Flow-Mediated Regulation of G-Protein Expression in Cocultured Vascular Smooth Muscle and Endothelial Cells

Eileen M. Redmond, Paul A. Cahill, James V. Sitzmann

Abstract—G-proteins have been implicated in the transduction of a number of flow-induced responses. We determined whether flow can modulate vascular endothelial or smooth muscle cell G-protein signaling. By use of a perfused transcapillary coculture system that permits the chronic exposure of cultured endothelial cells (ECs) and smooth muscle cells (SMCs) to physiological shear stresses, cocultures were exposed to stepwise increases in flow up to (1) 2 mL/min (low flow: 0.5 dyne/cm²), or (2) 44 mL/min (high flow: 15 dyne/cm²) and maintained for 72 hours before SMCs and ECs were harvested separately. Using Western blot analysis, EC Giα, expression was significantly increased (41±2.9%) by high-flow conditions compared with low-flow. The changes in G-protein expression were associated with a significant increase in endothelial nitric oxide synthase (eNOS) activity, elevated prostacyclin levels in the perfusing media, increased pertussis toxin–catalyzed ADP ribosylation of Giα substrates, and enhanced agonist-stimulated GTPase activity in cocultured ECs. In contrast, high flow induced a significant decrease in Giα1,5 expression (57±5%) in SMCs cocultured with ECs, an effect that was endothelium dependent, inhibited by indomethacin, and correlated with a decrease in pertussis toxin–catalyzed ADP ribosylation of Giα substrates, reduced agonist-stimulated GTPase activity, and enhanced basal and G-protein–stimulated adenyl cyclase activity. These data demonstrate that flow mediates selective changes in EC and SMC G-protein expression concomitant with changes in G-protein functionality and cellular signaling capacity. Moreover, flow-induced changes in SMC G-protein signaling capacity are endothelium dependent and require a cyclooxygenase product. G-protein modulation may thus represent an important mechanism whereby hemodynamic forces regulate vessel wall function. (Arterioscler Thromb Vasc Biol. 1998;18:75-83.)

Key Words: shear stress • flow • vascular endothelial cells • vascular smooth muscle • G-proteins

Mechanical forces associated with blood flow play an important role in the regulation of vascular tone, vascular remodeling, and the focal development of atherosclerotic lesions.1–3 ECs covering the inner surface of blood vessels are continuously exposed to this mechanical stress, which has two components: the tangentially acting shear stress and pressure (cyclic strain). ECs respond to increases in shear stress by releasing vasoactive mediators,1 including the vasodilators endothelium-derived relaxing factor, recently identified as NO, and PGI₂ and vasoconstrictors such as endothelin.4–6 Changes in shear stress also cause long-term alterations in vessel function through regulation of protein and gene expression.1–5,7 While endothelial responses to shear stress are important in physiological adaptation to the hemodynamic environment, they can also contribute to pathological conditions, eg, atherosclerosis and reperfusion injury.1–3 Thus, there is considerable evidence that hemodynamic forces can influence several aspects of EC biology that are critical to normal vessel wall function. However, there have been few studies of the subsequent consequences of sustained endothelial shear stress on the underlying smooth muscle, due in part to lack of an appropriate model.

In a similar manner to shear stress, ECs are exposed to cyclic strain that results from the pulsatile flow of blood. Cyclic strain increases the expression of endothelial substances including NO and cell adhesion molecule expression that may contribute to endothelial adhesion of monocytes on local vascular walls in vivo.9 Both tissue plasminogen activator and plasminogen activator inhibitor are enhanced after exposure of ECs to cyclic strain, with reactive oxygen species implicated in these processes.10 Cyclic strain also results in significant changes in EC signaling; namely, cAMP production,11,12 inositol triphosphate formation, diacylglycerol,13 and fibronectin production,14 all of which may contribute to the adaptation of the vasculature and in particular the endothelium, to changes in intravascular pressure. Vascular SMCs are also subjected to cyclic strain or stretch that results from the pulsatile flow of blood. Changes in SMC matrix/integrin interactions after cyclic strain have been demonstrated.15 Heterotrimeric G-proteins function as transducers of signals across the cell membrane by coupling diverse receptors to effectors and thus play a central role in signal transduction and cell biology.16,17 By definition, they consist of α, β, and γ subunits, each the product of distinct genes. α-Subunits bind and hydrolyze GTP and most obviously regulate the activity of the most studied effector response. Distinct subclasses of

