both CCAs were exposed (groups 1 to 5), and the left CCA (groups 1 to 4) was enclosed in a nonocclusive, biologically inert, silicone soft collar in accordance with Soma et al. Briefly, longitudinally split silicone collars (20-mm length, 1-mm wall thickness, 2×1-mm contact length, 4-mm internal diameter at the center, and 1.9 mm at either end) were placed around the left CCA, the external diameter of which was smaller than the bore of the collar endings.

Histology and Histomorphometric Analysis

The rabbits were euthanized after 14 days with an overdose of pentobarbital, and both CCAs from groups 1 to 5 were isolated and excised. The left CCAs from groups 1 to 4 were divided into 3 regions: a tract proximal to the collar, a midregion that had been surrounded by the collar, and a tract distal to the collar. The specimens were fixed for 2 hours in buffered formalin and embedded in paraffin. Cross sections (5-μm thickness) were cut and stained with hematoxylin and eosin and van Gieson-Weigert stain.

Histomorphometric analysis was performed by means of a semi-automatic image analyzer (Quantimet 500, Leica). The cross-sectional area of media and intima was measured at ×10 magnification. Neointimal growth was evaluated in terms of intimal area and intimal-to-medial area ratio.

Carotid Ultrasonography and Measurement of Shear Stress

Under isoflurane anesthesia and continuous hemodynamic monitoring, carotid ultrasonography was performed in each rabbit along both CCAs at days 0 (before and 20 minutes after surgical procedure), 3, 7, and 14 with an Esaote AU3 Partner linear array probe (imaging at 7.5 MHz) and spectral Doppler and color Doppler (at 5 MHz). In addition, the left CCA was divided into 3 main regions for Doppler analysis, as follows: (1) proximal to the collar (1 cm), (2) intermediate arterial segment surrounded by the collar, and (3) distal to the collar (1 cm). Blood velocities were measured with pulsed Doppler at an angle of 60°, and internal vessel diameters were obtained from 2-dimensional echocardiograms. Carotid blood flow was calculated as the product of the lumen area (πD^2/4) and the Doppler-derived time-velocity integral. To further confirm measurements obtained by the ultrasonographic velocimetric and B-mode procedures, blood flow at days 0 and 14 was determined by ultrasonic flowmetry based on the transit time principle (model 106, Transonic System). By assuming laminar flow conditions, shear stress (τ) at baseline and under increased blood flow conditions was calculated with the Hagen-Poiseuille approximation: \( \tau = \frac{8Q}{\pi r^4} \), where \( \mu \) is the viscosity of rabbit blood (considered to be constant and equal to 0.035 poise), \( Q \) is the blood flow (mL/s), and \( r \) is the radius (cm) of the carotid artery.

Statistical Analysis

Data are expressed as mean±SE. The responses under different experimental conditions were compared by using Student t test or 2-way ANOVA. If a significant F value was obtained, the Bonferroni test was used to assess specific differences between groups (Statview 4.02 statistical package). The level of statistical significance was set at \( P<0.05 \).

Results

Ligating the right internal carotid artery to increase carotid blood flow in the left CCA did not alter mean arterial pressure (91±6.5 versus 90±7.1 mm Hg after versus before ligation) and heart rate (235±18 versus 230±22 bpm) in conscious unrestrained rabbits. Therefore, the vascular responses to these procedures have to be attributed to changes in local hemodynamic conditions. It is also to be mentioned that with the rabbits under isoflurane anesthesia, blood flow was systematically found to be higher in the right CCA than in the left CCA (by ≈25%): as a consequence, in order to prevent this difference in baseline hemodynamics from confounding
the interpretation of the results of the study, it was elected to invariably place the collar on the same (left) side and, in turn, to ligate the internal carotid artery on the right side.

**Hemodynamic Responses to Increased Blood Flow**

No significant differences in hemodynamic parameters and arterial diameters between the different regions of the left CCA were observed, so the data from the proximal and distal segments are not shown, and only the midcollar figures are presented herein. Isoflurane anesthesia was used, and arterial blood pressure, heart rate, carotid diameter, mean flow velocity, mean blood flow, and mean wall shear stress remained unchanged during the entire experimental protocol in group 1 (collared, sham ligature) (Table 1) and in group 5 (sham control group). In groups 2, 3, and 4, the arterial blood pressure and heart rate were similar to those obtained in group 1 and did not significantly differ from those values obtained in conscious, nonrestrained rabbits.

