Adrenomedullin Receptor Antagonism by Calcitonin Gene-Related Peptide(8-37) Inhibits Carotid Artery Neointimal Hyperplasia After Balloon Injury

Koichi Shimizu, Hiroyuki Tanaka, Makoto Sunamori, Fumiaki Marumo, Masayoshi Shichiri

Abstract—Intimal injury by angioplasty results in a series of changes, including smooth muscle cell hyperplasia, that lead to vascular restenosis. Adrenomedullin, a potent vasodilator peptide, has natriuretic effects, and its plasma concentration is elevated in cardiovascular diseases. Adrenomedullin is secreted by endothelial and vascular smooth muscle cells, but its role in neointimal hyperplasia after balloon injury has not been previously described. We investigated the role of endogenous adrenomedullin in neointimal hyperplasia using an in vivo rat model of postinjury vascular restenosis. In the injured rats, bromodeoxyuridine-labeled nuclei in the media of untreated common carotid arteries were increased 2 days after injury, which were suppressed by in vivo treatment with the adrenomedullin receptor antagonist calcitonin gene-related peptide (CGRP)(8-37). Inhibition of neointimal hyperplasia by CGRP(8-37) was distinct at 7 and 14 days, whereas CGRP(1-37) had no effect. The expression of adrenomedullin in the media of both untreated and treated common carotid arteries was elevated at 2 days and further enhanced in hyperplastic intima of untreated common carotid arteries at 7 days. Our findings suggest a novel role for endogenous adrenomedullin in balloon injury–induced restenosis and indicate that CGRP(8-37) may be useful for the prevention of vascular restenosis. (Circ Res. 1999;85:1199-1205.)

Key Words: balloon angioplasty ■ adrenomedullin ■ CGRP(8-37) ■ remodeling ■ neointimal hyperplasia

Adrenomedullin is a potent, 52-amino acid vasorelaxant peptide originally isolated from human pheochromocytoma.1 It has an evolutionarily conserved structure and function among mammals,2 is part of the supergene family with calcitonin, calcitonin gene-related peptide (CGRP), and amylin, and has 24% amino acid homology with CGRP.3 Adrenomedullin is synthesized and secreted in a variety of tissues, including endothelial cells4 and vascular smooth muscle cells (VSMCs).5 CGRP, on the other hand, is not produced by vascular cells,6 but is densely distributed in the central nervous system,7 and it is released from nerve terminals.8 Whether adrenomedullin stimulates or inhibits mitogenesis depends on the particular cell type. Adrenomedullin stimulates the proliferation of fibroblasts9 and certain tumor cell lines,10 inhibits proliferation of rat mesangial cells,11,12 and prevents apoptosis of endothelial cells.13 It has recently been shown that adrenomedullin and, to a lesser degree, CGRP act as growth-promoting factors for rat VSMCs via protein tyrosine kinase-mediated extracellular signal–regulated kinase/mitogen-activated protein kinase activation.14

CGRPs are a group of peptides with vasodilatory action. Receptor activity–modifying proteins (RAMPs) have recently been shown to transport calcitonin receptor–coupled CGRP receptor, whereas RAMP2–transported receptors are adrenomedullin receptors.15,16 CGRP(8-37) is a truncated version of CGRP(1-37) and acts as a receptor antagonist of both adrenomedullin and CGRP(1-37).15 Although rat homologues of RAMPs and CRLR have not yet been cloned, rat VSMCs also express functional adrenomedullin receptors to which CGRP(8-37) binds with high affinity.14,17,18

VSMC hyperplasia after percutaneous transluminal coronary angioplasty (PTCA) is a component of vascular restenosis,19 and VSMCs constitute an important element of advanced occlusive atherosclerotic lesions in humans.20,21 Although the initial events, time course, and pathophysiological outcome of post-PTCA neointimal hyperplasia have been well characterized, restenosis remains resistant to therapy. Vasoactive factors such as endothelin-1, angiotensin II, catecholamines, and several cytokines are involved in the development of neointimal hyperplasia,22–24 but a similar role for adrenomedullin has not been previously described.

