Intramuscular gene transfer of CGRP inhibits neointimal hyperplasia after balloon injury in the rat abdominal aorta

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The invasive treatment of atherosclerosis is greatly limited by restenosis that occurs in >20% of patients after surgery. To investigate the effective pharmacological agents that prevent neointimal formation, studies have focused on the modulation of vascular smooth muscle cell (VSMC) proliferation and apoptosis. Targeting the VSMC cell cycle, gene transfer has provided promising results in preclinical experiments (2). However, approaches aimed at modulating VSMCs in atherosclerosis and restenosis are far from satisfactory and cannot be used for clinical applications. New agents with clear mechanisms of action, steady therapeutic effects with fewer side effects, and novel administration procedures are still major foci of cardiovascular research (26).

CGRP is a 37-amino acid neuropeptide with broad biological activity (1). It is considered the most potent intrinsic vasodilator in humans (3). Our recent studies (18, 27) and that of others (19) have revealed several new cell-protective roles of CGRP, including modulating immunoresponses, mediating ischemic preconditioning, and inhibiting VSMC proliferation. In particular, we have shown that CGRP and nitric oxide (NO) exhibit synergistic effects in inhibiting VSMC proliferation (32) and that NO also mediates a CGRP-induced vasorelaxant effect (33).

In addition to proliferation, apoptosis is also responsible for VSMC turnover in neointimal formation (30). VSMC apoptosis is modulated by many intrinsic agents, including vasoactive peptides, NO, reactive oxide species, and NF-κB (4, 8, 13, 17). Interestingly, NO and many vasoactive peptides, such as angiotensin II, endothelin, vasointestinal peptide, and adrenomedullin have opposite roles in modulating VSMC proliferation and apoptosis (8). For instance, adrenomedullin, a member of the CGRP superfamily, not only inhibits VSMC proliferation but also promotes apoptosis (8). However, it is unknown whether CGRP affects VSMC apoptosis.

The goal of the present study was to test the effect of CGRP on the rat model of restenosis induced by balloon injury and to investigate the underlying mechanism. The results from both in vivo and in vitro studies have shown that CGRP significantly reduces neointimal formation and promotes VSMC apoptosis. CGRP-induced apoptotic cell death is through an NO-mediated pathway and is associated with the upregulation of apoptotic proteins.

MATERIALS AND METHODS

Materials. Recombinant human α-CGRP (rh-α-CGRP), a truncated peptide of CGRP lacking the NH2-terminal 1–7 amino acid acting as a CGRP1 receptor antagonist (hCGRP8–37), and anti-CGRP antibody were purchased from Peninsula Laboratories (Belmont, CA). [Z-[1-[N-(2-aminoethyl)-N-(2-ammonoethyl)amino]diazen-1-ium-1,2-dioate (DETA-NONOate) was from Cayman Chemical (Ann Arbor, MI). 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was purchased from CalBiochem (La Jolla, CA). Thiorphan, N2-monomethyl-l-arginine (l-NMMA), staurosporin A, and 1H-[1,2,4]oxadiazolo[4,3-a]quinazolin-1-one (ODQ) were from Sigma (St. Louis, MO). Rp-cAMPS was purchased from RBI (Natick, MA). 2-(4-Carboxyphenyl)–4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (c-PTIO) was purchased from CalBiochem (La Jolla, CA). Anti-p53, anti-Bcl-2, anti-Bax, and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PCNA was purchased from Zymed (San Francisco, CA). Conjugates of secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). The DeadEnd fluorometric terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) system was purchased from Promega (Madison, WI). Enhanced chemilumines-

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ence detection reagents were from Pierce (Rockford, IL). Cell culture media and supplements were from Hyclone (South Logan, UT).

Balloon-injured rat abdominal aorta model. The treatment of the laboratory animals and experimental protocols were performed according to the guidelines of the Health Science Center of Peking University and were approved by the Institutional Authority for Laboratory Animal Care. Experiments were carried out in healthy male Wistar rats (280–320 g) obtained from the animal laboratory of the Health Science Center of Peking University. After the rats were anesthetized with 10% chloralhydrate (4 ml/kg body wt ip), a de amination, and the catheter withdrawn slowly to the diaphragm level. The balloon was passed through the abdominal aorta in this way six times. Fourteen days after balloon injury, the rats were euthanized, and the abdominal aortas were quickly removed for histological and immunological study. Skeletal muscles of the pelvic limbs were also removed and quickly fixed.

