Local Expression of C-Type Natriuretic Peptide Suppresses Inflammation, Eliminates Shear Stress–Induced Thrombosis, and Prevents Neointima Formation Through Enhanced Nitric Oxide Production in Rabbit Injured Carotid Arteries

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Abstract—We previously observed that adenovirus-mediated expression of C-type natriuretic peptide (CNP) markedly inhibits neointima formation after balloon injury in rat carotid arteries, suggesting that CNP has multiple effects over its modest inhibitory effect on cellular proliferation. We hypothesized that local expression of CNP might have antithrombotic and antiinflammatory effects. Balloon-injured rabbit carotid arteries were infected with an adenovirus expressing human CNP (AdCNP), human tissue factor pathway inhibitor (AdTFPI), or bacterial β-galactosidase (AdLacZ) or infused with saline. Seven days later, shear stress–induced thrombosis was evaluated by cyclic flow variation (CFV), reflecting recurrent cycles of thrombus formation and dislodgment. CFV was observed in all AdLacZ-infected and saline-infused arteries but not in arteries infected with AdCNP or AdTFPI even in the presence of epinephrine. Injury increased the expressions of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and infiltration of macrophages. However, these effects were markedly reduced in AdCNP-treated arteries but not in AdTFPI-infected ones. In AdCNP-infected arteries, injury-induced expression of inducible NO synthase (iNOS) was enhanced, leading to increased NO generation. Interestingly, when the enhanced NO production was inhibited, neither inhibitory effect was observed, and suppression of neointima formation by CNP was canceled. Our study demonstrates that overexpression of CNP shows antithrombotic and antiinflammatory effects and reduces neointima formation mainly through enhanced NO production. (Circ Res. 2002;91:1063-1069.)

Key Words: thrombosis ■ inflammation ■ nitric oxide ■ gene transfer

It is clinically important to inhibit thrombosis in atherosclerotic or balloon- and/or stent-treated arteries as a way of avoiding a serious outcome.1,2 However, because most antithrombotic agents, when administered systemically, can induce bleeding at locations far from their intended site of action, effective forms of local therapy are needed. Transfer of the genes of antithrombotic molecules into injured arteries could be such a local therapy without distant bleeding. In fact, when introduced by gene transfer, several molecules including hirudin,3 tissue plasminogen activator,4 cyclooxygenase,5 and tissue factor pathway inhibitor (TFPI)6–8 have been shown to have effective local antithrombotic actions. Searching for other effective molecules and investigating their modes of action are important to select better candidates for future clinical application and to increase our understanding of the molecular mechanisms underlying arterial thrombosis in vivo.

C-type natriuretic peptide (CNP), a secreted polypeptide consisting of 22 amino acids, binds to natriuretic peptide receptor B, which bears a guanylate cyclase, induces generation of cGMP, and exerts multiple effects.9 We have previously shown that adenovirus-mediated local expression of CNP leads to a potent inhibition of neointima formation in balloon-injured rat carotid arteries, although CNP shows a modest inhibitory effect on cell proliferation in vitro.10 CNP may exert various functions in arterial walls. Nitric oxide (NO), which also increases cGMP, produces diverse effects including suppression of platelet aggregation,11 as well as inhibition of adhesion of neutrophils and monocytes to vessel walls.12,13 These facts suggested to us that CNP might also have antithrombotic and antiinflammatory effects, and that those effects may contribute to its potent inhibitory effect on neointima formation. In this study, we found that local expression of CNP does indeed exert these effects. Moreover,
we found that CNP increases NO production by enhancing the expression of inducible NO synthase (iNOS), and that the antithrombotic and antiinflammatory effects, as well as the inhibitory effect on neointima formation of CNP, are largely NO-dependent.