In group 2 (data summarized in Table 2), ligature of the right internal carotid artery caused blood flow in the left CCA to markedly increase from 41.2 ± 5.1 mL/min to 117 ± 5.5 mL/min (+281%, *P* < 0.01) and did not significantly differ from those values obtained in group 1.

In group 3, which was experimentally identical to group 2 except for oral placebo administration, the hemodynamic and vascular responses to increased blood flow were, as expected, virtually identical to those obtained in group 2 and are not shown.

In group 4, L-NAME at the dose selected had no blood pressure–raising effects at any time. After ligature of the right internal carotid artery, blood flow in the left CCA and the calculated wall shear stress were found to be increased significantly on day 0 and stayed elevated through days 3, 7, and 14 (Table 3). Left CCA diameter was unaffected acutely after right internal carotid artery ligation, but at variance with groups 2 and 3, it failed to increase throughout the later period of observation.

In the right CCA from groups 2, 3, and 4, blood flow decreased by ~67% (from 59 ± 3.4 mL/min) 20 minutes after ligation of the homolateral internal carotid artery and remained unchanged at days 3, 7, and 14. The diameter increased by ~10% after the procedure (-8.0 ± 0.05 mm after versus before ligation) but tended to decrease toward control values at days 3, 7, and 14.

**Histological and Morphometric Analysis**

At harvest, all the carotid arteries were patent and did not show any abnormality on gross examination, except for a diffuse periadventitial fibrous thickening in the region surrounding the collar.

Left CCAs of group 1 and L-NAME–treated group 4 showed a severe degree of intimal hyperplasia in the region that had been surrounded by the collar (Figure 1A). Intimal hyperplasia consisted of a new growth of spindle-shaped cells and an extracellular matrix lined by a continuous layer of endothelial cells, either flat or swollen. Neither intimal

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**TABLE 1. Left CCA Hemodynamic Parameters in Rabbits Under Nonaltered Flow Conditions (Collared + Normal Shear Stress, Group 1)**

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Before</th>
<th>After</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>90 ± 4.5</td>
<td>89 ± 3.5</td>
<td>91 ± 5.5</td>
<td>92 ± 5.9</td>
<td>89 ± 5.4</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>255 ± 35</td>
<td>245 ± 25</td>
<td>251 ± 25</td>
<td>245 ± 20</td>
<td>255 ± 24</td>
</tr>
<tr>
<td>Blood flow, mL/min</td>
<td>42 ± 3.4</td>
<td>41 ± 5.4</td>
<td>42 ± 4.4</td>
<td>41 ± 3.1</td>
<td>42 ± 6.2</td>
</tr>
<tr>
<td>Midcollar diameter, mm</td>
<td>1.6 ± 0.05</td>
<td>1.6 ± 0.05</td>
<td>1.6 ± 0.05</td>
<td>1.6 ± 0.07</td>
<td>1.7 ± 0.09</td>
</tr>
<tr>
<td>Midcollar WSS, dyne/cm²</td>
<td>60.9 ± 4.2</td>
<td>61.3 ± 2.9</td>
<td>63.8 ± 4.8</td>
<td>57.9 ± 3.9</td>
<td>59.2 ± 4.7</td>
</tr>
</tbody>
</table>

Data are mean ± SE (n = 8). MAP indicates mean arterial pressure; HR, heart rate; and WSS, wall shear stress.

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**TABLE 2. Left CCA Hemodynamic Parameters in Nontreated Rabbits Under Increased Blood Flow Conditions (Collared + High Shear Stress, Group 2)**

