Pharmacologic inhibition of nitric oxide synthases and cyclooxygenases enhances intimal hyperplasia in balloon-injured rat carotid arteries

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Objective: Extensive proliferation and migration of smooth muscle cells (SMCs) contribute to development of fibromuscular intimal hyperplasia in response to balloon catheter–induced injury of the left carotid artery in Fischer 344 rats. The purpose of the present study was to test the hypothesis that endogenously generated nitric oxide (NO) and prostaglandins act synergistically to limit the extent of neointimal hyperplasia.

Methods: The left carotid artery of Fischer 344 rats was injured with a 2F balloon catheter. The following treatment was initiated 24 hours before arterial injury, and was continued for 2 weeks: N-nitro-L-arginine (L-NA; 10 mg/kg/d, in drinking water), indomethacin (1.5 mg/kg/d per gavage), and L-NA (10 mg/kg/d) plus indomethacin (1.5 mg/kg/d). After application of an overdose of pentobarbital animals were formalin-fixed. Subsequently, paraffin-embedded cross sections of the uninjured and injured carotid arteries were analyzed morphometrically. SMC proliferation was determined by incorporation of 5-bromo-2′-deoxyuridine.

Results: Two weeks after injury, L-NA caused a 1.29-fold ± 0.29-fold (mean ± SD; n = 14; P < .05) increase in the intima-media ratio, compared with control animals, whereas indomethacin had no effect. Combined treatment with L-NA plus indomethacin further increased intima-media ratio (1.65-fold ± 0.5-fold over control; n = 14; P < .05). SMC proliferation in the neointima of rats treated with L-NA and L-NA plus indomethacin was elevated. Furthermore, neointimal cell density (nuclei per square millimeter) was reduced after combined inhibition of cyclooxygenases and NO synthases.

Conclusion: The present results of pharmacologic NO synthase and cyclooxygenase inhibition suggest that NO and prostaglandins are part of an endogenous growth inhibitory mechanism that synergistically suppresses intimal thickening. (J Vasc Surg 2004;40:115-22.)

Clinical Relevance: The role of cyclooxygenase-1 (COX1) and cyclooxygenase-2 (COX2) during vascular recurrent stenosis and atherosclerosis is not clear yet. In particular, the effects of selective COX2 inhibitors on the frequency of cardiovascular events is still controversial. It is shown here in rats that the application of a non-selective COX inhibitor does not affect arterial stenosis. However, the concurrent inhibition of endogenous nitric oxide generation and COX1 or COX2 causes overshooting neointimal hyperplasia. These results suggest that increased vascular stenosis can result from administration of drugs that pharmacologically block 2 or more inhibitory pathways that normally counterbalance the effect of promotors of neointimal hyperplasia.

An important limitation of percutaneous transluminal coronary angioplasty is recurrent stenosis during the first months after initially successful interventions. A widely used experimental model to study arterial restenosis is neointimal hyperplasia, which develops in rats in response to balloon injury of the common carotid artery. Neointimal hyperplasia in rats is initiated by smooth muscle cell (SMC) replication in the media,1 followed by SMC migration toward the lumen2,3 and subsequent proliferation of neointimal SMCs for a limited time.4 After proliferation has ceased, further expansion of the neointima is predominantly due to synthesis and accumulation of extracellular matrix (ECM). ECM accumulation accounts for about 80% of the intimal volume between 2 and 12 weeks after injury.5 Thus far it is not known which mechanisms or mediators are responsible for the fact that neointimal hyperplasia eventually stops and even regresses in the rat model.

Candidates for endogenous inhibitors of neointimal hyperplasia are nitric oxide (NO) and prostaglandins. NO inhibits SMC proliferation and migration through cyclic guanosine monophosphate–dependent pathways.6-7 In vivo experimental evidence for an inhibitory function of NO has been provided by gene transfer studies that demonstrating that intimal hyperplasia is inhibited by overexpression of endothelial cell NO synthase (eNOS)8-10 and inducible NO synthase (iNOS).11 In addition, neointimal hyperplasia and constrictive arterial remodeling were increased in studies in mice deficient in iNOS, ecNOS, and...
neuronal NO synthase (nNOS), which supports the conclusion that NOS activity inhibits the response to vascular injury. In contrast, one study reported decreased intimal hyperplasia in iNOS knockout mice, which suggests that iNOS might also have the potential to promote intimal hyperplasia. Therefore, despite some controversy, NOS activity appears to be a likely mechanism for mediation of endogenous growth inhibitory effects in response to vascular injury.