**Materials and Methods**

**Preparation of Adenoviral Vectors**

Replication-defective E1\(^-\) and E3\(^-\) adenoviral vectors expressing human CNP (AdCNP),\(^{10}\) human TFPI (AdTFPI),\(^{6,7}\) or bacterial \(\beta\)-galactosidase (AdLacZ)\(^{7,10}\) under a CA promoter (comprising a cytomegalovirus enhancer and chicken \(\beta\)-actin promoter\(^{7}\)) were prepared, as described previously.\(^{6,7}\) The titer of the virus stock was assessed by a plaque-formation assay using 293 cells and expressed in plaque formation units (pfu).

**Animal Experiment**

All animals were treated under protocols approved by the institutional animal care committee. The experiments were carried out in accordance with the institutional Guidelines for Animal Experiments and the Law (No. 105) and Notification (No. 6) issued by the Japanese government. We used a total of 135 rabbits and 48 rats for this study. Japanese White rabbits (male, weighing 3750±250 g; Biotech Co Ltd, Saga, Japan) were treated, balloon-injured within their carotid arteries, and gene transferred, as previously described.\(^{6,7}\)

The injured arteries were infected with one of the aforementioned adenoviruses (0.2 mL of 7.5×10\(^3\) pfu/mL) or infused with saline for 20 minutes. Carotid arteries in rats (male Wistar, weighing 450 to 500 g; Kyudo Co Ltd, Saga, Japan) were also injured and infected, as previously described,\(^{10}\) and used for RT-PCR and immunohistostaining against various types of NOS. Expression of CNP and TFPI after gene transfer was as reported previously.\(^{6,7,10}\)

Seven days after injury plus gene transfer, the recurrent formation and dislodgment of thrombus under shear stress were examined by measuring carotid blood flow, as described previously.\(^6\) Reduction of \(\approx\)50\% in diameter, \(\approx\)75\% in area, and \(\approx\)55\% in blood flow was generated by a constrictor in 6 to 9 vessels for each group. Heart rate and femoral artery pressure were also monitored. Three vessels for each group were treated with one of the following agents: acetylsalicylate, an inhibitor of thromboxane formation (bolus, 1 to 4 mg/kg body weight [BW]), N\(^-\)nitro-L-arginine methyl ester (L-NNAME), an NOS inhibitor (bolus, 3 to 100 mg/kg BW), or epinephrine (infused, 0.2 to 1.0 \(\mu\)g/kg BW/min). In some other rabbits (6 to 8 vessels for each group), L-NNAME was added to the drinking water (500 mg/L) for 8 to 24 days from 1 day before injury until sacrifice.

**Histological Analyses**

Arteries were frozen, sectioned, fixed in cold acetone, then subjected to immunohistostaining using antibodies raised against rabbit intracellular adhesion molecule-1 (ICAM-1) or vascular cell adhesion molecule-1 (VCAM-1) (a generous gift from Dr M. Cybulsky, University of Toronto, Toronto, Ontario, Canada) or rabbit macrophages (RAM 11; Dako, Calif). Detection of iNOS, endothelial NOS (eNOS), or neural NOS (nNOS) (all purchased from Santa Cruz Biotechs) was performed in rat carotid arteries, because no antibodies capable of detecting those proteins in rabbits were available. Immunoreactive protein was visualized by use of a biotinylated second antibody, peroxidase-labeled streptavidin, and diaminobenzidine. The sections were faintly counterstained with hematoxylin. Semi-quantitative analyses of ICAM-1 and VCAM-1 were carried out morphometrically with the aid of an automated computer-based image analyzer (Digitizer KD 4600; Graphtec Corp) in 6 vessels for each group. A color threshold mask for immunostaining was defined to detect the brown color by sampling, and the same threshold was applied to all samples. The lesion area with brown color was recorded. Analyses were done in randomly selected sections from 3 blocks per vessel by a technician blinded to the treatment regimen. Infiltration of macrophages was assessed by counting the RAM 11–positive cells.