<table>
<thead>
<tr>
<th>Day 0</th>
<th>Before</th>
<th>After</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>91 ± 5.1</td>
<td>90 ± 4.3</td>
<td>90 ± 5.4</td>
<td>91 ± 5.6</td>
<td>89 ± 5.5</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>255 ± 25</td>
<td>250 ± 18</td>
<td>245 ± 20</td>
<td>245 ± 20</td>
<td>255 ± 18</td>
</tr>
<tr>
<td>Blood flow, mL/min</td>
<td>41.2 ± 3.4</td>
<td>79 ± 5.1*</td>
<td>76 ± 5.1*</td>
<td>87 ± 4.6*</td>
<td>92 ± 4.1†</td>
</tr>
<tr>
<td>Midcollar diameter, mm</td>
<td>1.6 ± 0.06</td>
<td>1.6 ± 0.06</td>
<td>2.05 ± 0.05*</td>
<td>2.05 ± 0.06*</td>
<td>2.1 ± 0.07*</td>
</tr>
<tr>
<td>Midcollar WSS, dyne/cm²</td>
<td>59.7 ± 5.5</td>
<td>117 ± 5.5*</td>
<td>61.9 ± 6.6</td>
<td>64.9 ± 6.4</td>
<td>68.8 ± 4.9</td>
</tr>
</tbody>
</table>

Data are mean ± SE (n = 8).

*P* < 0.01 vs values for day 0 (before); †P < 0.01 vs values for day 3.
inflammatory infiltrates nor medial lesions were observed. The carotid segments that were not enclosed in the collar did not show any intimal lesions. In groups 2 and 3, intimal hyperplasia was significantly reduced (Figure 1B). In the sham-operated left CCAs and right CCAs from all groups, no noticeable changes could be detected (Figure 1C).

On histomorphometric analysis, the neointimal areas were 0.15±0.03, 0.04±0.003, 0.05±0.006, and 0.026±0.002 mm² in groups 1, 2, 3, and 5, respectively; the corresponding values of the intima/media area ratio were 29±3%, 8±2.7%, 9±3.1%, and 3±0.7% (P<0.01 group 1 versus groups 2, 3, and 5 in all cases; Figure 2). In the L-NAME–treated animals of group 4, intimal hyperplasia was again present; its degree was similar to that observed in group 1 (neointimal area 0.16±0.03 mm², intima/media area ratio 30±3%; Figure 2).

Discussion

The major findings of the present study, in a model that allowed us to evaluate the effect of increased shear stress on the development of collar-induced intimal thickening in the presence of the original endothelium, are that (1) the increase in blood flow/shear stress prevents collar-induced intimal thickening, (2) this inhibitory effect is mediated by NO release, and (3) after 14 days, the increase in wall shear stress produces a significant enlargement of the left CCA, with the flow-dependent vasodilation also being mediated by NO release.

The inhibitory effect of high shear stress on the development of intimal hyperplasia has already been highlighted.6–8 However, these results were obtained in experimental models of intimal hyperplasia involving extensive denudation of the endothelium and significant trauma to the vessel wall. We report for the first time that this inhibitory effect also occurs in a model of intimal hyperplasia that develops under an original, nonregenerated, noninterrupted endothelium.

Although not definitively proven, the most likely explanation underlying our findings is that prevention of collar-induced intimal thickening by high flow was mediated by the endothelium. This is likely because the endothelial cell layer is the major sensor to changes in shear stress, and it subserves functions such as barrier regulation of permeability to platelet- and leukocyte-derived growth factors as well as production of growth-inhibitory substances,17,18 thus playing a key role in antagonizing neointimal formation.

This notion is strengthened by the second major result of the present study, ie, that the protective effect of high shear stress against neointimal formation is reversed by chronic inhibition of NO synthase. These data demonstrate that NO production is critical to the growth-inhibitory effect of high shear stress in collateral arteries. In addition, our results are in line with the suggestion that NO production is modulated by flow and that NO inhibits intimal hyperplasia in vivo. In fact, it has been reported that the gene expression of NO synthase is regulated by flow.9,10 Furthermore, it has also been shown that administration of L-arginine (the precursor of NO) or of synthetic NO donors or the transfer of constitutive NO synthase gene into smooth muscle cells is capable of inhibiting intimal thickening in balloon-injured arteries and vein grafts.3,4,20,21 Moreover, Cayatte et al22 reported that chronic inhibition of NO production by L-NAME accelerates neointimal formation in cholesterol-fed rabbits.

