

# Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

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*Arterioscler Thromb Vasc Biol.* 1998;18:1275-1280

doi: 10.1161/01.ATV.18.8.1275

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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# G Protein Signaling and Vein Graft Intimal Hyperplasia

## Reduction of Intimal Hyperplasia in Vein Grafts by a $G_{\beta\gamma}$ Inhibitor Suggests a Major Role of G Protein Signaling in Lesion Development

Mark G. Davies, Tam T.T. Huynh, Gregory J. Fulton, Robert J. Lefkowitz, Einar Svendsen, Per-Otto Hagen, Walter J. Koch

**Abstract**—Vein grafting results in the development of intimal hyperplasia with accompanying changes in guanine nucleotide-binding (G) protein expression and function. Several serum mitogens that act through G protein-coupled receptors, such as lysophosphatidic acid, stimulate proliferative pathways that are dependent on the G protein  $\beta\gamma$  subunit ( $G_{\beta\gamma}$ )—mediated activation of p21<sup>ras</sup>. This study examines the role of  $G_{\beta\gamma}$  signaling in intimal hyperplasia by targeting a gene encoding a specific  $G_{\beta\gamma}$  inhibitor in an experimental rabbit vein graft model. This inhibitor, the carboxyl terminus of the  $\beta$ -adrenergic receptor kinase ( $\beta$ ARK<sub>CT</sub>), contains a  $G_{\beta\gamma}$ -binding domain. Vein graft intimal hyperplasia was significantly reduced by 37% ( $P < 0.01$ ), and physiological studies demonstrated that the normal alterations in G protein coupling phenotypically seen in this model were blocked by  $\beta$ ARK<sub>CT</sub> treatment. Thus, it appears that  $G_{\beta\gamma}$ -mediated pathways play a major role in intimal hyperplasia and that targeting inhibitors of  $G_{\beta\gamma}$  signaling offers novel intraoperative therapeutic modalities to inhibit the development of vein graft intimal hyperplasia and subsequent vein graft failure. (*Arterioscler Thromb Vasc Biol.* 1998;18:1275-1280.)

**Key Words:** vein grafts ■ carboxyl terminus of  $\beta$ -adrenergic receptor kinase ■ gene transfer ■ G proteins ■ intimal hyperplasia

Vein grafts are the most common conduit used for surgical revascularization procedures. However, these grafts are susceptible to the development of intimal hyperplasia and accelerated atherosclerosis, which result in failure rates of 20% to 40% at 5 years.<sup>1,2</sup> Intimal hyperplasia is a chronic structural lesion that develops after vein graft implantation and leads to luminal stenosis and occlusion.<sup>3</sup> It may be defined as abnormal migration and proliferation of vascular smooth muscle cells with associated deposition of extracellular connective tissue matrix. The development of intimal hyperplasia in experimental vein grafts is also associated with increased or novel expression of G proteins ( $\alpha$  and  $\beta\gamma$  subunits) in vivo, which occurs simultaneously with phenotypically altered contractile function.<sup>4</sup> Experimental vein grafts possess increased concentrations of the G protein  $\alpha_s$ ,  $\alpha_{i2}$ ,  $\alpha_q$ , and  $\beta$  subunits.<sup>4,5</sup>  $G_{\alpha_{i3}}$  is detectable in vein grafts that display intimal hyperplasia but not in the native jugular veins.<sup>4,5</sup> Regarding the physiology of vein grafts, contractile responses become sensitive to pertussis toxin (PTx), which differs from the PTx-insensitive contractile response in native veins.<sup>4</sup> PTx ADP ribosylates the  $G_i/G_o$   $\alpha$  subunits, causing ablation of signaling.<sup>6</sup> Thus, G proteins appear to play a

critical role in the phenotypical alterations associated with vein grafts and intimal hyperplasia.

