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ROLE FOR Gβγ G-PROTEINS IN PROTEASE REGULATION DURING REMODELING OF THE MURINE FEMORAL ARTERY

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Abstract

Background—Intimal hyperplasia remains the principal lesion in the development of restenosis after vessel wall injury. G-protein coupled receptors are involved in smooth muscle cell proliferation but the role of Gβγ in arterial intimal hyperplasia has not been well defined. The aim of this study is to characterize the expression of Gβγ G-proteins in the developing intimal hyperplasia in a murine model and the impact of disruption of Gβγ signaling on intimal hyperplasia development.

Methods—The murine femoral wire injury model was employed. Specimens were perfusion-fixed and sections were stained with H&E and Movat's stains such that morphometry could be performed using an Image-Pro system. Additional specimens of femoral artery were also harvested and snap frozen for western blotting for the Gβγ expression and for western blotting and zymography to allow for the study of gelatinase and plasminogen activator expression and activation. Contralateral vessels were used as controls. Additional vessels were immersed in pluronic gel containing an adenovirus with the Gβγ inhibitor βARK_{CT}.

Results—The injured femoral arteries developed intimal hyperplasia, while sham vessels did not produce such a response. Cell proliferation peaked at 3–5 days and cell migration at 7 days after injury. There was a marked time-dependent increase in Gβγ over the 28 days following injury. Inhibition of Gβγ with βARK_{CT} inhibited cell proliferation, cell migration and the development of intimal hyperplasia. Inhibition of Gβγ decreased peak uPA activity and expression without increasing early PAI-1 activity and expression. Inhibition of Gβγ reduced peak MMP-2 activity at day 1 but not at day 7 and also reduced peak MMP-9 activity at day 3. Protein expression for both MMP-2 and MMP-9 was also transiently decreased. There were no changes in TIMP-1 and TIMP-2 expression and activity.

Conclusions—These data demonstrate a time-dependent increase in Gβγ G-protein expression following wire injury in the mouse. Inhibition of Gβγ alters cell proliferation and migration with associated changes in MMP-2, MMP-9 and uPA expression and activity.

Keywords

vascular smooth muscle; response to injury; proteases; G-proteins; arterial injury

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INTRODUCTION

Membrane signal transduction by vascular smooth muscle cells (VSMC) is necessary for vessel wall remodeling during atherogenesis in response to flow and after injury (1). G-protein-coupled receptors and receptor-linked tyrosine kinases are the major classes of membrane signal transduction proteins in vascular smooth muscle cells. G-proteins are heterotrimeric proteins consisting of $G\alpha$, $G\beta$ and $G\gamma$ protein subunits. Each component protein is a family of proteins. The $G\alpha$ family in smooth muscle cells consists of the $G\alpha_i$, $G\alpha_q$ and $G\alpha_s$. G-protein-coupled receptors are involved in smooth muscle cell proliferation but the role of G-proteins in arterial intimal hyperplasia has not been fully defined. We have previously reported the expression patterns of G-proteins in rabbit vein grafts and rat arterial balloon angioplasty models (2–7). Interference with $G\beta\gamma$ inhibited intimal hyperplasia in both models and inhibition of $G\alpha_i$ proteins inhibited intimal hyperplasia in the rat model (7).

We recently described the changes in vessel wall structure and examined the role of cell kinases in modulating the development of intimal hyperplasia in a murine model and demonstrated that femoral wire injury in the mouse induces a consistent model of intimal hyperplasia and that it is associated with a time-dependent increase in the activity of signaling kinases (ERK1/2, p38^{MAPK} and JNK) (8). There were also time-dependent alterations in the activity and expression of the protease/inhibitor combinations of MMP-2 / TIMP-2, MMP-9 / TIMP-1, and urokinase / PAI-1 after injury (8, 9). Interruption of the MAPK kinase activity decreased the intimal hyperplastic response and interrupted the expression and activity of the uPA / PAI-1, MMP-2 / TIMP-2 and MMP-9 / TIMP-1 pathways (8, 9).