Gene transfer with electroporation. The plasmid pcDNA3.1 containing the hCGRP gene was constructed in our laboratory as previously described (27). Plasmids were transferred into primary cultured VSMCs with the use of the calcium phosphate method to evaluate hCGRP expression in eukaryotic cells. Plasmids, with or without the hCGRP gene, were injected into the skeletal muscles of pelvic limbs right after the balloon-injury operation and before the rats regained consciousness. The doses of plasmids were 400 μg (2 μg/μl) for each limb. Immediately after plasmid injection, two stainless steel needle electrodes were placed at each side of the muscle, 10 mm apart and 5 mm deep. Square-wave electric pulses were introduced with the use of an Electro Square Porator (ECM 830, BTX), for 40 ms, and repeated 10 times. The electric field voltage was 200 V/cm and the frequency 1 Hz. These transcutaneous electric pulses facilitated the entrance of plasmids into skeletal muscle cells and prolonged the duration of target DNA expression.

Histological and immunohistochemical assays. Hematoxylin and eosin (HE) staining was carried out on 5-μm cross sections of abdominal aorta. The images were analyzed with Metaview software (Universal Imaging; Downingtown, PA). At least nine separate sections from each rat were used for quantification. For immunohistochemical assay, skeletal muscles, or aortic sections were incubated overnight with anti-CGRP (1:500) or anti-inducible NO synthase (iNOS; 1:1,000) antibody at 4°C. Sections incubated without the primary antibody, with otherwise identical conditions, were used as negative controls to verify the specificity of the antibody. After a PBS rinse, the sections were incubated with a biotinylated secondary antibody for 1 h and further incubated with streptavidin-conjugated horseradish peroxidase for 1 h. Diaminobenzedine and hydrogen peroxide were used for visualization. Sections were counterstained with hematoxylin before being mounted.

Western blot analysis. Tissues were lysed, homogenized under ultrasound, and boiled for 5 min. After quantification of the protein concentration with the use of the Bradford method, 30 μg of extracts were loaded onto 10% SDS-PAGE. Transferred proteins subsequently underwent blotting for indicated proteins. Kodak 1D Image Analysis Software was used to quantitatively analyze the protein bands.

Radioimmunoassay. Radioimmunoassay of CGRP was carried out after a procedure previously used in our laboratory (31). Briefly, the samples from culture supernatants, rat plasma, and skeletal muscles of pelvic limbs were collected and vacuum dried. Samples were incubated with anti-CGRP antibody in 4°C for 24 h. After the addition of 125I-labeled CGRP, samples were further incubated in 4°C for 24 h. The bound radioactivity of calibrators, controls, and samples was measured in duplicate by use of a gamma-scintillation counter (Beckman).

Cell culture. VSMCs were isolated from rat aorta and cultured in DMEM supplemented with 10% FBS. For all experiments, VSMCs within passages 4 and 5 were used at 80–90% confluence. VSMCs were cultivated in FBS-free medium for 12 to 24 h and were exposed to different concentrations of rh-αCGRP or DETA-NONOate for 72 h. Thiorphan, an endopeptidase inhibitor, was used to slow down the degradation of CGRP. hCGRP8–37, cAMP-PKA inhibitor, and NO blockers were added 1 h before CGRP.

Apoptosis detection. VSMCs were lysed and genomic DNA was extracted with the use of the phenol-chloroform method. Equal amounts of DNA were analyzed by use of agarose gel electrophoresis. For TUNEL assay, trypsinized cells were reattached on 0.01% polylysine-coated slides, fixed with 4% methanol-free formaldehyde solution, and fluorescence stained by following the procedure of the DeadEnd fluorometric TUNEL system. Tissue samples were frozen, embedded, cut into 7-μm sections, fixed in 4% paraformaldehyde solution, and stained in a procedure similar to that used for cells. Stained samples were analyzed under a fluorescence microscope, and at least 500 cells were counted in randomly selected views.

Statistics. The results are expressed as means ± SE. Data were analyzed with use of Prism software (GraphPad), one-way ANOVA, and Student-Newman-Keuls test for multiple comparisons between treatment groups. When the means between two groups were compared, unpaired Student’s t-test was used. P < 0.05 was considered significant.