The signaling mechanisms triggering the proliferation and migration of vascular smooth muscle cells are not well understood, but G proteins could be involved because several growth factors that induce mitogenesis act through membrane-embedded, G protein-coupled receptors. On stimulation, G proteins dissociate to the  $G_\alpha$  and  $G_{\beta\gamma}$  subunits, which can both lead to cellular signaling events.<sup>6,7</sup> Several G protein-coupled mitogens, acting primarily through PTx-sensitive G proteins, have been shown to specifically trigger intracellular signaling events via  $G_{\beta\gamma}$ , leading to proliferation through activation of p21<sup>ras</sup> (*ras*) and subsequent activation of the p42 and p44 mitogen-activated protein (MAP) kinases.<sup>8</sup> The  $\beta$ -adrenergic receptor kinase 1 ( $\beta$ ARK-1) is a  $G_{\beta\gamma}$ -dependent cytosolic enzyme that phosphorylates its activated receptor substrate after translocation to the cell membrane and binding to the membrane-anchored  $\beta\gamma$  subunits.<sup>9</sup> A peptide encoded by the carboxyl terminus of  $\beta$ ARK-1 ( $\beta$ ARK<sub>CT</sub>) contains the specific  $\beta\gamma$ -binding domain of the enzyme.<sup>10</sup> It was previously shown that when cells are transfected with a  $\beta$ ARK<sub>CT</sub> plasmid or when peptides con-

Received December 18, 1997; revision accepted February 26, 1998.

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Presented in part at the 52nd Annual Sessions of the American College of Surgeons, San Francisco, Calif, 1996, and published in abstract form (*Surg Forum.* 1996;47:341-345.)

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taining the G <sub>$\beta\gamma$</sub> -binding domain of  $\beta$ ARK<sub>CT</sub> are introduced into the cells, several G <sub>$\beta\gamma$</sub> -dependent processes are inhibited, including activation of *ras* and MAP kinase by several mitogens.<sup>8,11</sup> In addition, it has also been demonstrated that the peptide encoded by  $\beta$ ARK<sub>CT</sub> is specific for G <sub>$\beta\gamma$</sub>  and does not directly alter G <sub>$\alpha$</sub> -mediated responses.<sup>8,11</sup> We hypothesized that blockade of the G <sub>$\beta\gamma$</sub>  subunit would result in a reduction in intimal hyperplasia. This study examined the effect of  $\beta$ ARK<sub>CT</sub> on the formation of vein graft intimal hyperplasia and phenotypical functional alterations.

## Methods

### Experimental Design

Forty New Zealand White rabbits underwent carotid interposition vein bypass grafting. Before grafting, veins were incubated in heparinized Ringer's lactate (controls, n=18) or plasmid solutions containing either  $\beta$ ARK<sub>CT</sub> (n=14, 190  $\mu$ g/mL) or empty plasmid DNA (plasmid n=8, 190  $\mu$ g/mL) for 30 minutes at 37°C. These concentrations were shown to produce efficient transfection in vitro. Twenty-four vein grafts (controls n=10, plasmid n=6, and  $\beta$ ARK<sub>CT</sub> n=8) were harvested at 28 days by perfusion fixation. Intimal and medial dimensions of vein grafts were calculated by video morphometry. Sections were taken for scanning and transmission electron microscopy. Ten vein grafts (n=5, control and  $\beta$ ARK<sub>CT</sub>) were analyzed for in vitro contractile responses to norepinephrine and serotonin in the presence and absence of PTx to categorize G protein-receptor coupling. Six vein grafts (n=3, control and  $\beta$ ARK<sub>CT</sub>) were harvested at 3 days for  $\beta$ ARK<sub>CT</sub> protein and mRNA expression (reverse transcriptase-polymerase chain reaction [RT-PCR]). Animal care complied with Duke University Medical Center guidelines, the *Principles of Laboratory Animal Care* of the National Society for Medical Research,<sup>12</sup> and the *Guide for the Care and Use of Laboratory Animals* issued by the National Institutes of Health.<sup>13</sup>

### Transgene Constructs

Gene transfer to the experimental vein grafts was done using our previously described plasmid, which contains cDNA encoding the last 194 amino acid residues (Met-Gly<sup>495</sup>-Leu<sup>689</sup>) of bovine  $\beta$ ARK<sub>CT</sub> (pRK- $\beta$ ARK<sub>CT</sub>).<sup>8,10</sup> This peptide contains the experimentally determined (Gln<sup>546</sup>-Ser<sup>670</sup>) G <sub>$\beta\gamma$</sub> -binding domain. The empty pRK5 plasmid was used as the negative control as previously described.<sup>8,11</sup> Large-scale plasmid preparations of pRK5 and pRK- $\beta$ ARK<sub>CT</sub> were purified on Qiagen columns (Qiagen, Inc) before vein graft gene transfer.