The role of cyclooxygenase-1 (COX1) and cyclooxygenase-2 (COX2) during restenosis and atherosclerosis in vivo is even more difficult to define. A recent clinical trial suggested that use of COX2-selective inhibitors is associated with increased risk for cardiovascular events. In contrast, animal studies showed a protective effect of COX1 and COX2 inhibitors in experimental atherosclerosis. After balloon injury of carotid arteries in rats, which is an experimental model of neointimal hyperplasia without pronounced inflammation and lipid accumulation, COX inhibitors had no effect. These findings are in contrast to the pronounced anti-proliferative and anti-migratory effects of vasodilatory prostaglandins such as prostacyclin and prostaglandin E2 in vitro. Therefore, we hypothesize that in vivo, during fibroproliferative vessel remodeling, additional factors are required to support inhibitory effects of vasodilatory prostaglandins on neointimal hyperplasia. The synthetic pathways of eicosanoids and NO, and functionally important aspects in the context of vascular injury are summarized in Fig 1.

The goal of the present study was to investigate how simultaneous inhibition of COX1 and COX2, and iNOS, ecNOS, and nNOS by pharmacologic means affects neointimal hyperplasia in the balloon injury model in rats. For this purpose, use of specific inhibitors of ecNOS versus iNOS and COX1 versus COX2 were deliberately not used, because it was intended to determine the overall effect of NOS and COX during neointimal hyperplasia. Accordingly, Fischer 344 rats were treated with N-nitro-l-arginine (L-NA), an inhibitor of NO synthesis, and ecNOS, iNOS, and nNOS by pharmacologic means affects neointimal hyperplasia in the balloon injury model in rats. For this purpose, use of specific inhibitors of ecNOS versus iNOS and COX1 versus COX2 were deliberately not used, because it was intended to determine the overall effect of NOS and COX during neointimal hyperplasia. Accordingly, Fischer 344 rats were treated with N-nitro-l-arginine (L-NA), an inhibitor of NO synthesis, and ecNOS, iNOS, and nNOS, and indomethacin, an inhibitor of COX1 and COX2, either alone or in combination.

METHODS

Animals, surgery, and tissue preparation. Three-month-old male Fischer 344 rats (Simonsen Laboratories) received either normal tap water or tap water plus L-NA (Sigma), an inhibitor of NO synthesis. The water consumption was monitored and the L-NA concentration in the

**Fig 1.** Biosynthesis and function of eicosanoids and nitric oxide (NO) in vessel wall. Arachidonic acid is liberated from plasma membrane phospholipids mainly by cytosolic phospholipase A2 (PLA2). Arachidonic acid is subsequently converted by lipoxigenases into leukotrienes (not shown) or by cyclooxygenase-1 and cyclooxygenase-2 (COX1/2) into prostaglandin G2 (PG G2) and further into prostaglandin H2 (PG H2). PGH2 is the substrate of individual synthases generating various prostaglandins, such as PGE2, PGF2α, and thromboxane A2 (TXA2). The synthetic pathways of PGE1, PGD2, PGF2α, are not shown for reasons of simplicity. NO is synthesized from l-arginine by inducible (iNOS), endothelial (ec), and neuronal (n) nitric oxide synthases (NOS) via the intermediate N(G)-hydroxy-l-arginine (NOHA). The effects of eicosanoids and NO on vessel diameter, the synthetic smooth muscle cell (SMC) phenotype, and thrombocyte aggregation are indicated (arrows symbolize enhancement; lines with crossbar indicate inhibition). Synthetic SMC phenotype stands for SMC with high proliferative, migratory, and synthetic activity. TXA-S, Thromboxane synthase; PGI2-S, prostacyclin synthase; PGE-S, prostaglandin E synthase.
drinking water adjusted to ensure an L-NA dose of 10 mg/kg/d. In a previous study this dose of L-NA inhibited NO generation and decreased cyclic guanosine monophosphate levels in injured arteries. In addition, a suspension of indomethacin in 5% ethanol (~0.2 mL) was given by gavage once a day (1.5 mg/kg/d). The control animals and the L-NA–treated animals received the same amount of vehicle (5% ethanol) by gavage. In additional experiments hydralazine (13 mg/kg/d) was administered continuously by subcutaneous infusion via an Alzet mini-osmotic pump (Alza Corp) implanted subcutaneously in the neck of the animals. The drug treatment was initiated 24 hours before balloon injury. Balloon injury was performed as described.