The aim of this study is to characterize the expression of $G\beta\gamma$ in the developing intimal hyperplasia in a murine model and the consequences of disruption of this signaling on protease biology.

MATERIALS AND METHODS

Experimental Design

The murine femoral wire injury model was employed in which a micro wire is passed through a branch of the femoral artery and used to denude the common femoral artery (8, 9). Specimens were perfusion-fixed and sections were stained with H&E and Movat's stains such that morphometry could be performed using an Image-Pro system (Media Cybernetics, Inc., Bethesda, MD). Additional specimens of femoral artery were also harvested and snap frozen for western blotting to allow for the study of G-protein expression. Contralateral vessels were used as controls. Additional vessels were immersed in pluronic gel with an adenovirus containing the $G\beta\gamma$ inhibitor βARK_{CT} . Animal care and procedures were conducted at the University of Rochester Medical Center in accordance with state and federal laws and under protocols approved by the University of Rochester Animal Care and Use Committee. Animal care complied with the "Guide for the Care and Use of Laboratory Animals" issued by the Institute of Laboratory Animal Resources.

Femoral wire injury

Endoluminal injury to the common femoral artery was produced by 3 passages of a 0.25-mm-diameter angioplasty guidewire (Advanced Cardiovascular Systems) as previously described. Control sham-operated arteries underwent dissection, temporary clamping, arteriotomy, and ligation, without passage of the wire. Non-operated normal femoral arteries were used as additional controls. Based on the data derived from the control experiments, additional vessels were immersed in pluronic gel containing the adenovirus with $G\beta\gamma$ inhibitor βARK_{CT} as previously described (2–9). The vessels were harvested for either

western blotting to detect G-protein expression or final morphometry. Pluronic gel (1 ml of 30% F127 Pluronic gel; Sigma, St Louis, MO) was applied to the external surface of the femoral artery at the end of the procedure as previously described (8, 10, 11). The pluronic gel remains in a liquid form at 4°C and rapidly forms a gel on exposure to body temperature. It is dissipated within 4 hrs.

Adenoviral Infection

Adenoviral vectors were constructed by Welgen, Inc. (Worcester, MA) using purified plasmid encoding β ARK_{CT} obtained from Guthrie cDNA Resource center. Empty vector served as control. We have previously shown that we can obtain satisfactory transfection (~30% cells) of arterial tissue using adenoviral transfection of vector expressing β -galactosidase (12). The β -galactosidase staining lasts under 3 days and is evident throughout the vessel, with a relative gradient that is maximal with the adventitia and extends into the media. Both VSMC and fibroblasts stain with the vector.

Morphometry

Following perfusion fixation, each vessel was bisected and central segments from each of these halves were embedded in paraffin and cross sections were cut (10, 100 μ m apart). Standard morphometric measurements were performed on histological cross sections stained with Hematoxylin/Eosin and Movat's stains using a SPOT camera system. The luminal, intimal and medial areas of each vessel were determined using an Image-Pro system. Following determination of the dimensions of each vessel, a ratio of the intimal and medial areas was calculated.

Determination of in vivo proliferation and apoptosis

Labeling of proliferating VSMC in specimens was performed using the thymidine analogue 5-bromo-2-deoxyuridine (BrdU; Boehringer-Mannheim Corp., Indianapolis, IN; administered by Intraperitoneal injection 24 hours prior to sacrifice, 60 μ g/g body weight). The number of BrdU-stained VSMC nuclei was counted and the BrdU labeling index (% of unlabelled cells) calculated on 10 slides (100 μ m apart). Labeling of apoptotic VSMC on adjacent sections from each vessel specimen was evaluated using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL; Boehringer-Mannheim Corp.) after processing the tissue according to the manufacturer's guidelines on 10 slides (100 μ m apart). Stained VSMC nuclei were counted and the TUNEL labeling index (% of unlabelled cells) calculated.