### Analysis of $\beta$ ARK<sub>CT</sub> Transgene Expression

Three-day vein grafts were used for analysis of specific transgene expression.  $\beta$ ARK<sub>CT</sub> mRNA expression was determined by standard methods of RT-PCR<sup>14</sup> with an RT-PCR kit using TaqPlus DNA polymerase (Stratagene, Inc). Total RNA was first isolated using the single-step reagent RNeasy (Qiagen, Inc)<sup>15</sup> and treated with DNase I to eliminate any possible plasmid contamination. A  $\beta$ ARK<sub>CT</sub> primer set was used to specifically amplify  $\beta$ ARK<sub>CT</sub> mRNA. The primers were as follows: sense primer (corresponding to the start of  $\beta$ ARK<sub>CT</sub>), 5'-GAATTCGCCGCCACCATGGG-3'; and antisense primer (corresponding to the  $\beta$  globin-untranslated region linked to the end of the  $\beta$ ARK<sub>CT</sub> cDNA<sup>11</sup>), 5'-GGAACAAAGGAACCTTTAATAG-3'. This primer set amplifies a 670-bp fragment corresponding to  $\beta$ ARK<sub>CT</sub> mRNA.

### Operative Procedure

Anesthesia was induced and maintained with subcutaneously injected ketamine hydrochloride (60 mg/kg, Ketaset, Bristol Laboratories) and xylazine (6 mg/kg, Anased, Lloyd Laboratories). Antibiotic prophylaxis with 30 000 IU/kg of benzathine and procaine penicillin (Durapen, Vedco, Inc) was given intramuscularly at the time of induction. Surgery was performed using an operating microscope (JKH 1402, Edward Weck, Inc) under sterile conditions.

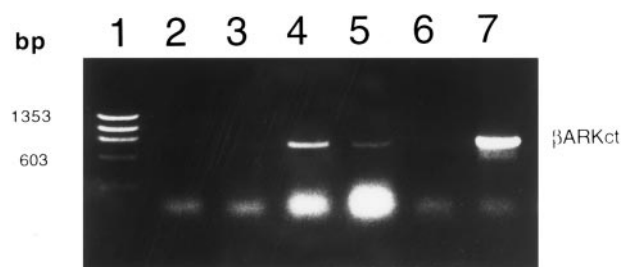
After exposure through a midline longitudinal neck incision, the right external jugular vein was identified, its branches were coagulated by diathermy at a distance from the vein to minimize injury, and the vein was then excised. After excision, the vein was incubated in a heparinized Ringer's lactate solution (5 IU/mL, heparin, Elkins-Sinn, Inc) containing either  $\beta$ ARK<sub>CT</sub> (n=14, 190  $\mu$ g/mL) or empty plasmid DNA (plasmid n=8, 190  $\mu$ g/mL) for 30 minutes at 37°C. Ex vivo, a cannula was placed atraumatically into a branch of the jugular vein, and the incubation solution was introduced once into the lumen of the vein without developing any distension pressure to ensure luminal and adventitial exposure. The right common carotid artery was identified and dissected, and both proximal and distal control was obtained. Heparin (200 IU/kg) was administered intravenously. A proximal longitudinal arteriotomy was made, and one end of the reversed jugular vein was anastomosed to the artery in an end-to-side manner using a continuous 10-0 microvascular monofilament nylon suture (Ethilon, Ethicon, Inc, Somerville, NJ). The distal anastomosis was performed in a similar manner. Throughout the procedure, care was taken to avoid unnecessary instrumentation of the vein graft. The right common carotid artery was ligated and divided between the two anastomoses with 4-0 silk sutures, and the wound was closed in layers.

### Morphology

Vein grafts were harvested 28 days after surgery. After isolation and systemic heparinization (200 IU/kg IV), the vein grafts were perfusion fixed in situ at 80 mm Hg with an initial infusion of HBSS (Gibco Laboratories, Life Technologies, Inc), followed by 2% glutaraldehyde made up in 0.1 mmol/L cacodylate buffer (pH 7.2) supplemented with 0.1 mmol/L sucrose to give an osmolality of  $\approx$ 300 mOsm. After 60 minutes, the specimen was removed and immersed in the glutaraldehyde fixative for an additional 24 hours. Cross sections from the middle portion of the vein graft were processed for light microscopy. With the use of standard histological procedures, each specimen was stained with a modified Masson's trichrome and Verhoeff's elastic tissue stain and dimensional analysis was performed by video morphometry (Innovision 150, American Innovision, Inc). The intima and media were delineated by identification of the demarcation between the crisscross orientation of the intimal hyperplastic smooth muscle cells and circular smooth muscle cells of the media, and the outer limit of the media was defined by the interface between the circular smooth muscle cells of the media and the connective tissue of the adventitia. The thickness of each layer was also determined. A ratio of the intimal and medial areas (intimal area/[intimal+medial areas]) and an index of luminal diameter to cross-sectional wall thickness (luminal diameter/cross-sectional wall thickness) were calculated.