RESULTS

CGRP gene transfer inhibited neointimal formation in vivo. Intimal thickness and the ratio of intima to media were significantly increased in rat abdominal aortas 14 days after balloon injury. CGRP gene transfer largely reversed the neointimal formation. The ratio of intima to media in normal, balloon-injured, and balloon-injured with CGRP gene transfer aortas was 5.2 ± 0.9%, 52.7 ± 5.4%, and 29.2 ± 3.7%, respectively (P < 0.05). Therefore, CGRP gene transfer inhibited neointimal thickening by 40% (Fig. 1, A–C). The pcDNA3.1 plasmid containing the hCGRP gene was transferred into cultured VSMCs to test its expression in eukaryocytes (Fig. 2A). Immunohistochemical assay revealed CGRP-positive cells at the site of the in vivo gene transfer (Fig. 2B). In the CGRP gene transfer group, the level of CGRP locally and in circulation was also greatly increased and lasted for 14–28 days (Fig. 2, C and D). This observation further confirmed the effectiveness of in vivo gene transfer with electroporation, as described previously (27).

CGRP-induced VSMC apoptosis in vivo. TUNEL assay revealed few apoptotic VSMCs in neointima 14 days after balloon injury. However, with CGRP gene transfer, apoptotic cells were found in the neointima of injured rat aortas (Fig. 3). These data suggest that in addition to its antiproliferative effect, CGRP also induces apoptosis in VSMCs, which may contribute to its therapeutic effect in neointimal formation.

CGRP modulated expression of proliferation and apoptosis-related proteins in vivo. The expressions of some important proliferative and apoptotic factors in balloon-injured aortic walls were detected by Western blot analysis. The p53 protein level was increased, and PCNA and Bcl-2 levels were decreased 14 days after CGRP gene transfer (P < 0.05). No change was found in the level of Bax (Fig. 4, A–E). These data further indicate that CGRP may affect VSMC proliferation and apoptosis through differentially regulating the expression of growth/apoptosis-related genes.

CGRP increased apoptosis in cultured VSMCs. To ascertain the effect of CGRP on VSMC apoptosis, we examined DNA
fragmentation by using agarose gel electrophoresis and TUNEL assay. Increased DNA fragmentation in VSMCs with CGRP treatment for 72 h was dose dependent (Fig. 5A). To quantitatively analyze VSMC apoptosis, TUNEL assay was carried out, and apoptotic cells were stained with green fluorescein (Fig. 5B). As in the electrophoresis results, CGRP markedly increased the population of TUNEL-positive cells, from 13.6 ± 3.3% to 26.0 ± 5.7% (P < 0.05). The effect of

Fig. 1. CGRP gene transfer inhibits neointimal formation in rat aortas 14 days after balloon injury. A: hematoxylin and eosin staining of rat aortic wall. 1–3: normal aorta, balloon-injured aorta with gene transfer of pcDNA3.1 vector, and balloon-injured plus CGRP gene transfer aorta (magnification ×400), respectively. B and C: quantitation of neointimal formation by measuring the thickness of intima and media and their ratio (n = 9–12). *P < 0.05 vs. normal; #P < 0.05 vs. pcDNA3.1.

Fig. 2. Determination of CGRP gene transfer. A: radioimmunoassay of CGRP released from cultured rat vascular smooth muscle cells (VSMCs) 24 h after CGRP gene transfer; n = 3, *P < 0.05 vs. control. B: immunohistochemical analysis of CGRP expression in skeletal muscles 14 days after in vivo gene transfer with electroporation. 1–4: negative control, pcDNA3.1 vector, CGRP gene transfer (magnification ×200), and CGRP gene transfer (magnification ×100), respectively. C and D: time course of CGRP expression in skeletal muscles of pelvic limbs (C) and plasma CGRP levels (D) from rats after in vivo gene transfer; n = 3–6, *P < 0.05 vs. pcDNA3.1.
CGRP was largely prevented by 1 μM hCGRP8–37, an antagonist of the CGRP1 receptor (Fig. 5C).

Because stimulation of CGRP activates the cAMP-PKA pathway in many cell types (1, 33), we examined the potential involvement of cAMP-PKA signaling in CGRP-mediated cell apoptosis. Rp-cAMPS (11 μM), specific cAMP-PKA blocker, significantly attenuated CGRP-induced apoptosis in cultured VSMCs (Fig. 5D). These data are consistent with those from in vivo studies showing VSMC apoptosis in neointima after CGRP gene transfer (Fig. 3).