These findings prompted us to consider the potential role of adrenomedullin as a previously unappreciated endogenous factor responsible for the development and/or maintenance of

Received July 29, 1999; accepted September 21, 1999.
From the Department of Cardiothoracic Surgery (K.S., H.T., M. Sunamori) and the Second Department of Internal Medicine (F.M., M. Shichiri), Tokyo Medical and Dental University, Tokyo, Japan.
Correspondence to Masayoshi Shichiri, MD, Second Department of Internal Medicine, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8519, Japan, E-mail mshichiri.med2@med.tmd.ac.jp
© 1999 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org
Figure 1. Photomicrographs showing representative cross sections of the common carotid arteries 2 days after balloon injury. A, C, and E, Untreated arteries; B, D, and F, arteries treated with CGRP(8-37). A and B, Immunohistochemical staining for adrenomedullin. C through F, BrdU labeling. Magnification, ×400 (A through D), and ×100 (E and F).

Figure 2. Photomicrographs showing representative cross sections of the common carotid arteries 7 days after balloon injury. A and B, Untreated arteries; C and D, arteries treated with CGRP(8-37); E and F, arteries treated with CGRP(1-37). Magnification, ×100 (A, C, and E) and ×400 (B, D, and F). Arrows indicate internal elastic laminae.
vascular restenosis. The present study is designed to assess adrenomedullin/CGRP receptor antagonism by CGRP(8-37) as a treatment for neointimal hyperplasia after vascular injury.

Materials and Methods

Common Carotid Artery Balloon Angioplasty

Left common carotid artery balloon injury was performed aseptically in anesthetized animals (sodium pentobarbital, 50 mg/kg IP) as described previously. Deendothelialization of the left common carotid artery was accomplished by withdrawal and advancement of the inflated balloon 3 times, with rotation of the catheter 120° after each withdrawal. Two, 7, or 14 days after balloon injury, the animals were euthanized, and the right and left common carotid arteries were excised and each divided into two 5-mm segments. One segment was fixed with 10% formaldehyde for morphometric analysis, whereas the other was frozen at -80°C for immunohistochemistry.

Drug Treatment

Drugs were infused after balloon injury using independent miniaturized osmotic pumps implanted in dorsal and ventral subcutaneous pockets. A pump in the ventral lower pocket delivered bromodeoxyuridine (BrdU; 32 μmol/kg body weight per minute), and the other, in the dorsal pocket, delivered CGRP(8-37), CGRP(1-37), or distilled water. Three groups of rats received CGRP(8-37) as a 25 pmol/kg per minute continuous subcutaneous infusion until euthanasia at 2 (n=5), 7 (n=6), or 14 days (n=5) and 60 nmol/kg subcutaneous bolus injections once daily for 3 days, commencing immediately after balloon injury. Four groups of rats served as controls: 6 rats received an equal volume (bolus and infusion) of CGRP(1-37) and were euthanized at 7 days; the other 3 groups received an equal volume of distilled water until euthanized at 2 (n=5), 7 (n=6), or 14 days (n=5), respectively.

Morphometric Analysis

Two carotid artery tissue segments from each animal were stained with hematoxylin and eosin. The internal elastic lamina was used as the border to distinguish the neointima from the media. The mean cross-sectional area of the intima was calculated by subtracting the mean area of the lumen from the mean area enclosed by the internal elastic lamina. The mean cross-sectional area of the media was calculated by subtracting the mean area enclosed by the internal elastic lamina from the mean area enclosed by the external elastic lamina.

Immunohistochemistry for Adrenomedullin and BrdU

Sections of frozen arterial tissue were stained immunohistochemically with either monoclonal mouse anti-BrdU (DAKO Corp; 1:30 dilution) or polyclonal rabbit anti-adrenomedullin (Peptide Institute; 1:100 dilution). Omission of primary antibody and incubation with type- and class-matched irrelevant immunoglobulin served as negative controls. The ratio of BrdU-positive nuclei to the total number of VSMCs served as a BrdU labeling index.