### In Vitro Contractile Studies

For these studies, the animals were anesthetized, the original incision was reopened, and the jugular vein and vein graft were isolated. The middle part of each vessel was sectioned in situ into four 5-mm segments and excised. These rings were suspended immediately from 2 stainless steel hooks in 5-mL organ baths containing oxygenated Krebs solution (122 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgCl<sub>2</sub>, 2.5 mmol/L CaCl<sub>2</sub>, 15.4 mmol/L NaHCO<sub>3</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, and 5.5 mmol/L glucose, maintained at 37°C and bubbled with a mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>). One hook was fixed to the bottom of the bath, and the other was connected to a force transducer (Myograph F-60, Narco Bio-Systems). The isometric responses of the tissue were recorded on a multichannel polygraph (Physiograph Mk111-S, Narco Bio-Systems). The tissues were then placed under 0.5 g of tension and allowed to equilibrate in physiological Krebs solution for 1 hour. During the equilibration period, the Krebs solution was replaced every 15 minutes. After equilibration, the resting tension was adjusted in 0.25-g increments from 0.25 to 2.5 g, and the maximal response to a modified oxygenated Krebs solution (60 mmol/L KCl, 66.7 mmol/L NaCl, 1.2 mmol/L MgCl<sub>2</sub>, 2.5 mmol/L CaCl<sub>2</sub>, 15.4 mmol/L NaHCO<sub>3</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, and 5.5 mmol/L glucose) was measured at each resting tension to establish a length-tension relation. On the basis of



**Figure 1.** RT-PCR results from 3-day-old vein grafts treated with empty pRK5 and pRK- $\beta$ ARK<sub>CT</sub>. Lane 1,  $\phi$ X174HaeIII-digested DNA markers with 2 of the size marker positions listed at left; lanes 2 and 3, two control vein grafts transfected with pRK5 (plasmid); lanes 4 and 5, two vein grafts transfected with pRK- $\beta$ ARK<sub>CT</sub>; lane 6, negative control for PCR; and lane 7, amplification of the positive control pRK- $\beta$ ARK<sub>CT</sub>-purified plasmid. This gel displays two each of the four 3-day-old vein grafts tested by RT-PCR for transgene expression.

these results, the optimal resting tension for each ring (the tension at which the response to the modified Krebs solution was maximal) was determined, and the ring was set at this tension for subsequent studies. Norepinephrine ( $10^{-9}$  to  $10^{-4}$  mol/L) was added cumulatively in half-molar increments, and the isometric tension developed by the tissue was measured. After washout and reequilibration, dose-response curves were obtained for serotonin ( $10^{-9}$  to  $10^{-4}$  mol/L). The responses to each agonist were assessed in the presence and absence of PTx (100 ng/mL preincubated for 60 minutes).<sup>4</sup> All compounds were obtained from Sigma Chemical Company.

### Data and Statistical Analyses

The  $EC_{50}$  value, the concentration for the half-maximal response, for each agonist in each ring was calculated by logistic analysis and is expressed as  $-\log_{10}[EC_{50}]$ .<sup>16</sup> All data are presented as mean  $\pm$  SEM, and statistical differences between groups were tested by ANOVA with post hoc Tukey-Kramer multiple-comparison tests (functional studies) and with a Kruskal-Wallis nonparametric ANOVA with post hoc Dunn's multiple-comparison tests (morphometric data).