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α-subunits involved in signal transduction include Giα, Goq, and Gsα.16,17 Recent evidence suggests that inhibitory G-proteins (Giα1,2,3) and Goq may play a role in both agonist- and shear stress–induced activation of NOS and COX–1, the enzymes responsible for the formation of NO and prostanooids, respectively, in ECs.18–20 Stimulation of these G-proteins triggers a number of signal-transduction cascades, including activation of K⁺ channels, phospholipase C, phospholipase A₂, and adenylyl cyclase.16,17,21,22 Few studies have investigated the possibility of flow-mediated regulation of vascular cell G-protein expression, which may play an important role in vessel adaptation to blood flow. To address this question, we used a perfused transcapillary coculture system, previously developed in our laboratory, which permits the chronic exposure of ECs and SMCs to graded changes in pulsatile flow within the range of physiological shear stresses.23 We report here that increased flow mediates selective and cell-specific alterations in vascular cell G-protein expression that correlate with changes in cell-signaling capacity and G-protein functionality.

Methods

Materials

Antibodies specific for Giα, Goq, and Gsα,16,17 were purchased from New England Nuclear. Antisera against Goq and Goq were purchased from Santa Cruz Biotechnology, Inc. Anti-rabbit IgG (horseradish peroxidase-linked) and ECL detection system were obtained from Amersham, [α-32P]ATP (800 Ci/mmol), [32P]NAD (800 Ci/mmol), and [α32P]GTP (30 Ci/mmol) were obtained from New England Nuclear. L-NAME, indomethacin, Dowex (AG 50 WX4, 200 to 400 mesh), and alumina were purchased from Sigma Chemical Company. All other chemicals were of the highest purity commercially available.

Cell Culture

Vascular SMCs

Rat superior mesenteric artery SMCs were isolated and cultured as previously described.24 Briefly, superior mesenteric arteries of male Sprague-Dawley rats weighing between 150 and 175 g (Charles River Wilmington, Mass) were stripped of fat and connective tissue and digested in minimal essential medium containing 0.7 mg/mL collagenase (type IA, Sigma), 0.25 mg/mL elastase (type III, Sigma), 0.4 mg/mL soybean trypsin inhibitor, and 1 mg/mL bovine serum albumin for 60 minutes at 37°C. Dissociated vascular SMCs were seeded into conventional plastic tissue-culture plates (Falcon) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, plus 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO₂, 95% air. Cells were routinely subcultured after treatment for 10 minutes with 0.125% trypsin-EDTA at 37°C. Cells between passages 5 and 9 were used in either monocultures or cocultures as described below.

<table>
<thead>
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<th>Selected Abbreviations and Acronyms</th>
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<tr>
<td>COX = cyclooxygenase</td>
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<td>EC = endothelial cell</td>
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<td>eNOS = endothelial NOS</td>
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<tr>
<td>L-NAME = Nω-nitro-L-arginine-methyl ester</td>
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<td>NO = nitric oxide</td>
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<td>NOS = NO synthase</td>
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<td>6-keto-PGF1α = 6-ketoprostaglandin F1α</td>
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<td>PGI2 = prostacyclin</td>
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<td>SMC = smooth muscle cell</td>
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Figure 1. Diagrammatic representation of the perfused transcapillary coculture model showing the media reservoir, flow path, and the capillary bundle (a); cocultured SMCs and ECs on semipermeable capillaries (b); and the experimental protocol (c). Cocultures (ECs and SMCs) or monocultures (SMCs grown in standard CO₂ incubator and consisted of an enclosed bundle of 150 semipermeable, Proenectin–F–coated polyethylene capillaries (“bioreactor,” capillary length 9.6 cm; internal diameter 330 μm/L; wall thickness 50 μm/L; pore size 0.3 μm/L; extracapillary surface area 164 cm²; lumenal surface area 124 cm²) through which media from a reservoir is pumped in a pulsatile fashion, at a chosen flow rate, via silicone rubber tubing (Fig 1). By altering the flow rate, a shear stress range of 0.05 to 15 dyne/cm² can be achieved in this system.25

Vascular ECs

Bovine aortic ECs (ECs), repository No. AG07680B, were obtained from the NIA Cell Culture Repository, Cornell Institute for Medical Research. These cells tested positive for the EC-specific von Willebrand factor and angiotensin-1 converting enzyme activity. They tested negative for α-smooth muscle actin. ECs were maintained under the same conditions as the SMCs above. ECs were used in cocultures, as described below, between passage 12 and 15.