Three vessels for each group were examined under the scanning electron microscope, as described previously.\(^7\)

**Reverse Transcription–Polymerase Chain Reaction of iNOS**

Rat carotid arteries were balloon-injured and infected with AdLacZ or AdCNP or infused with saline and harvested 3 days later (4 vessels for each group). Total RNA was extracted from the vessels, then reverse-transcribed using a Ready-to-Go reverse transcription–polymerase chain reaction (RT-PCR) kit (Pharmacia Biotech) with oligo(dT) primers. Then, the sequence of iNOS was amplified by PCR (annealing at 55°C for 1 minute, extension at 72°C for 1 minute, 30 cycles), generating a 367-bp product. The sense primer for rat iNOS was 5’-AAATGCGAGGATGGTTCCG-3’ at location 3094 to 3113. The antisense primer was 5’-GTCTTTGCTTGGG-GCTCC-3’ at location 3459 to 3440 (Genebank D14051). Primers for rat GAPDH,\(^{15}\) 5’-AATGCATCCTGACACCCAATGC-3’ (sense), and 5’-GGAGGCATGGAGCCATGAGTC-3’ (antisense) were also included in each reaction (annealing at 55°C for 1 minute of 25 cycles) as an internal control, resulting in a product of 555 bp. The data were densitometrically analyzed using NIH Image.

**NO Production**

To measure in vivo NO production, segments of rabbit carotid arteries (2 cm in length, 6 vessels for each group) were harvested 7 days after injury plus gene transfer, then placed in 1 mL of serum-free DMEM at 37°C for 24 hours. Media were then assayed for nitrates using a Griess reagent\(^{16}\) and a chemiluminescence analyzer (Sievers 270B).

**Statistical Analysis**

Data are expressed as mean±SEM. Statistical analysis of differences was performed using a two-way ANOVA and Fisher’s multiple comparison test (\(P<0.05\) being considered significant).

**Results**

**Elimination of Shear Stress–Induced Thrombosis in AdCNP-Infected Arteries Even in the Presence of Catecholamine**

We investigated whether local expression of CNP would effectively inhibit shear stress–induced thrombosis in injured arteries. Seven days after balloon injury (plus gene transfer), in which injury-induced inflammatory response reaches a submaximal level, we added shear stress using a constrictor and measured cyclic flow variation (CFV), which is thought to reflect recurrent cycles of thrombus formation and dislodgment.\(^{6,17}\) There were no significant differences among the arteries tested in terms of the change in carotid blood flow before (41.6±8.1\%, 41.3±6.9\%, 39.4±6.4\%, and 40.5±6.5\% in AdLacZ-, AdTFPI-, and AdCNP-infected and saline-infused groups, respectively; \(P>0.05\)) and immediately after the establishment of stenosis (20±5.3\%, 18.7±3.3\%, 17.1±2.8\%, and 18.5±3.2\%, respectively; \(P>0.05\)). After stenosis, CFV was observed in all AdLacZ-infected (12.4±4 cycles/h). Figures 1A and 1C) and in saline-infused arteries (12.0±2.4 cycles/h). In contrast, no CFV was detectable in AdCNP-infected (Figures 1B and 1E) or AdTFPI-infected arteries (Figure 1D). This inhibitory effect of CNP was intact in the presence of epinephrine at up to 1.0 \(\mu\)g/kg BW/min (Figure 1B). When this dose of epinephrine was exceeded, the rabbits died. The CFV seen in AdLacZ-infected arteries disappeared with acetylsalicylate
systemic bolus injection); however, it reappeared with infusion of epinephrine (Figure 1A).

Seven days after injury plus gene transfer, arteries exposed to shear stress were subjected to electron microscopic analysis. The luminal surface of AdLacZ-infected arteries was covered with a large quantity of aggregated platelets and fibrin in which many erythrocytes were entrapped (Figure 2, right). In contrast, in AdCNP-infected arteries (Figure 2, left), as well as in AdTFPI-infected arteries, there was only a monolayer of spread-out platelets, with neither platelet aggregation nor fibrin formation being observed.