## Results

### Transgene Expression

Successful transfection of the vein grafts was demonstrable 3 days after surgery.  $\beta$ ARK<sub>CT</sub> mRNA was specifically amplified from DNase I-treated total RNA using RT-PCR from vein grafts treated with pRK- $\beta$ ARK<sub>CT</sub>, whereas control grafts treated with the empty pRK5 plasmid showed no transgene expression at 3 days (Figure 1). No pRK5 and pRK- $\beta$ ARK<sub>CT</sub> RNA was detectable at 5 and 7 days, suggesting transient

**TABLE 1. Dimensional Analysis**

	Control	Plasmid	$\beta$ ARK <sub>CT</sub>	P
Lumen, mm <sup>2</sup>	20.5 $\pm$ 1.5	28.6 $\pm$ 4.01	16.6 $\pm$ 2.33*	0.02
Intima, mm <sup>2</sup>	1.14 $\pm$ 0.09	1.29 $\pm$ 0.12	0.62 $\pm$ 0.03*	0.01
Media, mm <sup>2</sup>	1.08 $\pm$ 0.11	1.29 $\pm$ 0.17	1.12 $\pm$ 0.10	0.18
Intimal ratio	0.52 $\pm$ 0.02	0.54 $\pm$ 0.02	0.36 $\pm$ 0.02†	0.02
Luminal index	39.4 $\pm$ 2.6	44.2 $\pm$ 3.1	37.8 $\pm$ 3.9	0.4

Area of the lumen and the intimal and medial layers from control (n=10), plasmid treated (n=6), and  $\beta$ ARK<sub>CT</sub> treated (n=8) grafts. The intimal ratio (intimal area/[intimal+medial areas]) and luminal index (luminal diameter/cross-sectional wall thickness) are also shown. Values are mean  $\pm$  SEM. Statistical analysis was done by Kruskal-Wallis nonparametric ANOVA with post hoc Dunn's multiple-comparison tests.

\* $P < 0.05$  vs plasmid.

† $P < 0.05$  vs control.

expression of the vector (data not shown). Because the amount of tissue available was small, protein immunoblotting for  $\beta$ ARK<sub>CT</sub> peptide expression was not possible.

### Intimal Hyperplasia

All animals survived to 28 days, and all grafts were patent at harvest. The jugular vein of the rabbit consists of a single layer of endothelial cells and 1 or 2 layers of smooth muscle cells in a loose, connective tissue matrix (histological data not shown). Microscopically, the luminal surfaces of the vein grafts from each group were covered by a layer of intact endothelial cells, beneath which lay a hyperplastic intima with the smooth muscle cells arranged in a crisscross pattern with little extracellular matrix (Figure 2). The medial smooth muscle cells in the grafts from each group appeared slender, were arranged in a circular pattern, and contained a greater amount of extracellular matrix, suggestive of medial hypertrophy. At 28 days, there was a significant (36%) reduction in intimal thickness in  $\beta$ ARK<sub>CT</sub> vein grafts ( $45 \pm 4$   $\mu$ m, n=8) compared with either plasmid ( $69 \pm 3$   $\mu$ m, n=6) or control ( $70 \pm 4$   $\mu$ m, n=10) vein grafts without a significant change in medial thickness. Results of dimensional analysis of the control and treated groups are shown in Table 1. There was a 52% decrease in intimal area (Table 1), whereas the medial area was unchanged in the  $\beta$ ARK<sub>CT</sub>-treated vein grafts compared with the plasmid-treated grafts (Table 1). The intimal ratio was significantly reduced in the  $\beta$ ARK<sub>CT</sub> vein



**Figure 2.** Composite photomicrograph showing a cross section from the wall of a vein graft from the control (A), plasmid (B), and  $\beta$ ARK<sub>CT</sub> (C) groups. At 28 days, there was a significant (36%) reduction in intimal thickness in  $\beta$ ARK<sub>CT</sub>-treated vein grafts ( $45 \pm 4$   $\mu$ m) compared with either plasmid ( $69 \pm 3$   $\mu$ m) or control ( $70 \pm 4$   $\mu$ m) vein grafts without a significant change in medial thickness ( $70 \pm 4$ ,  $65 \pm 5$ , and  $77 \pm 3$   $\mu$ m, respectively). H indicates intimal hyperplasia; M, media. Arrowheads indicate demarcation between intimal hyperplasia and media. Modified Masson's trichrome and Verhoeff's elastic tissue stains, magnification  $\times 250$ .

**TABLE 2. Sensitivity of Contractile Responses**

	Norepinephrine	Norepinephrine With PTx	Serotonin	Serotonin With PTx
Control	6.00±0.09	5.16±0.09*	6.34±0.10	5.54±0.26*
$\beta$ ARK <sub>CT</sub>	5.91±0.19	5.81±0.18	6.57±0.10	6.55±0.13

Data are mean±SEM. Contractile sensitivity is shown as  $-\log EC_{50}$ .

\* $P < 0.01$  compared with corresponding vein graft not treated with PTx (ANOVA). There was no difference in the maximal contractile responses to the two agonists in the different preparations when they were normalized to their responses to the 60-mmol/L KCl solution.