**NO pathway was involved in CGRP proapoptotic signaling.** Because of cross-talk between the CGRP and NO signaling pathways, we examined the possible modulatory effect of NO on CGRP-mediated cell apoptosis. As revealed by Western blot analysis and immunostaining, CGRP gene transfer markedly increased the iNOS protein level in balloon-injured aortic

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**Fig. 3.** Terminal deoxynucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay of CGRP-induced VSMC apoptosis in neointima of rat aortas 14 days after balloon injury. In normal and balloon-injured aortas with pcDNA3.1 gene transfer, no apoptotic cells were found in the intima. However, with CGRP gene transfer, the intima of balloon-injured aortas showed TUNEL-positive staining of apoptotic cells (magnification ×630). Arrows indicate the apoptotic cells in neointima.

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**Fig. 4.** Western blot analysis of growth/apoptosis-related proteins in rat aortic walls 14 days after balloon injury. A: PCNA (n = 3–6); B: p53 (n = 3–6); C: Bcl-2 (n = 4–6); D: Bax (n = 3); E: actin (n = 3). *P < 0.05 vs. normal; #P < 0.05 vs. pcDNA3.1.
walls (Fig. 6). In cultured VSMCs, a long-lasting NO donor, DETA NONOate, had no additive effect with CGRP in promoting VSMC apoptosis (Fig. 7A). Furthermore, L-NMMA (an NOS inhibitor), ODQ (a cGMP inhibitor), and c-PTIO (an NO scavenger), all at the concentration of 100 μM, remarkably decreased CGRP-induced apoptosis (Fig. 7B). These data indicate that CGRP may induce iNOS expression and NO formation in VSMCs, which, in turn, mediates the pro-apoptotic effect of CGRP.

**DISCUSSION**

CGRP is a neuropeptide with various protective effects in the cardiovascular system. The present study demonstrated, for the first time, that intramuscular delivery of the CGRP gene markedly reduced the thickness of neointima and induced VSMC apoptosis in an in vivo restenosis model. In cultured VSMCs, CGRP decreased cell proliferation and increased apoptosis. In addition, CGRP modulated the expression of PCNA, p53, and Bcl-2. These effects were mediated by the CGRP₁ receptor via a cAMP-PKA- and NO-dependent signaling pathway. Therefore, these results reveal a novel mechanism by which CGRP controls VSMC proliferation and apoptosis. CGRP gene transfer may be considered as a therapeutic approach for proliferative vascular diseases.

VSMC proliferation contributes to vascular remodeling seen in various cardiovascular diseases, such as atherosclerosis and restenosis. Many growth factors and intrinsic vasoactive peptides have been implicated in VSMC proliferation (7). CGRP is one of the important modulators of cardiovascular function by its altering VSMC contractility and phenotype turnover (1). Our previous studies have indicated an anti-proliferative effect of CGRP in cultured rat VSMCs. As well, NO interacts with CGRP at the second messenger level and shows a synergistic effect with CGRP in inhibiting VSMC proliferation (32). Here, results of our in vivo study shows that the inhibitory effect of CGRP on neointimal hyperplasia is associated with the down-regulation of PCNA, which suggests that VSMC proliferation is a target of the action. However, in a previous study (24), bioactive CGRP failed to inhibit neointimal formation 7 days after injury of rat carotid arteries. hCGRP₈–₃₇, acting as an
Apoptosis is closely linked to proliferation and also plays a key role in the pathogenesis of vascular diseases (16). Decreased apoptosis in VSMCs in the neointima contributes to neointimal hyperplasia and restenosis (20, 21). Therefore, hunting for proapoptotic agents becomes pivotal. NO is one of the candidates that has been studied intensively. In addition to its antiproliferative effect, NO also induces apoptosis in cultured VSMCs (13). Moreover, iNOS mediates cytokine-induced VSMC apoptosis in vitro (9). However, the results of an in vivo study indicated that reduced neointimal formation by NO is due to its inhibiting proliferation but not increasing apoptosis (12). The present results support a proapoptotic role of NO in rat VSMCs, because inhibitors of the NO signaling pathway fully block CGRP-induced apoptosis in cultured VSMCs, and the effects of CGRP and NO on apoptosis are similar but not additive. Moreover, we detected increased iNOS expression in balloon-injured aortic walls with CGRP gene transfer (Fig. 6). Previous studies (11, 14) have also suggested that the cAMP pathway elevates the iNOS level in VSMCs (11, 14). CGRP-induced VSMC relaxation may also involve an inducible NO pathway (6, 22). Therefore, CGRP directly induces iNOS expression and subsequent NO production in VSMCs. It can be inferred that the beneficial effect of CGRP in restenosis is mediated, at least in part, by the induction of iNOS expression and NO formation.