Enzyme Immunoassay for CGRP/CGRP(8-37)

Arterial blood samples were obtained via the femoral artery 24 and 48 hours after balloon injury for determination of plasma immunoreactive CGRP/CGRP(8-37) using an enzyme immunoassay (Penin-
sular Laboratories). The polyclonal anti-human CGRP antibody cross-reacts fully with both rat CGRP and human CGRP(8-37), but not with adrenomedullin, amylin, calcitonin, somatostatin, or other related peptides.

**Statistical Analysis**

Data are expressed as mean±SEM. Differences between groups were examined for statistical significance using the unpaired Student t test.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

**Effect of CGRP(8-37) on Neointimal Hyperplasia**

None of the experimental animals died or developed thrombosis. Uninjured arteries maintained an intimal monolayer of endothelial cells and an intact medial layer. In rats with injured arteries, intimal thickening was not observed at 2 days (Figure 1), whereas marked neointimal hyperplasia developed in untreated arteries within 7 days (Figure 2A and 2B), which further progressed at 14 days (Figure 3A and 3B). Administration of CGRP(8-37) markedly reduced the number of BrdU-labeled cells (Figure 1D and 1F). The labeling index as calculated from the percentage of BrdU-labeled cells relative to the total number of cells revealed marked suppression of DNA replication by CGRP(8-37) (untreated, 63±9%; versus CGRP(8-37) treated, 34±8%; P<0.05, n=5). At 7 days, BrdU-positive nuclei were seen mostly in the neo-intima of untreated arteries, but again markedly reduced in CGRP(8-37)-treated arteries (Figure 5). Quantification of BrdU-positive nuclei showed a 68% decrease in the neo-intima of CGRP(8-37)-treated arteries, although the number of BrdU-labeled nuclei remained unchanged in the media. However, despite the marked decrease in the absolute number of BrdU-labeled cells in the neo-intima of treated cells in the neo-intima of untreated arteries, the BrdU labeling index in untreated and CGRP(8-37)-treated sections was not different within the neo-intima (76±7% versus 62±6%; P=0.184, n=6) and media (52±4% versus 47±7%; P=0.451, n=6).

**Expression of Adrenomedullin**

Immunohistochemical staining showed intense adrenomedullin expression in the medial VSMCs of both the untreated and CGRP(8-37)-treated arteries as early as 2 days (Figure 1A and 1B). Adrenomedullin was expressed to a lesser extent in fibroblasts in the adventitia (Figure 1A and 1B). Adrenomedullin staining was detected diffusely throughout the thickened neo-intima most intensely at 7 days (Figure 6B) and less but still intensely at 14 days (Figure 6C), appearing as abundant, fine granules in nearly all neointimal cells. Adrenomedullin staining was detected at a lesser intensity in the untreated media (Figure 6B and 6C) and injured, CGRP(8-37)-treated neo-intima (Figure 6E and 6F), and minimal staining was detected in treated media (Figure 6E and 6F) and uninjured endothelium (Figure 6D).

**Plasma CGRP/CGRP(8-37) Concentrations**

Plasma CGRP/CGRP(8-37)-like immunoreactivity before injury was 0.54±0.23 ng/mL, and untreated animals showed slightly increased levels after injury (0.92±0.09 ng/mL at 24 hours, 0.86±0.16 ng/mL at 48 hours) representing endogenous CGRP levels. In CGRP(8-37)-treated animals, the values were markedly higher at 3.26±0.84 ng/mL at 24 hours and 19.47±4.75 ng/mL at 48 hours.

**Discussion**

We have demonstrated in the present study that administration of CGRP(8-37), an adrenomedullin receptor antagonist, suppressed neointimal hyperplasia of the rat carotid arteries suppress or enhance neointimal hyperplasia (Figure 2E and 2F). The suppressive effect of CGRP(8-37) on neointimal hyperplasia was also clear even at 14 days; the neo-intima:media cross-sectional area ratio was 47% less in the CGRP(8-37)-treated group (Figures 3C and 3D and 4B).