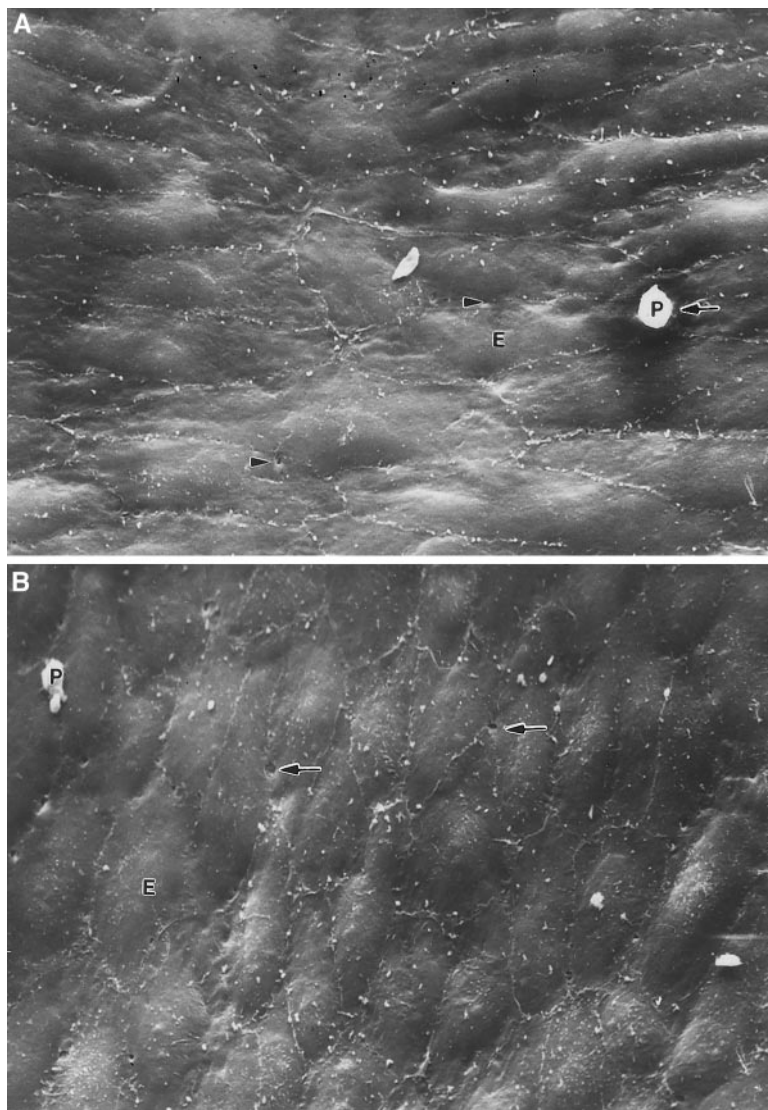
grafts ( $P < 0.01$ ,  $0.36 \pm 0.02$ , mean±SEM) compared with either the plasmid ( $0.54 \pm 0.02$ ) or control ( $0.52 \pm 0.02$ ) vein grafts. The luminal area of the  $\beta$ ARK<sub>CT</sub>-treated vein grafts was 41% less than the plasmid-treated vein grafts, whereas the luminal indices were not significantly different for the control, plasmid, and  $\beta$ ARK<sub>CT</sub> vein grafts, suggesting that there has been no negative modulation of remodeling in the vein grafts.

### Contractile Function of Experimental Vein Grafts

Control and  $\beta$ ARK<sub>CT</sub>-treated vein grafts responded with concentration-dependent contractions to the agonists norepinephrine and serotonin. In the presence of PTx at concentrations sufficient to produce 100% ADP ribosylation of G proteins,<sup>4</sup> the contractile responses in control vein grafts to norepinephrine ( $P < 0.01$ ) and serotonin ( $P < 0.01$ ) were significantly reduced compared with untreated control vein grafts (Table 2). This is the typical functional alteration seen in experimental vein grafts, because native veins do not have a PTx-sensitive component in their contractile responses to these G protein-coupled agonists. In contrast, the responses of the  $\beta$ ARK<sub>CT</sub>-treated vein grafts to norepinephrine and serotonin were unchanged in the presence of PTx, indicating the loss of a  $G_{\alpha i}$  component (Table 2).

