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Original article

# Endogenous CGRP protects against neointimal hyperplasia following wire-induced vascular injury

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ABSTRACT

Neointimal hyperplasia is the primary lesion underlying atherosclerosis and restenosis after percutaneous coronary intervention. Calcitonin gene-related peptide (CGRP) is produced by alternative splicing of the primary transcript of the calcitonin/CGRP gene. Originally identified as a strongly vasodilatory neuropeptide, CGRP is now known to be a pleiotropic peptide widely distributed in various organs and tissues. Our aim was to investigate the possibility that CGRP acts as an endogenous vasoprotective molecule.

We compared the effect of CGRP deficiency on neointimal formation after wire-induced vascular injury in wild-type and CGRP knockout (CGRP−/−) mice. We found that neointimal formation after vascular injury was markedly enhanced in CGRP−/− mice, which also showed a higher degree of oxidative stress, as indicated by reduced expression of nitric oxide synthase, increased expression of p47phox, and elevated levels of 4HNE, as well as greater infiltration of macrophages. In addition, CGRP-deficiency led to increased vascular smooth muscle cell (VSMC) proliferation within the neointima. By contrast, bone marrow-derived cells had little or no effect on neointimal formation in CGRP−/− mice. In vitro analysis showed that CGRP-treatment suppressed VSMC proliferation, migration, and ERK1/2 activity.

These results clearly demonstrate that endogenous CGRP suppresses the oxidative stress and VSMC proliferation induced by vascular injury. As a vasoprotective molecule, CGRP could be an important therapeutic target in cardiovascular disease.



## 1. Introduction

Neointimal hyperplasia is the primary lesion underlying atherosclerosis and restenosis after percutaneous coronary intervention [1]. Accumulating evidence now shows that inflammatory cell infiltration [2,3], proliferation/migration of vascular smooth muscle cells (VSMCs) [4,5], and deposition of extracellular matrix [6] all contribute to the pathogenesis of neointimal hyperplasia. Moreover, inflammatory cell infiltration of the neointima is intimately related to oxidative stress [7], and several lines of evidence indicate that NADPH oxidase plays a crucial role in the production of reactive oxygen species (ROS) within the neointima [8,9].

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide produced by alternative splicing of the primary transcript of the calcitonin/CGRP gene [10]. CGRP is widely distributed in the central and peripheral nervous systems and has been shown to exert a variety of effects within the cardiovascular system [11], including vasodilation, protection of endothelial cell function, and inhibition of VSMC proliferation [12–14]. CGRP also has pleiotropic functions that have been implicated in the regulation of cell proliferation [15] and differentiation [16]. The potent microvascular vasodilatory action of CGRP and its wide distribution in the periphery ensure that it is in a prime position to protect tissues from injury in several disease models [17,18], and to play a variety of roles under normal physiological conditions [19,20].

Based on its structural homology and similar vasodilatory effects, CGRP has been classified as an adrenomedullin (AM) family peptide. Endogenous AM has been shown to exert a protective effect during vascular response to injury [21]. For example, AM inhibits angiotensin II-induced proliferation and migration of VSMCs [22], possibly by inhibiting ROS production. Like AM, CGRP inhibits angiotensin II-induced VSMC proliferation through inactivation of the extracellular

*Abbreviations:* CGRP, Calcitonin gene-related peptide; VSMC, Vascular smooth muscle cell; EC, Endothelial cell; ROS, Reactive oxygen species; AM, Adrenomedullin; BMT, Bone marrow transplantation.

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signal-regulated protein kinase 1/2 (ERK1/2) signaling pathway [23] and inhibits hypoxia-induced proliferation of pulmonary artery SMCs via the ERK1/2/p27/c-fos/c-myc pathway [14].

To investigate the possibility that CGRP serves as an endogenous vasoprotective substance, in the present study we used CGRP knockout (CGRP−/−) mice for assessing the effect of CGRP deficiency on neointimal formation after vascular injury. We then tested whether inflammatory cell infiltration and ROS are involved in the development of neointimal hyperplasia, and because bone marrow-derived cells reportedly contribute to neointimal formation [24], we also tested a bone marrow transplantation (BMT) model using CGRP−/− mice. Finally, we investigated the effects of CGRP on the proliferation and migration of VSMCs.

## 2. Materials and methods

### 2.1. Animals

We generated CGRP knockout mice using a targeting DNA construct that replaced exon 5 encoding a CGRP-specific region [25]. C57BL/6 pure background mice were used for bone marrow transplantation (BMT) experiments. In other experiments, 129/Sv×57BL/6 hybrid background mice were used. Male CGRP−/− mice and their wild-type (WT) littermates mice were maintained under specific pathogen-free conditions in an environmentally controlled clean room at the Division of Laboratory Animal Research, Department of Life Science, Research Center for Human and Environmental Sciences, Shinshu University. All animal experiments were conducted in accordance with the ethical guidelines of Shinshu University.

### 2.2. Wire-induced vascular injury

Wire-induced vascular injury of the right femoral artery was produced as described previously by Sata et al. [26]. We confirmed that this procedure induced reproducible neointimal formation in 12- to 16-week-old 129/Sv×C57BL/6 mice. Some CGRP−/− mice underwent isolation of the femoral artery without wire-injury (sham-operated group).