En Face SMC Migration Assay

Femoral arteries were perfusion-fixed as described above, and the vessels were opened longitudinally and pinned down onto agar plates with the luminal surface facing upward. Arteries were stained with hematoxylin for 10 seconds and incubated in Scott's blue for 2 minutes. Migrated VSMCs were identified as the hematoxylin-stained cells on the luminal surface of the endothelium-denuded area. The length of the femoral artery was visualized under a microscope at $\times 100$ magnification, and the total number of VSMC above and below the internal elastic lamina were counted and results expressed as % cells on the luminal surface of the artery.

Immunoblotting

Following wire injury femoral arteries were rapidly retrieved, cleaned once in ice-cold dPBS and then placed in ice-cold kinase buffer (200 μ l; 25 mM Hepes, pH 7.5, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 100 mM benzamidine, 0.1% 2-mercaptoethanol, 1% Triton X-100, 1 mM pepstatin A, 2 mg/ml leupeptin and 20 kallikrein inhibitor units/ml

aprotinin). Protein determinations were performed using a bicinchonic acid assay (Bio-Rad Laboratories, Inc., Richmond, CA) with bovine serum albumen as the standard. Equal amounts of protein lysates (20 μ g) were loaded onto a 10% polyacrylamide gel, separated by electrophoresis and then transferred to a nitrocellulose membrane (Bio-Rad) (13). The membrane was blocked in 5% non-fat milk. The blot was then immunostained with antibodies against the target proteins. Protein bands were visualized using an anti-mouse IgG horseradish peroxidase-conjugated antibody followed by enhanced chemi-luminescence (ECL, Amersham Bioscience, Piscataway, NJ) using the manufacturer's protocols. Band intensity was measured using Gel-Imaging software (Kodak, Rochester, NY). All experiments were performed at least three times and normalized to GAPDH levels.

Plasminogen Zymography

Casein Zymography was performed as described previously (14) with minor modifications. Equal amounts of proteins were mixed with SDS sample buffer without reducing agent and subjected to 12.5% SDS-PAGE containing casein (0.25 mg/ml) and plasminogen (20 μ g/ml). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min each at room temperature to remove SDS and then incubated for 18 hrs at 37°C in buffer containing 100 mM Glycine, pH 8.0. The proteins in the gel were stained with 0.2% Coomassie Brilliant Blue in 30% Methanol: 10% Acetic acid solution for 2–3 hrs and then destained in 30% Methanol: 10% acetic acid.

Reverse Plasminogen Zymography

Reverse Plasminogen Zymography was performed as described previously (14) with minor modifications. Equal amounts of proteins were mixed with SDS sample buffer without reducing agent and subjected to 12.5% SDS-PAGE containing casein (0.25 mg/ml), plasminogen (20 μ g/ml) and u-PA (0.03 U/ml). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min each at room temperature to remove SDS and then incubated for 18 hrs at 37°C in buffer containing 100 mM Lysine, pH 8.0. The proteins in the gel were stained with 0.2% Coomassie Brilliant Blue in 30% Methanol: 10% Acetic acid solution for 2–3 hrs and then destained in 30% Methanol: 10% acetic acid.

Gelatin Zymography

For assay of MMP-2 and MMP-9 activity, vessels were isolated and snap frozen after rinsing in ice cold PBS. Segments were then pulverized on liquid nitrogen using a mortar and pestle and extracted with a guanidine based buffer (0.05 M TRIS, pH 7.5, 0.01 M CaCl_2 , 2.0 M Guanidine and 0.2% Triton X-100). The suspension was centrifuged and dialyzed against 0.05 M TRIS and 0.2% Triton for 48 hours. A BCA protein assay (Pierce, Rockford, IL) was performed and 10 μ g protein was loaded per lane. Gelatin zymography for matrix metalloproteinases (MMPs) was performed as described previously (15).