### Electron Microscopy of Vein Grafts

Scanning electron microscopy of both control vein grafts and vein grafts transfected with empty plasmid showed the luminal surface to be lined with sharply outlined endothelial



**Figure 3.** A, Scanning electron micrograph from a vein graft transfected with plasmid (pRK5). Endothelial cells are sharply outlined with well-defined cell borders. Stomata are small and few (arrowheads). Single polymorphonuclear neutrophils can be seen (arrow). Magnification  $\times 640$ . B, Scanning electron micrograph from a vein graft transfected with  $\beta$ ARK<sub>CT</sub>. Endothelial cell junctions are sharply outlined. Some well-defined junctional stomata are shown (arrows). The morphological features are similar to those of the plasmid-transfected vein graft. One single, somewhat deformed polymorphonuclear neutrophil is in the upper left corner. Original magnification  $\times 640$ .

cells with well-defined cell borders (Figure 3A). Occasional junctional stomata were noted. Transmission electron microscopy of these vein grafts confirmed the presence of well-formed endothelial cells, beneath which were well-developed smooth muscle cells of both contractile (cytoplasm predominantly filled with contractile filaments) and synthetic (cytoplasm filled with synthetic organelles) phenotypes in a loose, connective tissue matrix. No inflammatory cells or evidence of apoptosis was identified in these grafts. Scanning electron microscopy of vein grafts transfected with  $\beta$ ARK<sub>CT</sub> showed a similar picture for the control and plasmid-transfected vein grafts with well-preserved, normal-appearing endothelial cells with occasional stomata at their junctions on the luminal surface (Figure 3B). Transmission electron microscopy showed an ultrastructural pattern similar to that of the control and plasmid-transfected vein grafts.

### Discussion

This study revealed 3 novel findings in the area of surgical and gene-transfer manipulations of vein grafts. First, we showed for the first time that plasmid DNA can be transiently transferred to an experimental vein graft. Second, we found that the transfection of a  $G_{\beta\gamma}$  inhibitor ( $\beta$ ARK<sub>CT</sub>) results in a significant in vivo biological effect (ie, reduction in intimal hyperplasia up to 28 days). Finally,  $\beta$ ARK<sub>CT</sub> transfection resulted in alterations in functional coupling of  $G_{\alpha}$  subunits to vascular agonists.

There is considerable interest in modulating the development of vein graft intimal hyperplasia by molecular means. To date, transfection of vein grafts with adenovirus has been demonstrated to have limited efficacy. LeClerc et al<sup>17</sup> showed that  $\beta$ -galactosidase can be introduced into the endothelium of vein grafts at the time of operation but that the viral transfection is short-lived and is lost by 7 days. Chen et al<sup>18</sup> demonstrated that the vascular cell adhesion molecule can be transfected into porcine vein grafts at the time of implantation but, again, with limited duration of transfection. Mann et al<sup>19</sup> demonstrated that liposome-delivered antisense oligonucleotide can achieve effective delivery of anti-cyclin/cdck2, with a significant decrease in intimal hyperplasia, but there was an increase in medial hypertrophy compared with controls. We have demonstrated that pluronic gel delivery of *c-myc* antisense oligonucleotide will decrease vein graft intimal hyperplasia without changing the medial response.<sup>20</sup> This study is the first to demonstrate plasmid transfection into a vein graft with a sustained biological effect. Efficient transfer of DNA was determined by  $\beta$ ARK<sub>CT</sub> mRNA amplification in 3-day-old vein grafts. Only the vein grafts treated with the  $\beta$ ARK<sub>CT</sub> plasmid showed transgene expression. DNase I was added to the isolated total RNA to eliminate any possibility of plasmid contamination.

Activation of both G protein-coupled and protein tyrosine kinase-coupled receptors results in a sequential cascade of phosphorylation reactions that converge at the MAP kinase step in the phosphorylation cascade.<sup>7,21-23</sup> Intraoperative transfection of vein grafts with  $\beta$ ARK<sub>CT</sub> resulted in a 36% reduction in intimal hyperplasia at 28 days. The marked decrease in intimal hyperplasia appears to be the result of modulation of G protein signaling activity in the vein graft