Systolic arterial blood pressure was measured in conscious, restrained rats by tail-cuff plethysmography (Norco Biosystems). The measurement was performed twice before the drug treatment to obtain the baseline value for each rat, and subsequently at 3, 7, and 13 days after balloon injury.

After 14 days the animals were sacrificed, fixed by perfusion with 10% neutral buffered formalin (pH 7.4) at 100 mm Hg. Two pieces of each carotid artery were embedded in paraffin for histologic analysis. The rats received 50 mg of 5-bromo-2’-deoxyuridine (BrdU) subcutaneously 24 hours before they were sacrificed. Subsequently, paraffin-embedded cross sections were used for immunostaining and for morphometric measurements, as described. Because the extent of intimal hyperplasia varied between independent experiments, data derived from individual experiments were subsequently normalized to medial areas (intima-media [i/m] ratio) and presented as increase of i/m ratio over the respective controls. This analysis enabled pooling of data from 3 independent experiments.

All surgical procedures were performed according to the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 86-23, revised 1985), and were approved by the University of Washington, Seattle.

Immunocytochemistry. Rabbit antiserum to the core protein of human versican was generously provided by Dr Richard Le Baron (University of Texas at San Antonio), and was used at 1:100 dilution in phosphate-buffered saline solution plus 0.1% bovine serum albumin. Sections to be stained for the proteoglycan core protein of versican were digested with chondroitin ABC lyase (ICN Biomedicals) at 200 mU/mL in 0.1 mol/L of Tris-acetate, pH 7.3, for 1 hour at 37°C. BrdU incorporation was detected with a specific monoclonal antibody to BrdU (Boehringer Mannheim). BrdU-positive and BrdU-negative nuclei were counted under a microscope, and the proliferative index (percent BrdU-positive cells) was calculated.

Measurement of 6-keto-prostaglandin F1α. In preliminary experiments we determined whether the dose of indomethacin (1.5 mg/kg/d) effectively inhibited prostacyclin synthesis. After 3 days of treatment, rats were sacrificed with an overdose of pentoobarbital. Subsequently blood and the thoracic aorta were harvested. Plasma was obtained by routine centrifugation and used directly for determination of 6-keto-prostaglandin F1α (6-keto-PGF1α) concentrations, the degradation product of prostacyclin, with the TiterZyme enzyme immunoassay kit (PerSeptive Diagnostics). The 6-keto-PGF1α plasma concentration in indomethacin-treated animals was reduced to 200 ± 15 pg/mL, compared with 1000 ± 212 pg/mL in untreated animals. In addition, the aorta was chopped into pieces of approximately equal size, which were subsequently placed in serum-free tissue culture medium. After 3 initial washes with serum-free medium, 1 mL of serum-free medium was added either with or without phorbol-12-myristate-13-acetate (PMA, 10 μmol/L), which induces COX-2 activity and prostacyclin release. After 6 hours the medium was collected and the amount of 6-keto-PGF1α determined. Subsequently the pieces of the carotid artery were assayed for protein content, and the release of 6-keto-PGF1α per milligram of protein was calculated. In control animals, a 3.8-fold ± 0.3-fold (mean ± SD; n = 3) stimulation of 6-keto-PGF1α release over unstimulated controls was induced by PMA, which was completely abolished in vessels of indomethacin-treated animals (1.1-fold ± 0.2-fold stimulation over controls; n = 3). Therefore it was concluded that 1.5 mg of indomethacin per kilogram per day was an appropriate dose to inhibit COX activity in the present experimental system.

Reagents. All reagents and chemicals were purchased from Sigma, unless otherwise stated.

Statistical analysis. Data are expressed as mean ± SD. Statistical analysis was performed with analysis of variance and Dunnet post hoc test.

RESULTS

Intimal hyperplasia at 14 days after balloon injury is enhanced by simultaneous treatment with L-NA and indomethacin. To characterize the neointima 14 days after injury, 3 independent experiments were performed (Table, group 1). Fig 2, A, shows representative cross sections, and Fig 2, B, shows original data (square millimeters per cross section) of intimal hyperplasia obtained from 1 of 3 experiments. Fig 3, A, shows the increase over controls of i/m ratios from the 3 independent experiments. At 2 weeks after injury the inhibition of NOS by L-NA induced a slight increase in i/m ratio by a factor of 1.29 ± 0.29 (n = 14; P < .05 vs control). The treatment with indomethacin alone had no effect on i/m ratio. However, L-NA plus indomethacin caused a dramatic further increase in i/m ratio that was higher than after treatment with L-NA alone (factor of 1.65 ± 0.53; n = 14; P < .05 vs L-NA). The use of the i/m ratio was justified because the medial area of the injured arteries was not altered by the drug treatment (Fig 3, B) at 14 days. Furthermore, the medial areas of the contralateral uninjured carotid arteries were not changed in any of the groups at 14 days after injury (data not shown).