**Effect of CGRP(8-37) on DNA Replication**

In the next step, we assayed DNA replication in medial VSMCs at 2 days by BrdU incorporation. BrdU-labeled nuclei were already abundant at 2 days in the media of injured, untreated arteries (Figure 1C and 1E), whereas CGRP(8-37) markedly reduced the number of BrdU-labeled cells (Figure 1D and 1F). The labeling index as calculated from the percentage of BrdU-labeled cells relative to the total number of cells revealed marked suppression of DNA replication by CGRP(8-37) (untreated, 63±9%; versus CGRP(8-37) treated, 34±8%; P<0.05, n=5). At 7 days, BrdU-positive nuclei were seen mostly in the neo-intima of untreated arteries, but again markedly reduced in CGRP(8-37)-treated arteries (Figure 5). Quantification of BrdU-positive nuclei showed a 68% decrease in the neo-intima of CGRP(8-37)-treated arteries, although the number of BrdU-labeled nuclei remained unchanged in the media. However, despite the marked decrease in the absolute number of BrdU-labeled cells in the neo-intima of treated cells in the neo-intima of untreated arteries, the BrdU labeling index in untreated and CGRP(8-37)-treated sections was not different within the neo-intima (76±7% versus 62±6%; P=0.184, n=6) and media (52±4% versus 47±7%; P=0.451, n=6).

**Expression of Adrenomedullin**

Immunohistochemical staining showed intense adrenomedullin expression in the medial VSMCs of both the untreated and CGRP(8-37)-treated arteries as early as 2 days (Figure 1A and 1B). Adrenomedullin was expressed to a lesser extent in fibroblasts in the adventitia (Figure 1A and 1B). Adrenomedullin staining was detected diffusely throughout the thickened neo-intima most intensely at 7 days (Figure 6B) and less but still intensely at 14 days (Figure 6C), appearing as abundant, fine granules in nearly all neointimal cells. Adrenomedullin staining was detected at a lesser intensity in the untreated media (Figure 6B and 6C) and injured, CGRP(8-37)-treated neo-intima (Figure 6E and 6F), and minimal staining was detected in treated media (Figure 6E and 6F) and uninjured endothelium (Figure 6D).

**Plasma CGRP/CGRP(8-37) Concentrations**

Plasma CGRP/CGRP(8-37)-like immunoreactivity before injury was 0.54±0.23 ng/mL, and untreated animals showed slightly increased levels after injury (0.92±0.09 ng/mL at 24 hours, 0.86±0.16 ng/mL at 48 hours) representing endogenous CGRP levels. In CGRP(8-37)-treated animals, the values were markedly higher at 3.26±0.84 ng/mL at 24 hours and 19.47±4.75 ng/mL at 48 hours.

**Discussion**

We have demonstrated in the present study that administration of CGRP(8-37), an adrenomedullin receptor antagonist, suppressed neointimal hyperplasia of the rat carotid arteries suppress or enhance neointimal hyperplasia (Figure 2E and 2F). The suppressive effect of CGRP(8-37) on neointimal hyperplasia was also clear even at 14 days; the neo-intima:media cross-sectional area ratio was 47% less in the CGRP(8-37)-treated group (Figures 3C and 3D and 4B).
after balloon injury. Neointimal hyperplasia developed within 7 days and was dramatically suppressed by CGRP(8-37); at 7 days after injury, the neointimal cross-sectional area was reduced by 61%, whereas that of the media was unaltered. BrdU-positive nuclei, mostly observed in the neointima of untreated arteries, were markedly reduced in CGRP(8-37)-treated arteries, whereas the number of BrdU-labeled nuclei in the media was unchanged between the 2 groups. Labeling index of BrdU-positive nuclei of neointimal VSMCs was 76% in untreated arteries, suggesting that the markedly increased total number of neointimal cells is mainly a result of VSMC proliferation.