smooth muscle cells. These findings suggest that  $G_{\beta\gamma}$  is critical in the proliferation of smooth muscle cells required for intimal hyperplasia formation. This is a significant finding, because most of the attention is given to tyrosine kinase receptor-activating growth factors, such as platelet-derived growth factor, fibroblast growth factor, and insulin. However, in light of our findings, the role of activated G protein-coupled receptors, which subsequently leads to  $G_{\beta\gamma}$ -mediated mitogenesis, should be further defined. This hypothesis is supported by our previous findings that  $G_i$ -coupled receptors, such as lysophosphatidic acid,  $\alpha_2$ -adrenergic, and M2 muscarinic cholinergic receptors, activate the *ras*-MAP kinase pathway through  $G_{\beta\gamma}$ , whereas  $G_{\alpha_s}$ -coupled and  $G_{\alpha_q}$ -coupled receptors do not.<sup>8,24</sup> In addition, we have also shown that a tyrosine kinase growth factor, insulin growth factor 1, can activate the *ras*-MAP kinase pathway through  $G_{\beta\gamma}$ .<sup>25</sup> Other agents that can couple and activate  $G_{\alpha_i}$  include thrombin and angiotensin II (both of which can also couple to  $G_{\alpha_q}$ ). Both of these agents have been implicated in stimulating growth. We cannot directly state that  $G_{\beta\gamma}$  inhibits MAP kinase activity, because we have not been able, to date, to demonstrate significant MAP kinase activity at early time points in either control or transfected vein grafts. Thus, several candidate potential G protein-coupled receptors exist that might direct vein graft intimal hyperplasia and whose signaling would be inhibited by the  $\beta$ ARK<sub>CT</sub>.

One difference in our approach to inhibiting vascular smooth muscle proliferation is that it is targeted to a specific pathway. Approaches such as antisense oligonucleotides<sup>18,26</sup> or herpesvirus thymidine kinase gene delivery are nonselective inhibitors of mitogenesis and may affect other cell types.<sup>27</sup>  $\beta$ ARK<sub>CT</sub> targets 1 common pathway that is apparently shared by several mitogens. Our findings suggest a critical role for  $G_{\beta\gamma}$  signaling in intimal hyperplasia.

Functional responses of vascular smooth muscle cells to vasoactive agonists may be representative of "stimulus-response coupling" and may broadly define the status of many of the common extracellular signals, surface receptor systems, and intracellular regulatory mechanisms present. The increases in G proteins after vein grafting<sup>4</sup> are associated with the development of enhanced PTx-sensitive contractile responses that are known to be coupled to  $G_{\alpha_i}$  proteins.<sup>28,29</sup> Native veins do not have PTx-sensitive contractile responses. Although  $\beta$ ARK<sub>CT</sub> does not affect  $G_{\alpha}$ -mediated events, intraoperative transfection with  $\beta$ ARK<sub>CT</sub> abolished the sensitivity of the contractile responses of the vein graft to PTx. This suggests that the contractile responses in the  $\beta$ ARK<sub>CT</sub>-transfected vein grafts are mediated predominantly by PTx-insensitive  $G_{\alpha}$  subunits (ie,  $G_{\alpha_q}$ , which is more like native veins). The mechanism of G protein-coupling change is not known, and additional experiments will be required to determine whether this is a property of specific  $G_{\beta\gamma}$  inhibition or another mechanism, possibly dealing with the loss of vascular smooth muscle "buildup." Nonetheless, our results indicate that transfection of vein grafts with a plasmid containing a  $G_{\beta\gamma}$  inhibitor produces a significant biological effect with the potential to have a large impact in the surgical vein graft arena.

## Conclusions

This study shows, for the first time, that plasmid-directed local gene transfer can be achieved with a biological effect. In addition, this is the first demonstration of  $\beta$ ARK<sub>CT</sub>-mediated inhibition of G $\beta\gamma$  function in vivo and suggests that targeting G protein pathways may offer new intraoperative therapeutic modalities to reduce the development of vein graft intimal hyperplasia and subsequent vein graft failure.

## Acknowledgments

This study was supported by the US Public Health Service [grants HL15448 (P.-O.H.) and TW04810 (R.T.L.)]. Gregory J. Fulton held a Trinity College Dublin Postgraduate Scholarship. Einar Svendsen was supported by the Blix Family Foundation for Medical Research and the Astri and Edvard Riisoens Legacy. The technical assistance of L. Barber, E. Tomhave, and C. Skaer (Duke University) and A.-M. Sandsbakk-Austarheim and T. Foldnes Gulbrandsen (University of Bergen) is greatly appreciated. Microsutures were a gift of Ethicon, Inc, Somerville, NJ.

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