With respect to proliferation of neointimal SMCs, in rats treated with L-NA and with L-NA plus indomethacin intimal proliferation remained slightly elevated at 14 days compared with controls (control, 8% ± 0.7%, n = 19;
### Experimental groups

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*Group 1 consisted of three individual experiments (data shown in Figs 2 and 3). Treatment was initiated 24 hours before balloon injury. Indomethacin was given PG as suspension, and L-NA PO in drinking water.

†Hydralazine was used to lower blood pressure in experimental group 2, and was administered with an Alzet osmotic minipump in addition to L-NA PO and indomethacin PG (data shown in Fig 4).

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**Fig 2.** Carotid arteries 14 days after balloon injury. **A,** Cross sections of balloon-injured carotid arteries stained with hematoxylin-cosin 14 days after injury. a, Untreated control; b, N-nitro-L-arginine (L-NA, 10 mg/kg/d); c, indomethacin (1.5 mg/kg/d); d, indomethacin (1.5 mg/kg/d) plus L-NA (10 mg/kg/d). Arrows indicate internal elastic lamina. Original magnification ×40. **B,** Original measurements of intimal areas per cross section derived from one representative experiment. Control animals (n = 4) received vehicle; additional rats were treated with N-nitro-L-arginine (L-NA, 10 mg/kg/d; n = 5), indomethacin (1.5 mg/kg/d; n = 6), or L-NA plus indomethacin (n = 5). *P < .05.
L-NA, 11.1% ± 4.2%, n = 14; L-NA plus indomethacin, 11.5% ± 3.5%, n = 14; *P < .05 vs control). In addition, the number of intimal SMCs per cross section was significantly increased over controls in rats treated with L-NA plus indomethacin at 14 days (132% of control values; n = 14; *P < .05), but not in the other groups.

In additional experiments (Table, group 3), a higher indomethacin dose (3 mg/kg/d) was used in combination with the same dose of L-NA (10 mg/kg/d). Neointimal hyperplasia was almost doubled with this treatment (intimal area: controls, 0.054 ± 0.012 mm², n = 5; indomethacin plus L-NA, 0.092 ± 0.023 mm², n = 5; *P < .05).

The increase in neointimal hyperplasia in response to treatment with L-NA and indomethacin is blood pressure–independent. Mean systolic blood pressure was increased over controls in all 3 groups (Fig 3, C). To analyze whether this increase in blood pressure might be involved in the effect on neointimal hyperplasia, blood pressure was lowered with subcutaneous infusion of hydralazine (Table, group 2). In response to hydralazine infusion, blood pressure no longer differed significantly between treatment groups (controls, 107 ± 17 mm Hg, n = 8; L-NA, 122.5 ± 10 mm Hg, n = 6; L-NA plus indomethacin, 121 ± 11 mm Hg, n = 6; *P < .05; Fig 4, B). However, morphometric analysis still revealed a similar increase in i/m ratio in response to L-NA and to L-NA plus indomethacin in the hydralazine–treated animals (Fig 4, A), compared with those without pharmacologic lowering of blood pressure (compare with Fig 3, A). The increase of i/m ratio was again strongest in the group receiving L-NA plus indomethacin. A group of animals receiving indomethacin alone plus hydralazine was not included, because indomethacin alone had no effect on neointimal hyperplasia (Figs 2 and 3).

Another reason to assume that the increase in arterial blood pressure is not crucial for the observed synergistic enhancement of neointimal hyperplasia in response to L-NA and to L-NA plus indomethacin is that blood pres-
Intimal hyperplasia at 14 days and mean arterial blood pressure in rats receiving subcutaneous infusion of 13 mg/kg/24 hr of hydralazine in addition to N-nitro-L-arginine (L-NA, 10 mg/kg/d) or L-NA plus indomethacin (1.5 mg/kg/d). A, Fold increase of intima-media ratio over balloon-injured controls. B, Mean arterial blood pressure; controls, n = 8; L-NA, n = 6, indomethacin plus L-NA, n = 6. *P < .05.