### 2.3. Histology and immunohistochemistry

The femoral arteries were excised from each mouse, fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The arteries were then cut into 5-μm sections, which were stained with hematoxylin-eosin (HE) and Elastica Van Gieson (EVG). For immunohistochemical analysis, arterial sections were incubated with rat anti-mouse CD31 (BD Pharmingen, San Jose, CA), mouse anti-human α-smooth muscle actin (Dako), rat anti-mouse F4/80 (Serotec), rat anti-mouse 4-hydroxy-2-nonenal (4HNE) (NOF Corporation), rabbit anti-rat p67-phox (Upstate), mouse anti-rat PCNA (Dako), or mouse IgG2a (negative control) (Dako). DAPI (Invitrogen) was used to stain the nuclei.

For the evaluation of re-endothelialization after the wire-injury, endothelial cells (ECs) were identified by immunostaining with anti-CD31 antibody. The percentage of CD31-positive length to lumen perimeter in the femoral section was evaluated. Representative 1 section from 6 cross-sections from each mouse was used to calculate the percentage.

### 2.4. RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from tissues or cells using TRIZOL Reagent (Invitrogen, Carlsbad, CA), after which the sample was treated with DNA-Free (Ambion, Austin, TX) to remove contaminating DNA, and subjected to reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Semiquantitative RT-PCR was then carried out using Ex Taq DNA polymerase (Takara, Japan). Quantitative real-time RT-PCR was carried out using an Applied Biosystems 7300 real time PCR System (Applied Biosystems) with SYBR green (Toyobo, Japan) or Realtime PCR Master Mix (Toyobo) and TaqMan probe (MBL). Values were normalized to mouse GAPDH (Pre-Developed TaqMan®assay reagents, Applied Biosystems). Primers and probes are listed in Table 1.

### 2.5. Bone marrow transplantation

BMT model mice were produced as described previously [27]. For the study of BMT model mice, C57BL/6 pure background mice were generated by speed congenic method. Whole bone marrow cells were harvested from WT and CGRP−/− mice by flushing their femurs with PBS. Red blood cells were lysed by incubation in ACK buffer (150 mmol/L NH4Cl, 10 mmol/L KHCO3, 0.1 mmol/L EDTA, pH 7.2) for 20 min at 0 °C. The remaining cells were washed 3 times with PBS and resuspended in 1 mL of PBS. Recipient mice (C57BL/6 background; purchased from Charles River Laboratories Japan, Inc.; male, 7 to 8 weeks old) were lethally irradiated with a total dose of 9 Gy (MBR-155R2, Hitachi, Japan) and injected with bone marrow cells via the tail vein. Using this protocol, we produced 2 types of BMT mice: WT to WT ($BMT^{WT\rightarrow WT}$) and CGRP−/− to WT ($BMT^{CGRP-/-\rightarrow WT}$). After 8 weeks, all BMT mice underwent wire-induced vascular injury as described previously [27].

### 2.6. Cell culture

#### 2.6.1. Macrophages

WT and CGRP−/− mice were intraperitoneally injected with 2 ml of 3% thioglycolate medium (DIFCO) (Biobrás, Montes Claros, Brazil). After 3 days, macrophages were harvested by peritoneal lavage using cold PBS. The cells were counted, centrifuged and resuspended at a concentration of $1\times10^6$/mL in RPMI 1640 (Invitrogen) medium

**Table 1**
Primers and probes used for quantitative real-time RT-PCR.

| | | |
|---|---|---|
| CRLR | Forward | AGGCGTTTACCTGCACACACT |
| | Reverse | CAGGAAGCAGAGGAAACCCC |
| | Probe | ATCGTGGTGGCTGTGTTTGCGGAG |
| RAMP1 | Forward | GCACTGGTGGTCTGGAGGA |
| | Reverse | CCCTCATCACCTGGGATACCT |
| | Probe | CAAGCGCACAGAGGGCATCGTG |
| CGRP | Forward | GGAGCAGGAGGAAGAGCAG |
| | Reverse | TGCCAGCCGATGGGTCACA |
| TNF-α | Forward | ACGGCATGGATCTCAAAGAC |
| | Reverse | AGATAGCAAATCGGCTGACG |
| MCP-1 | Forward | GCAGTTAACGCCCCACTCA |
| | Reverse | CCTACTCATTGGGATCATCTTGCT |
| F4/80 | Forward | GATGAATTCCCGTGTTGTTGGT |
| | Reverse | ACATCAGTGTTCCAGGAGACACA |
| CCR2 | Forward | GCTCAACTTGGCCATCTCTGA |
| | Reverse | AGACCCACTCATTTGCAGCAT |
| CD68 | Forward | TGGCGGTGGAATACAATGTG |
| | Reverse | GAGATGAATTCTGCGCCATGA |
| TGFβ1 | Forward | CCCGAAGCGGACTACTATGC |
| | Reverse | TAGATGGCGTTGTTGCGGT |
| IL-6 | Forward | CCCAATTTCCAATGCTCTCC |
| | Reverse | TGAATTGGATGGTCTTGGTCC |
| IL-1β | Forward | TCTCACAGCAGCACATCAAC |
| | Reverse | TCGTTGCTTGGTTCTCCTTG |
| eNOS | Forward | AGGCACTGCTGAGCCGAGT |
| | Reverse | TTCTCCAGTTGTTCCACAGCC |
| P22 phox | Forward | GGCCATTGCCAGTGTGATCT |
| | Reverse | GCTCAATGGGAGTCCACTGC |
| P47 phox | Forward | ATCCTATCTGGAGCCCCTTGA |
| | Reverse | CACCTGCGTAGTTGGGATCC |
| P67 phox | Forward | CAGACCCAAAACCCCAGAAA |
| | Reverse | AAAGCCAAACAATACGCGGT |