Several protein factors, such as p53, Bcl-2, and Bax, have been implicated in vascular apoptosis. p53 is a well-known adrenomedullin receptor antagonist, effectively reduces the proliferation of VSMCs in neointima. CGRP and adrenomedullin belong to the same superfamily and have similar roles in VSMC contractility (28) and cell proliferation in vitro (15). The discrepancy is likely due to different delivery methods, times of observation, and types of arteries in the two studies. Recently, we found that CGRP downregulated transforming growth factor-β (TGF-β) expression in neointima (W. Wang, unpublished observation). Because TGF-β contributes to neointimal formation by modulating the differentiation and proliferation of vascular cells, these findings add TGF-β as a new target of the anti-proliferative effect of CGRP.

Although inspiring effects of gene therapy have been observed in experimental restenosis animal models, very few effects have been confirmed by clinical trials. Among many obstacles, the delivery methods are crucial. Viral vectors are widely used in experimental studies of gene transfer in restenosis (25). However, they are less used in clinical trials because of the safety concern. The present study used naked plasmid DNA for gene transfer under electroporation, a newly developed technique that greatly increases transfection efficiency (5). Previously, we (27) demonstrated that gene transfer of CGRP into the skeletal muscle greatly increased the level of plasma CGRP and subsequently improved the symptoms of Type I diabetes in mice. Thus we adopted intramuscular gene transfer in this study to examine the role of CGRP in neointimal formation induced by balloon injury.

Fig. 6. Effect of CGRP gene transfer on inducible nitric oxide (NO) synthase (iNOS) expression in rat aortas 14 days after balloon injury. A: Western blot analysis of iNOS protein level in aortic walls. B: immunohistochemical staining of iNOS in aortic walls. 1–4: negative control, normal aortas, balloon-injured aortas with pcDNA3.1 transfer, and balloon-injured aortas with CGRP gene transfer (magnification ×200).

Fig. 7. Involvement of the NO pathway in CGRP-induced VSMC apoptosis. A: effect of the NO donor [2-(2-aminoethyl)amino]-diazen-1-ium-1,2-dioate (DETA-NONOate; 10 μM) on VSMC apoptosis. Cultured VSMCs were incubated with CGRP or DETA-NONOate for 72 h (n = 4, total cell number >500). B: effect of NO inhibitors on CGRP-induced VSMC apoptosis. Nω-nitro-l-arginine (l-NMMA), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), or 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-2-oxide (c-PTIO), all at the concentration of 100 μM, were added 1 h before CGRP. Apoptotic cells were detected by the use of TUNEL assay. Results are shown as the amount of increase over control group, n = 4, total cell number >500. *P < 0.05 vs. control; #P < 0.05 vs. CGRP 0.1 μM.
proapoptotic protein that plays a vital role in the apoptosis of many cell types, including VSMCs (23). The therapeutic effect of p53 gene transfer has been demonstrated in the human saphenous vein (10). In the present study, the level of p53 protein was upregulated by CGRP, which suggests that p53 may be involved in CGRP-induced VSMC apoptosis. In addition, the Bcl-2 and Bax proteins belong to one superfamily but regulate apoptosis in opposite ways; their ratio is considered an important determinant in controlling the initiation of the mitochondrial pathway of apoptosis (29). Indeed, we have shown the decrease of the Bcl-2-to-Bax ratio after CGRP treatment in vivo, which indicates that Bcl-2/Bax proteins are likely involved in the pro-apoptotic effect of CGRP. Thus, these apoptotic proteins, as well as PCNA, a proliferation marker, are mediators of NO in modulating VSMC apoptosis and proliferation. It is likely that these proteins are the downstream factors of the CGRP-NO-apoptosis pathway.

In summary, the present study provides evidence that CGRP inhibits balloon injury-induced neointimal formation and induces VSMC apoptosis in rat abdominal aortas. Some proapoptotic proteins, including p53 and Bcl-2, are involved in this process. In vitro studies further support the proapoptotic effect of CGRP in VSMCs. Both the cAMP-PKA and NO signaling pathways mediate the pro-apoptotic effect of CGRP. These findings reveal not only a novel regulatory mechanism of VSMC apoptosis but also potential therapeutic targets for proliferative vascular disorders, particularly restenosis.

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