**Injury-Induced Inflammation Is Reduced in AdCNP-Treated Arteries**

We hypothesized that CNP might have an antiinflammatory effect in addition to its antithrombotic action. To test this idea, we examined macrophage infiltration and the expression levels of the inflammatory cytokine-regulated adhesion molecules, ICAM-1 and VCAM-1. The expressions of these molecules were detectable 1 day after injury and reached submaximal levels around 7 days after injury, as previously reported in rabbit arteries. Hence, having treated arteries in various ways, we examined them on the seventh day after injury. Representative immunohistostainings are shown in Figure 3. In saline-infused and AdLacZ-infected arteries, ICAM-1 and VCAM-1 were readily detectable in the medial layers. Interestingly, in AdTFPI-infected arteries, similar expressions of those molecules were observed. In contrast, in AdCNP-infected arteries, the expressions of ICAM-1 and VCAM-1 were substantially reduced in terms of both staining area and intensity. Likewise, macrophage infiltration was reduced in AdCNP-treated arteries.

The immunohistostaining results were analyzed in a semi-quantitative fashion (Figure 4). There was no significant difference in terms of the staining areas for ICAM-1 and VCAM-1 or the number of infiltrated macrophages among saline-, AdLacZ-, and AdTFPI-treated arteries. However, all three variables showed significant reductions in AdCNP-treated arteries.

**Increased iNOS Expression and NO Generation in AdCNP-Infected Arteries**

Because there have been two reports that cGMP upregulates the gene expression for inflammatory cytokine-induced iNOS in smooth muscle cells in vitro, we examined the hypothesis that iNOS expression would be enhanced in AdCNP-treated arteries in vivo. For immunohistostaining analyses against various types of NOS in arteries 7 days after balloon injury and gene transfer, we used balloon-injured rat carotid arteries for the reason stated in Materials and Methods. As shown in Figure 5, iNOS protein, but not eNOS or nNOS protein, was indeed detectable only in the media of the AdCNP-infected arteries. It should also be noted that neointima formation (Figures 5A through 5D) was suppressed in AdCNP-treated rat carotid arteries, as previously observed. To confirm enhanced expression of iNOS in the AdCNP-
infected arteries, we semiquantitated iNOS mRNA by RT-PCR assay using total RNA extracted from rat arteries 3 days after injury and gene transfer. A densitometric analysis shows that iNOS mRNA level was increased 3 times in AdLacZ-infected or saline-infused injured arteries compared with that in intact arteries but 17 times in the AdCNP-treated ones (Figures 5E and 5F).

Next, we quantitated the NO generated from rabbit carotid arteries ex vivo (7 days after injury and gene transfer) (Figure 6). Balloon injury itself increased NO generation (195±32% versus the basal level). In AdCNP-infected arteries, NO generation was further enhanced (305±27%, P<0.05), but no such enhancement was observed in either AdLacZ- (187±27%) or AdTFPI-infected arteries (182±25%).

**Antithrombotic and Antiinflammatory Effects of CNP Are Dependent on NO**

Having established that CNP significantly enhances iNOS expression and NO production in vivo, we tested whether the antithrombotic and antiinflammatory effects of CNP might be dependent on NO. In rabbits in which L-NAME, an NOS inhibitor, was injected via the ear vein, CFV did appear in AdCNP-infected arteries (Figure 1E), whereas L-NAME had no effect on the suppression of CFV achieved using acetyl-salicylate (Figure 1C) or TFPI (Figure 1D).

L-NAME was given to rabbits via their drinking water for 8 days (starting 1 day before injury and gene transfer) (Figure 6). Balloon injury itself increased NO generation (195±32% versus the basal level). In AdCNP-infected arteries, NO generation was further enhanced (305±27%, P<0.05), but no such enhancement was observed in either AdLacZ- (187±27%) or AdTFPI-infected arteries (182±25%).

L-NAME was given to rabbits via their drinking water for 8 days (starting 1 day before injury). NO production from the AdCNP-infected arteries was indeed reduced (176±21%) to the level similar to those from AdLacZ- or AdTFPI-infected and saline-infused arteries (Figure 6). Interestingly, NO production from AdLacZ-infected arteries was not significantly altered with L-NAME in our setting. Under L-NAME treatment, the expressions of ICAM-1 and VCAM-1 and the infiltration of macrophages in AdCNP-treated arteries were markedly increased and reached levels that were indistinguishable from those seen in saline-infused or AdLacZ-infected arteries (Figures 3 and 4), in which the staining levels were not changed by treatment with L-NAME (Figure 4). These results were in accordance with the amount of NO produced from the arteries (Figure 6).