Perfused Transcapillary Cultures

Cocultures of SMCs and ECs were established essentially as described in detail previously.23 The CELLMAX QUAD Artificial Capillary Cell Culture System (Cellco, Inc) was used. This apparatus was maintained in a standard CO₂ incubator and consisted of an enclosed bundle of 150 semipermeable, Proenectin–F–coated polyethylene capillaries (“bioreactor,” capillary length 9.6 cm; internal diameter 330 μm/L; wall thickness 50 μm/L; pore size 0.3 μm/L; extracapillary surface area 164 cm²; lumenal surface area 124 cm²) through which media from a reservoir is pumped in a pulsatile fashion, at a chosen flow rate, via silicone rubber tubing (Fig 1). By altering the flow rate, a shear stress range of 0.05 to 15 dyne/cm² can be achieved in this system.25

Seeding of SMCs and ECs

SMCs (∼3.2×10⁶ cells) in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics were first seeded into the extracapillary space via the side ports. The SMCs were allowed to establish themselves in multilayers on the outer surface of the capillaries for 14 to 17 days. After this period, ECs (∼3×10⁶ cells) were seeded into the lumenal spaces of the capillary bundle as detailed previously.25 In some cases, the addition of ECs to the bioreactor was omitted, thus resulting in monocultured SMCs.

The experimental protocol followed is shown diagrammatically in Fig 1. A series of perfused transcapillary cultures was examined in parallel. The SMC/EC cocultures or the SMC monocultures were designated as either “low flow” or “high flow.” The low-flow group
was exposed to a constant flow rate of 2 mL/min throughout the experiment, corresponding to a shear stress of 0.5 dyne/cm². The high-flow group was exposed to stepwise increases in flow up to 44 mL/min, maintained for 72 hours, corresponding to a shear stress of 15 dyne/cm². Where indicated, high-flow cocultures were treated with L-NAME (100 μmol/L) or indomethacin (10 μmol/L) throughout the last 72-hour period.

Cell Harvest

At the end of the experimental period, SMCs and ECs were separately harvested, without trypsin, essentially as described previously. Briefly, ECs were first removed via the end ports by repeated back flushing with Hanks’ balanced salts solution. The cartridge was cut open using a pipe cutter to access the SMCs. In this way, separate, pure populations of cells could be obtained for further analysis.

Particulate Fraction Preparation

Harvested SMCs and ECs were pelleted by low-speed centrifugation. The cell pellet was resuspended in 3 mL buffer A (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 0.05% bactein, protease inhibitor cocktail [Boehringer], pH 7.4), sonicated, and centrifuged at 30 000g for 30 minutes at 4°C. The resulting pellet was resuspended in buffer A at a concentration of ≥1 mg/mL and stored at −70°C. Protein was measured by the method of Bradford using bovine serum albumin as a standard.

NOS Activity

NOS activity, in particulate fractions prepared from ECs and SMCs, was measured by determining the conversion of L-[14C]arginine to L-[14C]citrulline essentially based on a method of Bredt and Snyder and described in detail previously.

PGI₂ Measurement by Radioimmunoassay

The stable metabolite of PGI₂, 6-keto-PGF₁α, was determined in samples of perfusate from the cocultures by direct radioimmunoassay, using standard techniques and as described in detail previously. Antisera used for 6-keto-PGF₁α had a cross-reactivity of <0.1% for other common prostaglandins. Values are expressed as picograms 6-keto-PGF₁α per milliliter perfusate per minute.

Western Blotting

Membrane proteins (15 to 40 μg per lane) were separated on a 10% SDS-polyacrylamide gel as described previously. After SDS-polyacrylamide gel electrophoresis, the separated proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-C, Amersham) using a transphor electroblotter unit ( Hoefer Scientific Instruments) at 100 V for 2 hours. After transfer, the membranes were incubated for 2 hours in blocking solution containing 50 mmol/L Tris base (pH 7.6), 4 mmol/L MgCl₂, and 14 mmol/L NaCl (TBS) supplemented with 5% nonfat dry milk, 0.1% Tween 20 and 2 mg/mL sodium azide. The membranes were then washed twice three times for 5 minutes each with TBS containing 0.1% (vol/vol) Tween 20 and 2% (vol/vol) NP-40. The membranes were incubated with the specific antisera in TBS-Tween 20, 0.02% (wt/vol) for 1 hour at room temperature with gentle rocking. After washing the blots three times for 10 minutes, they were incubated with the secondary antibody solution (horseradish peroxidase conjugated), diluted 1:5000 in TBS-Tween 20, for 40 minutes at room temperature with gentle agitation. The blots were finally washed three times for 10 minutes each before they were processed using the ECL detection system (Amersham), as described by the manufacturer. Blots were then covered in cellophane and exposed to Hyperfilm-ECL (Amersham) for 15 to 30 seconds. Equal protein loading was confirmed by India ink staining of protein in each lane of the same blot. The signal intensity (integral volume) of the appropriate bands on the autoradiogram was analyzed using a Personal Densitometer (Molecular Dynamics) and the Imagequant software package (Biosoft).