**DISCUSSION**

After removal of the endothelium during balloon injury, iNOS is strongly upregulated in medial and intimal SMCs.24–27 iNOS can be detected as early as 1 day after injury in the medial SMCs, and up to 2 weeks in the intimal SMCs. Similarly, COX2 is expressed in intimal SMCs after balloon injury. Beginning as early as 2 hours after injury, the expression of COX2 remains increased for at least 2 weeks.28–30 Taken together, both iNOS and COX2 are induced after balloon injury, and are expressed for at least 2 weeks after injury. However, the functional relevance of the co-expression of these enzymes during neointimal hyperplasia has not been investigated.

At 14 days after injury, inhibition of COX1 and COX2 by indomethacin had no effect on SMC proliferation and intimal hyperplasia, which confirms the results of previous studies that showed no effect of the COX inhibitors aspirin and flurbiprofen.18,19 The administration of L-NA, however, caused an increase in intimal thickening (1.29-fold) and intimal SMC proliferation. These findings also are in line with those of previous studies that showed that gene transfer of ecNOS8–10 or iNOS11 or the administration of L-arginine31 inhibited neointimal thickening. However, the most important result of the present study is that simultaneous inhibition of NOS and COX synergistically increased neointimal hyperplasia.

The rise in mean arterial blood pressure in the animals treated with L-NA and with L-NA plus indomethacin must be considered a potential mechanism that causes enhanced intimal expansion and SMC proliferation. However, the increase in pressure (≤30 mm Hg) did not lead to increased medial areas in the uninjured contralateral vessels. Furthermore, no difference was detected between blood pressure in rats treated with L-NA and those treated with L-NA plus indomethacin, although neointimal hyperplasia was dramatically increased in rats treated simultaneously with both inhibitors. In addition, subcutaneous infusion of hydralazine (15 mg/kg/d) was used to reduce blood pressure in all groups. After hydralazine infusion, blood pressure in animals treated with L-NA and with L-NA plus indomethacin was not significantly different from that in control animals that received only hydralazine, but treatment with L-NA plus indomethacin still caused the same increase in i/m ratio as observed in the experiments without hydralazine. These data support the conclusion that NO and prostanoids have synergistic inhibitory effects on intimal hyperplasia that are independent of elevation of blood pressure.

On the basis of the present data, possible mechanisms responsible for enhanced intimal hyperplasia after treatment with L-NA plus indomethacin include increased proliferation of intimal SMCs, which was found in the L-NA and the L-NA plus indomethacin groups at 14 days, and increased accumulation of ECM, which was detected by a significant decrease in intimal cell density at 14 days after combined drug treatment. Versican, a large, extracellular chondroitin sulfate proteoglycan, is induced within the first
days after balloon injury in rats, and accumulates in the neointima. Versican possesses numerous large chondroitin sulfate chains, and binds to hyaluronan. This interaction leads to formation of large, highly hydrated networks, which contribute substantially to the volume occupied by the ECM. Furthermore, versican supports proliferation and migration of vascular SMCs. Therefore versican is thought to promote intimal expansion during early stages of neointimal hyperplasia. In addition to the rat model, versican is believed to serve similar functions in lesions in human beings. As shown at immunostaining, the accumulation of versican was increased in the luminal part of the neointima in animals treated with L-NA plus indomethacin, which invites the hypothesis that increased versican accumulation might be causally involved in the effect of L-NA and indomethacin on neointimal hyperplasia. The observation that NO and prostaglandins are involved in the regulation of neointimal ECM accumulation is in line with studies that showed that both NO and prostaglandins reduce expression of various ECM proteins, for example, fibronectin, collagen, and proteoglycans, in other experimental models. Whether versican expression by SMCs is directly repressed by NO and prostaglandins needs to be addressed in future studies. Based on the literature and the present findings, it is likely that control of neointimal SMC proliferation and migration, and inhibition of ECM accumulation mediate an inhibitory effect of NO and prostaglandins on neointimal hyperplasia. With respect to the pathophysiology of neointimal hyperplasia, these data suggest that endogenously produced NO and prostaglandins cooperate to limit the extent of neointimal hyperplasia.

In conclusion, pharmacologic inhibition of NOS and COX synergistically enhance neointimal hyperplasia. Inasmuch as many pharmacologic interventions modulate the activity of NOS or COX, possible effects with respect to vascular remodeling may have to be considered.

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REFERENCES

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