**CNP Suppresses Neointimal Formation Largely Through NO Production**

Twenty-three days after injury (plus gene transfer), we histologically examined neointima formation in the rabbit carotid arteries. As shown in Figure 7, neointima formation was significantly reduced in AdCNP-treated arteries in an NO-dependent manner. Similar results were also obtained in balloon-injured rat carotid arteries (data not shown).

**Discussion**

In our previous study, we found that adenovirus-mediated expression of CNP has a potent inhibitory effect on neointima formation in vivo despite its only moderate inhibitory effect on cellular proliferation in vitro. We therefore assumed that
CNP has multiple actions. In the present study, we tested the hypothesis that local expression of CNP may show antithrombotic and antiinflammatory effects in injured arteries. We found that CNP eliminates shear stress–induced thrombosis even in the presence of submaximal doses of catecholamine (Figures 1 and 2), and that CNP greatly suppresses the injury-evoked expression of the adhesion molecules ICAM-1 and VCAM-1, as well as macrophage infiltration (Figures 3 and 4), suggesting that CNP can have an antiinflammatory effect. Furthermore, we observed that CNP increases iNOS gene expression (Figure 5) and leads to an enhanced production of NO (Figure 6), although the detailed mechanism of how CNP (and cGMP) enhances the transcription of iNOS is not elucidated. A similar finding that CNP augments the transcriptional activation of iNOS induced by inflammatory cytokines (a combination of interleukin-1 [IL-1] and tumor necrosis factor-α [TNF-α]) and hence the production of NO has been reported in rat aortic smooth muscle cells in vitro.20

Our study shows for the first time that this effect indeed occurs in vivo. Finally, we found that all inhibitory effects of CNP were canceled when NO production was blocked (Figures 1, 3, 4, 6, and 7), indicating that CNP exerts its therapeutic effects largely through enhancing NO generation. Our results show that local expression of CNP in injured arteries enhances iNOS gene expression and NO production and that this in turn inhibits inflammation and shear stress–induced thrombosis and suppresses neointima formation.

Although both CNP and TFPI eliminate shear stress–induced thrombosis, their actions are different. Overexpression of CNP enhances iNOS gene expression leading to an increased production of NO and thus suppresses thrombosis in an NO-dependent manner (Figure 1E). In contrast, the antithrombotic effect observed in AdTFPI-infected arteries was not affected at all by an inhibition of NO production (Figure 1D). Overexpression of TFPI did not show antiinflammatory effects. NO, with its lipophilic and free radical properties, diffuses three-dimensionally and rapidly away from the blood vessel wall into the blood. The details of the mechanisms by which NO inhibits inflammation and thrombosis are not yet fully elucidated. However, we do know that...
by activating soluble guanylate cyclase in the cytoplasm, NO increases cGMP in platelets and inhibits fibrinogen binding to the surface receptors as well as the phosphorylation of myosin light chain.\textsuperscript{21} It has also been reported that NO suppresses factor XIII activity\textsuperscript{22} and mediates cyclooxygenase activation,\textsuperscript{23} although the other steps underlying these responses remain unknown. Several agents have been reported to work synergistically with NO, including prostaglandin E,\textsuperscript{24} tissue-type plasminogen activator,\textsuperscript{24} and adenosine.\textsuperscript{25} It is possible that CNP directly increases cGMP and inhibits platelet activation through its binding to the CNP receptor of the platelets, although it is not known whether rabbit platelets actually have a receptor for CNP.