Reverse Gelatin Zymography

Gelatin Zymography was performed as described previously (16) with minor modifications. Equal amounts of proteins were mixed with SDS sample buffer without reducing agent and subjected to 7.5% SDS-PAGE containing gelatin (2.5 mg/ml) and pro-MMP-2 (1.2 μ g/ml) or pro-MMP-9 (1.2 μ g/ml). After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min each at room temperature to remove SDS and then incubated for 18 hrs at 37°C in buffer containing Tris-HCl (40 mM), CaCl_2 (10 mM) and NaCl (50 mM). The proteins in the gel were stained with 0.2% Coomassie Brilliant Blue in 30% Methanol: 10% Acetic acid solution for 2–3 hrs and then destained in 30% Methanol: 10% acetic acid. TIMP activity was detected as blue bands against a clear background of MMP digestion.

Data and Statistical Analysis

All data are presented as the mean $\pm$ standard error of the mean (s.e.m.) of six observations and statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate. A p-value less than 0.05 was regarded as significant. Non-significant p-values were expressed as p=ns.

RESULTS

G-protein Expression

Following injury, there was a marked time-dependent increase in G $\beta\gamma$ over the 28 days, peaking at day 7 (Fig 1). We did not see any significant change in the sham-operated arteries. Transfection with β ARK_{CT} did not alter G $\beta\gamma$ expression.

Modulation of cell kinetics

Vascular smooth muscle cell apoptosis using TUNEL as a marker peaked within 1–3 days after injury (Fig 2A), and vascular smooth muscle cell proliferation using BrdU as a marker peaked at 3–5 days after injury (Fig 2B). Vascular smooth muscle cell migration peaked at 7 days after injury (Fig 2C). Inhibition of G $\beta\gamma$ by adenoviral transfection of β ARK_{CT} inhibited both cell proliferation and migration (Fig 2B and 2C). However, apoptosis was increased following adenoviral transfection with β ARK_{CT} (Fig 2A). We detected apoptosis in the adventitia and in the media early and in the intima and media late.

Morphological Response

The injured femoral arteries develop intimal hyperplasia, which is maximal at 28 days. Sham-operated vessels did not produce such a response. When the vessels were encased in pluronic gel in vivo, there was no significant change in intimal hyperplasia development. Transfection with β ARK_{CT} to inhibit G $\beta\gamma$ reduced intimal hyperplasia development by 81% (Fig 2D). The percent area of the wall composed of proteoglycans increased after balloon injury and this increase was decreased by transfection with β ARK_{CT}. (E) There was no difference in the inflammatory infiltrate between the empty and β ARK_{CT} vectors.

Protease Response

uPA activity peaked at 7 days while PAI-1 activity was decreased early from 0 to 3 days and returned to baseline thereafter. There were time-dependent changes in expression of uPA and PAI-1 by western blotting after injury. PAI-1 expression was increased early from 0 to 3 days and returned to baseline thereafter. In contrast, uPA protein showed a steady increase that rose at day 7 and peaked at day 28. Inhibition of G $\beta\gamma$ decreased peak uPA activity and expression but did not alter the early peaks in PAI-1 activity and expression (Fig 3).

MMP-2 activity increased significantly at day 1 and peaked again at day 7 and then showed a continual decline in activity to day 56. MMP-9 activity peaked early at day 3 and declined thereafter. Protein expression for both MMP-2 and MMP-9 increased significantly after injury and were both maximal at day 14. Inhibition of G $\beta\gamma$ reduced peak MMP-2 activity at day 1 and at day 7 and also reduced peak MMP-9 activity at day 3 (Fig 4). Protein expression for both MMP-2 and MMP-9 was also decreased (Fig 4). There was an initial decrease in TIMP-1 and TIMP-2 expression and activity after injury until day 5. Both expression and activation of TIMP-1 and TIMP-2 gradually increased thereafter, to level out by day 21. Following inhibition of G $\beta\gamma$, there was no change in TIMP-1 and TIMP-2 expression and activity after injury (Fig 4).