The third significant result of the present study is that a sustained increase in blood flow (3 to 14 days) produces a significant enlargement of the CCA, which tends to normalize shear stress, and that this effect is also related to NO production because the enlargement of the artery was not observed in animals chronically treated with a NO synthase inhibitor. This may be of relevance in the debate concerning the effects of increased blood flow on vascular remodeling: in rats the increase in shear stress has been reported to induce aortic expansive remodeling 2 months after an aortocaval fistula is opened,13 whereas in adult rabbits the increase in blood flow in the right CCA by left-to-right carotid anastomosis would not induce any compensatory enlargement after 2 months.12 On the other hand, Tronc et al23 have reported that NO synthase inhibition by N'G-nitro-L-arginine blocks the arterial dilation induced by increased blood flow in experimental arteriovenous fistulas in adult rabbits. These controversial results could depend on differences in the technical conditions under which data have been collected (ultrasonography versus histomorphometric analysis) and/or in the experimental protocol, species used, and timing of observations. Our findings are in favor of the occurrence of significant dilation in response to enhanced blood flow/shear stress, although they also indicate that the process may have some degree of latency for the following reason: despite the immediate increase in wall shear stress after internal carotid artery ligation, the vasodilator effect was not immediately apparent and could only be detected ≥3 days later. This is in accordance with data reported in the literature and could be due to development of a Venturi effect within the left carotid axis in the early phases after ligation of the contralateral internal carotid artery.13

### Table 3. Left CCA Hemodynamic Parameters in L-NAME–Treated Rabbits Under Increased Blood Flow Conditions (Collared + High Shear Stress + L-NAME Treatment, Group 4)

<table>
<thead>
<tr>
<th>Day</th>
<th>Before</th>
<th>After</th>
<th>Day 3</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mm Hg</td>
<td>91±5.2</td>
<td>91±5.2</td>
<td>90±6.4</td>
<td>92±7.5</td>
<td>89±4.5</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>250±15</td>
<td>250±15</td>
<td>255±20</td>
<td>245±16</td>
<td>251±12</td>
</tr>
<tr>
<td>Blood flow, mL/min</td>
<td>42±4.5</td>
<td>78.7±5.4*</td>
<td>77±4.7*</td>
<td>88±5.4*</td>
<td>92±3.9†</td>
</tr>
<tr>
<td>Midcollar diameter, mm</td>
<td>1.7±0.06</td>
<td>1.7±0.05</td>
<td>1.7±0.06</td>
<td>1.7±0.07</td>
<td>1.7±0.08</td>
</tr>
<tr>
<td>Midcollar WSS, dyne/cm²</td>
<td>57.3±4.4</td>
<td>96.4±3.7*</td>
<td>107.2±5.8*</td>
<td>117.8±4.8*</td>
<td>127.1±6.5†</td>
</tr>
</tbody>
</table>

Data are mean±SE.

*P<0.01 vs values for day 0 (before); †P<0.01 vs values for day 3.
A few additional aspects of our experiments are worthy of comment. First, chronic oral administration of L-NAME (160 μg/mL in drinking water) had no significant effect on systemic blood pressure measured in the anesthetized rabbit for up to 14 days of observation. These results are at variance with those reported in rats during chronic administration or in rabbits after acute intravenous administration. However, our data are consistent with previous reports showing that chronic oral administration of L-NAME at similar doses in rabbits has no effect on blood pressure. These discrepancies may be due to interspecies differences and/or to different routes of administration of L-NAME.

Second, among the possible limitations of the present study, it is to be mentioned that we based shear stress estimation on measurement of blood flow and arterial diameter by using a Hagen-Poiseuille flow approximation according to which shear stress is directly proportional to blood flow and inversely proportional to the cube of the vessel radius; although known not to be strictly valid for pulsatile flow, this approximation is widely accepted and used in the literature to calculate shear stress. A further problem relates to the fact that NO synthase inhibition by L-NAME is not specific to an isoform; because 2 major isoforms, constitutive and inducible, have been described, the isoform of NO synthase involved in the processes examined in our experiments remains to be determined.

In conclusion, our results demonstrate that in a rabbit model of intimal hyperplasia that develops in the presence of the original nonmechanically lesioned endothelium, a chronic increase in blood flow/shear stress (1) inhibits intimal thickening and (2) produces a significant flow-dependent enlargement of the left CCA. Both effects are mediated by stimulation of NO production.

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References


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