Adenyllyl Cyclase Assay

Adenyllyl cyclase activity was assessed by measuring the conversion of [32P]ATP to [32P]cAMP, as previously described. Samples (150 μl) routinely contained ~50 μg protein, 1 mmol/L ATP, 0.5 μCi [32P]ATP, 0.4 mg/mL theophylline, 2.2 mg/mL creatine phosphate, 0.2 mg/mL creatine phosphokinase, 10 μmol/L GTP, and 4 mmol/L MgCl₂ in TME buffer (50 mmol/L Tris-HCl, 2 mmol/L MgCl₂, 1 mmol/L EDTA, pH 7.4). Assays were ended after 20 minutes at 37°C by addition of 100 μL stopping solution (10 mmol/L Tris-HCl, 2% SDS, 2 mg/mL ATP, and 0.5 μg/mL cAMP, pH 7.5). The [32P]cAMP formed was separated from the [32P]ATP by sequential chromatography on Dowex and neutral alumina. Chromatographic yields were 75% to 90%. Specific activities were expressed as picomoles cAMP formed per milligram of protein per minute.

Pertussis Toxin–Catalyzed ADP Ribosylation

G-protein substrates of pertussis toxin were assayed using pertussis toxin-catalyzed incorporation of [32P]ADP-ribose from [32P]NAD, as previously described. Membranes (40 to 60 μg) were resuspended in 100 μL of 100 mmol/L Tris-HCl (pH 8.0), containing 5 mmol/L DTT, 10 mmol/L thymidine, 6 mmol/L MgCl₂, 2 mmol/L GTP, 2.5 mmol/L ATP, and 10 μmol/L [32P]NAD. Pertussis toxin (100 μg/mL) was activated by prior incubation in 50 mmol/L HEPES (pH 8.0), containing 20 mmol/L DTT, 0.125% SDS, and 0.1 mg/mL BSA for 30 minutes at room temperature. After addition of activated pertussis toxin (20 μg/mL), the membrane preparations were incubated for 90 minutes at 30°C. The ADP ribosylation reactions were stopped by centrifugation at 15,000g for 3 minutes and the pellet was resuspended in SDS-sample buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% (vol/vol) glycerol, and 5% (vol/vol) β-mercaptoethanol. The samples were boiled for 10 minutes before being resolved on a 10% SDS-polyacrylamide gel. Gels were dried on cellophane and exposed to Kodak XAR-5 film with an intensifying screen at −70°C for 1 to 3 days.

GTPase Assay

A modification of the method described by Weber and Macleod was used to measure release of [32P]GTP from [γ-32P]GTP. The assay system contained [γ-32P]GTP (~50 000 cpm), 5 mmol/L MgCl₂, 0.1 mmol/L EGTA, 50 μmol/L NaCl, 4 mmol/L creatine phosphate, 5 U phosphocreatine kinase, 0.1 mmol/L ATP, 0.5 μmol/L GTP, 1 mmol/L DTT, 33 mmol/L Tris-HCl, pH 7.4, and 10 to 15μg of membrane protein in a total volume of 150 μL. The assay was initiated by addition of membranes to the reaction mixture and incubating for 15 minutes at 22°C in the absence or presence of norepinephrine (10 μmol/L). The reaction was terminated with 500 μL of ice-cold 5% activated charcoal in 20 mmol/L phosphoric acid, and the mixture was centrifuged for 10 minutes at 12,000g at 4°C. The liberated [32P]GTP was determined by counting the supernatant for 1 minute in a liquid scintillation counter. High affinity GTPase activity was calculated as the difference between total and nonspecific hydrolysis (determined in the presence of 100 μmol/L GTP).

Statistics

The data shown are the mean ± SEM. Statistical significance was estimated using the following analysis: Unpaired Student’s t test for comparison of two groups; Wilcoxon signed rank test for the densitometric data. A value of P <0.05 was considered significant.