Injury is known to cause medial VSMC proliferation occurring as early as 2 days. In the present study, CGRP(8-37) markedly reduced the number of BrdU-labeled nuclei in medial VSMCs at 2 days, indicating its antiproliferative effect. Migration of VSMCs from the media to the intima begins at ~2 to 3 days. However, adrenomedullin is reported to act as an antimigratory factor in cultured rat VSMCs. Accordingly, it is difficult to consider the role of CGRP(8-37) as an antagonist to the migration of medial VSMCs. Between 7 and 14 days, neointimal thickening progresses with VSMC proliferation and extracellular matrix accumulation. In the present study, although the inhibitory effect was still distinct at day 14, the density of neointimal VSMCs per mm² was not different between the 2 groups, suggesting that the difference in neointimal size was not due to a marked increase in the tissue matrix component. Therefore, it is difficult to ascribe the difference in neointimal thickening at day 14 to the suppressive effect of CGRP(8-37) on matrix expansion. Taken together, the results indicate that marked inhibition of neointimal hyperplasia by CGRP(8-37) is mainly due to its VSMC antiproliferative effect.

To our knowledge, in vivo expression of adrenomedullin after balloon injury has not been previously demonstrated. The intense expression of adrenomedullin in the medial VSMCs as early as 2 days after balloon injury was followed by intense neointimal expression and less intense expression on the medial VSMCs at 7 days. These results suggest a paracrine role of endogenous adrenomedullin in the event of vascular injury. Explanted rat VSMCs in culture are reported to express high-affinity adrenomedul-
CGRP receptors \( (K=0.3\times10^{-8} \text{ mol/L}) \) to which CGRP(8-37) competitively binds.\(^{16}\) CGRP has been shown to bind to the adrenomedullin receptor with a lower affinity\(^{18}\) and to induce proliferation of cultured quiescent VSMCs only at high concentrations \( (10^{-7}-10^{-6} \text{ mol/L}) \).\(^ {14}\) However, the present enzyme immunoassay revealed a low plasma CGRP concentration \( (0.54 \text{ ng/mL}=0.5\times10^{-10} \text{ mol/L}) \). These data suggest a limited role for endogenous CGRP in VSMC proliferation. This notion is further supported by the observation that administration of CGRP for 7 days did not enhance neointimal thickening. Administration of CGRP(8-37) caused a marked increase in plasma CGRP(8-37) level and is considered to have antagonized the binding of endogenous adrenomedullin. Considered together, our results suggest that postinjury neointimal hyperplasia is more likely due to the action of actively produced endogenous adrenomedullin on the injured tissue and that CGRP(8-37), which may antagonize both adrenomedullin and CGRP receptors, likely mediates its biological effects mainly through the adrenomedullin receptor.

Several agents previously demonstrated to be effective in animal models were later found to have a poor effect in humans.\(^ {29}\) This disparity underscores the complexity of interspecies differences in the pathophysiology of arterial hyperplasia. Our study is the first demonstration of the beneficial effects of an adrenomedullin receptor antagonist in an animal model of vascular restenosis after balloon injury. Although the efficacy of adrenomedullin receptor antagonism should be further investigated before CGRP(8-37) is considered as a candidate for adjunct therapy to PTCA in humans, our findings point to a novel therapeutic strategy for the prevention of neointimal hyperplasia.

**Acknowledgment**

This study was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan and by a Grant from Japan Cardiovascular Research Foundation.

---

**References**


---

**Figure 6.** Photomicrographs showing immunohistochemical staining for adrenomedullin 7 days after injury. A, Untreated (omission of primary antibody); B, untreated at 7 days; C, untreated at 14 days; D, uninjured normal vessel; E, treated with CGRP(8-37) at 7 days; F, treated with CGRP(8-37) at 14 days. Magnification, \( \times 400 \). Arrows indicate internal elastic lamina.
Adrenomedullin Receptor Antagonism by Calcitonin Gene-Related Peptide(8-37) Inhibits Carotid Artery Neointimal Hyperplasia After Balloon Injury
Koichi Shimizu, Hiroyuki Tanaka, Makoto Sunamori, Fumiaki Marumo and Masayoshi Shichiri

Circ Res. 1999;85:1199-1205
doi: 10.1161/01.RES.85.12.1199

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/85/12/1199

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/1999/12/03/85.12.1199.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/