DISCUSSION

This study confirms that injury to the arterial wall results in an increase in G β γ expression and that inhibition of G β γ results in a decrease in cell proliferation and cell migration. The inhibition of G β γ markedly decreased development of intimal hyperplasia. Associated with these changes in cell kinetics and morphology, the early expression and activity of the proteases uPA, MMP-2 and MMP-9 were also decreased without an increase in their respective inhibitors.

We have previously demonstrated that there are time-dependent changes in G-protein expression after vein grafting in the rabbit and balloon injury in the rat. In the current study, we noted that the relative expression of G β γ increased steadily over time with the increase in smooth muscle mass. Exposure to the G β γ inhibitor β ARK_{CT} did not reduce the relative expression of G β γ . Several inhibitor studies have strengthened the contention that G-proteins are involved in the genesis of intimal hyperplasia (4–6, 17). Specific blockade of the G β γ -subunit in balloon-injured rat carotids with a single exposure to the G β γ inhibitor β ARK_{CT} reduced intimal hyperplasia (6). We have seen a similar response in rabbit vein grafts (5). The development of intimal hyperplasia is the result of cell migration, cell proliferation and the deposition of extracellular matrix. The origins of the cells in the intimal hyperplastic response are multifactorial (1). The VSMC may be alternated phenotypes of the existing VSMC (conversion from contractile to synthetic phenotypes), they may be dedifferentiated fibroblasts that migrated in from the adventitia or they may arise from circulating mesenchymal cells which are recruited into the vessel wall. G β γ proteins have been shown to be involved in each of these cell types and thus β ARK_{CT} may have altered one or more of these cells' appearance into the intimal hyperplasia. This report confirms these findings but examines the mechanisms of the G β γ inhibition. Inhibition of G β γ has been shown to inhibit the proliferation and migration of VSMC in vitro in response to a variety of G-protein coupled agonists. In the current study, we noted a decrease in both cell proliferation and cell migration in the injured vessels after transfection with G β γ inhibitor β ARK_{CT}. The decrease in cell proliferation has been shown in other arterial injury studies (6). Changes in cell migration and invasion have been noted in models of cancer metastases, but this is the first report in a vascular model. However, we also noted an increase in cell apoptosis after transfection, which is a novel finding. This finding is in contrast to other recent data that demonstrated that overexpression of G β γ will increase UVB-induced apoptosis, while inhibition of G β γ with β ARK_{CT} decreased the apoptotic response (18).

uPA and PAI-1 expression is linked to wound healing and inflammation. Elevated uPA and decreased PAI-1 levels are predictors for angiographic coronary restenosis (19). In a previous report, we saw a steady rise in uPA protein, which stabilized at 14 days, and a peak in uPA activity at 7 days in this model (8). Both responses were blunted by the presence of MAPK inhibitors. A similar pattern is seen after injury to the rat carotid artery (20) and tranexamic acid, a plasmin inhibitor, will inhibit smooth muscle cell migration after carotid balloon injury (21). If uPA is placed in a pluronic gel around an injured carotid, there is a significant increase in the earlier development of intimal hyperplasia due to enhanced migration and proliferation of smooth muscle cells (22). Increased expression of PAI-1 will promote proliferation when VSMCs are exposed to tumor necrosis factor (23). Overexpression of uPA or a deficiency of PAI-1 in transgenic mice has been shown to promote neointimal formation in response to transmural electrical injury. A lack of uPA leads to a reduction in neointimal hyperplasia (24). Inhibition of G β γ decreased peak uPA activity and expression without altering the early peaks in PAI-1 activity and expression, resulting in a change in the relative activities, which may in part explain the decrease in intimal hyperplasia.