The link between inflammation in injured arterial walls and thrombosis is well recognized.\textsuperscript{26} VCAM-1 and ICAM-1 are adhesion molecules for various classes of leukocytes and are well-characterized markers of cellular activation and inflammation. The expression of such adhesion molecules on smooth muscle cells should facilitate transmigration of activated leukocytes and monocytes/macrophages into the vessel wall. Activated macrophages release soluble mediators of inflammation, such as IL-1 and TNF-α, which then further stimulate the expression of adhesion molecules in arterial-wall cells.\textsuperscript{27} Importantly, in our AdCNP-treated arteries, the expressions of VCAM-1 and ICAM-1 and the number of infiltrated macrophages were all markedly reduced, indicating that CNP suppresses inflammation. This antiinflammatory effect of CNP should contribute to the inhibition of thrombosis as well as to the suppression of neointima formation in AdCNP-treated arteries. Our finding that these antiinflammatory and antithrombotic effects of CNP are dependent on NO (Figures 1, 3, and 4) is consistent with the results of a previous in vitro study using smooth muscle cells from human aorta and saphenous vein.\textsuperscript{28} In that study, NO donors such as 3-morpholinosydnonimine and sodium nitroprusside, but not cGMP analogues, were shown to inhibit the IL-1-induced expression of ICAM-1 as well as the interferon-γ-induced expression of VCAM-1, in part, through inhibition of nuclear factor-κB.

The role of iNOS on neointima formation is not clear and is even controversial. Adenovirus-mediated gene transfer of iNOS inhibited neointima formation in injured rat and pig arteries.\textsuperscript{29} However, in iNOS knockout mice, VCAM-1 expression and neointima formation after periaortic injury were not increased, rather reduced, suggesting that iNOS expression after injury may promote inflammation and cell proliferation, leading to increased neointima formation.\textsuperscript{30} As the authors discussed,\textsuperscript{30} the discrepant results between these studies may be due to the difference in the amount of NO. NO enhances proliferation of vascular smooth muscle cells at low concentrations (likely achieved by injury-induced iNOS expression) but shows an antiproliferative effect at higher concentrations (probably achieved by iNOS gene transfer).\textsuperscript{31}

There remains a possibility that iNOS knockout mice would compensate by activating other kinds of NOS, and that NO production in arteries might be somewhat enhanced. On the other hand, too much NO could be harmful and might accelerate atherosclerosis, because NO can react with superoxide and form potent oxidants.\textsuperscript{32} Furthermore, it should be noted that iNOS can generate superoxide by its reductase domain, independent of NO production.\textsuperscript{33} In view of all of this, it would be interesting to measure NO (together with superoxide) produced from arteries subjected to gene transfer of either iNOS, eNOS, or CNP.

It was recently reported that adenovirus-mediated expression of CNP in balloon-injured rabbit vein grafts promotes reendothelialization and reduces luminal thrombus formation.\textsuperscript{34} We also observed that reendothelialization evaluated 7 days after injury by the same method (Evans Blue staining) as they used\textsuperscript{34} was accelerated in AdCNP-infected carotid arteries (35.9±4.6% [n=6] of luminal area was reendothelialized) compared with that in AdlacZ-infected ones (25.6±4.9%). However, this shows that a large area of arterial wall was not yet covered with endothelium at the time at which we performed the CFV study. When we tested 3 days after injury plus gene transfer, CFV was never observed in the AdCNP-treated arteries (data not shown). Thus, accelerated reendothelialization most likely plays only a minor role in the antithrombotic and antiinflammatory effects we observed in this study.

In summary, we found that local expression of CNP in injured arteries enhances iNOS gene expression and NO production and thereby reduces inflammation and eliminates shear stress–induced thrombosis. These NO-dependent antiinflammatory and antithrombotic effects of CNP may well contribute to its potent inhibitory effect on neointima formation and increase the potential clinical value of the local gene transfer of CNP after angioplasty. Which molecule is likely to be the most beneficial among ones including NOS, TFPI, hirudin, tissue plasminogen activator, cyclooxygenase, and CNP and whether any combination of those antithrombotic molecules would produce an additional therapeutic effect are interesting questions.

**References**


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