MMP-2 is closely associated with cell migration and is a target of uPA. In a recent study, we demonstrated that MMP-2 was unregulated after injury and that there is decreased VSMC migration when MMP activity is reduced (9). In contrast to MMP-2, MMP-9 is not constitutively expressed in VSMC but is induced after arterial injury and this increase in activity coincides with the onset of cell proliferation (14, 25–27). We have shown that the stress kinases play a role in MMP-9 expression and that peak MMP-9 expression and activity is associated with VSMC proliferation within the vessel wall (8). MMP inhibitors decrease smooth muscle cell proliferation and migration (9, 21, 25). Inhibition of G β γ reduced peak MMP-2 activity at day 1 and also reduced peak MMP-9 activity at day 3. Both these time points are associated with cell proliferation. This change in protease activity is associated with a decrease in cell proliferation.

The regulation of TIMPs is distinctly different from MMPs. The inhibitor associated with MMP-9 is TIMP-1. We have reported a decrease in TIMP-1 expression and activity after injury in this model (9). Arterial injury in TIMP-1^{-/-} mice demonstrates an enhanced response confirming the important role of TIMP-1 in moderating the neointimal response once injury has been initiated (28, 29). TIMP-2 is more closely associated with MMP-2 and TIMP-2 is constitutively expressed in VSMC and the vessel wall. TIMP-1 and TIMP-2 overexpression in the injured rat carotid will induce similar results, namely decreased intimal hyperplasia formation (30–33). This is equivalent to studies using exogenous MMP inhibitors (9, 21, 25). However, following inhibition of G β γ , there were no significant changes in TIMP-1 and TIMP-2 expression and activity after injury.

There are multiple methods used to transfect blood vessels. These can be intraluminal, transmural or adventitial. The agents can be viral, plasmid or siRNA and the carriers can be liquid, polymer and coatings. The current vector is a basic viral transfection system. Other systems have a greater efficacy. The current study uses a pluronic gel that allows concentration of an agent at the adventitial site and with its surfactant component it allows intracellular transport. It may not be a practical clinical delivery system. The PREVENT III study verified the possibility of delivering molecular therapeutics to a vein graft both intraluminally and adventitially ex vivo (34).

In conclusion, these data demonstrate that femoral wire injury in the mouse induces a consistent model of intimal hyperplasia and that it is associated with a time dependent increase in G-protein expression. G-proteins are integral to both smooth muscle cell proliferation and migration in response to injury.

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Figure 1.

Time course of $G\beta\gamma$ expression normalized to α -actin following wire injury in the murine femoral artery. Values are the mean $\pm$ s.e.m. ($n=6$ per group). Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

Figure 2.

Changes in cell apoptosis (**A**) cell proliferation (**B**) and cell migration (**C**) in wire-injured femoral vessels in the presence of pluronic gel with and without β ARK_{CT}. (**D**) A comparison of the effect of pluronic gel with and without β ARK_{CT} on intimal hyperplasia development at 14 days after wire injury. (**E**) Changes in the area of cells, collagen, proteoglycan and elastin in wire-injured femoral vessels in the presence of pluronic gel with and without β ARK_{CT}. Values are the mean $\pm$ s.e.m. (n=6 per group). Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

Figure 3.

Alterations in the peak expression (**A**) and peak activity (**B**) of uPA at day 7 and PAI-1 at day 3 in wire-injured femoral vessels in the presence and absence of $\beta\text{ARK}_{\text{CT}}$. Values are the mean $\pm$ s.e.m. (n=6 per group) (8, 9). Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.

Figure 4.

Alterations in the peak expression (**A**) at day 14 and peak activity at day 7 (**B**) of MMP-2 / TIMP-2 and MMP-9 / TIMP-1 in wire-injured femoral vessels in the presence and absence of $\beta\text{ARK}_{\text{CT}}$. Values are the mean $\pm$ s.e.m. (n=6 per group) (8, 9). Statistical differences between groups were tested with a Kruskal-Wallis non-parametric test with post hoc Dunn's multiple comparison correction, where appropriate.