Results

Effect of Increased Flow on Cocultured eNOS Activity and PGI₂ Levels (6-Keto-PGF₁α)

Previous studies have shown that ECs exposed to shear stress increase their NO and PGI₂ production. We therefore determined whether cocultured ECs exposed to sustained pulsatile flow mimicked this effect. ECs exposed to high flow (44 mL/min, shear stress ~15 dyne/cm²) exhib-
ited a significant increase, compared with low flow (2 mL/min, shear stress 0.5 dyne/cm²), in eNOS from 1.75±0.3 to 3.85±0.5 pmol [14C]citrulline per minute per milligram, n = 7, P < .05. Treatment of cocultures with 100 μmol/L L-NAME significantly inhibited the flow-induced increases in eNOS activity (60±5%, P < .05, n = 3).

Similarly, 6-keto-PGF₁α levels were significantly elevated in the conditioned media under high-flow conditions compared with low flow (2.20±0.3 to 3.7±0.7 pg 6-keto-PGF₁α per milliliter perfusate per minute, P < .05). In addition, pretreatment of the EC/SMC cocultures with 10 μmol/L indomethacin, a COX inhibitor, significantly decreased the levels of 6-keto-PGF₁α in conditioned media under high-flow conditions (3.7±0.7 to 0.69±0.1 pg 6-keto-PGF₁α per milliliter perfusate per minute, P < .05, n = 3).

Effect of Increased Flow on Cocultured EC G-Protein Expression
Using Western Blot analysis, ECs cocultured with SMCs expressed Giα₁–2, Giα₃, Gaq, and Gsα proteins under both low-flow (2 mL/min, shear stress ≈0.5 dyne/cm²) and high-flow (44 mL/min, shear stress ≈15 dyne/cm²) conditions. In addition, the detection of these antigens was linear with respect to protein concentrations for each G-protein examined (data not shown). In cocultured ECs exposed to high-flow conditions, Giα₃ protein expression was significantly enhanced compared with cocultured ECs exposed to low-flow conditions (Fig 2). Cumulative densitometric analysis of Giα₃ protein expression in ECs exposed to high flow demonstrated a 41±2.9% increase compared with low flow. However, inhibition of eNOS or COX activity with L-NAME (100 μmol/L) and indomethacin (10 μmol/L), respectively, failed to reverse the flow-induced increase in Giα₃ protein expression (Fig 3). The specificity of the flow-induced increase in Giα₃ protein expression was apparent because there was no significant effect of increased flow on cocultured ECs Giα₁–2, Gaq, or Gsα expression (Fig 4a, 4b, and 4c).

Effect of Increased Flow on Cocultured SMC G-Protein Expression
The effect of increased flow on SMC G-protein expression was determined in SMCs that had been cultured either in the presence (cocultures) or absence (monocultures) of ECs. In this way it was possible to assess the role, if any, of the endothelium in mediating a change in SMC G-protein expression. Using Western Blot analysis, SMCs cocultured with ECs and cultured alone expressed Giα₁–2, Giα₃, Gaq, and Gsα proteins under both low-flow (2 mL/min, shear stress ≈0.5 dyne/cm²) and high-flow (44 mL/min, shear stress ≈15 dyne/cm²) conditions.
and high-flow (44 mL/min, shear stress ≈15 dyne/cm²) conditions. In addition, the detection of these antigens was linear with respect to SMC protein concentrations for each G-protein examined (data not shown). Giα1–2 expression was significantly decreased (57 ± 5%) in cocultured SMCs exposed to high flow compared with cocultured SMCs exposed to low flow (Fig 5). This appeared to be an endothelium-dependent effect, as there was no effect of flow on Giα1–2 in SMCs cultured in the absence of ECs (Fig 6). In addition, the flow-induced decrease in cocultured SMC Giα1–2 expression was completely reversed by indomethacin (10 μmol/L), whereas L-NAME (100 μmol/L) had no effect (Fig 6). There was no effect of flow on Gia3, Gaq, or Gsα expression in either cocultured SMCs or SMCs cultured alone in the absence of ECs (Fig 7).

**Figure 5.** Effect of increased flow on cocultured SMC Giα1–2 protein expression. SMCs cultured in the presence of ECs (cocultured SMCs) were exposed to either low flow (shear stress 0.5 dyne/cm²) or high flow (shear stress 15 dyne/cm²), and Western blot analysis was performed on particulate fractions, as described in “Methods.” A representative Western blot is shown, together with the cumulative densitometric data of eight separate experiments. *P<.05 versus low flow.

**Figure 6.** Effect of L-NAME (100 μmol/L) and indomethacin (Indo, 10 μmol/L) on the flow-induced decrease in cocultured SMC Giα1–2. Cocultured SMCs or SMCs cultured in the absence of ECs (monocultured SMCs) were exposed to low flow or high flow in the absence or presence of L-NAME (100 μmol/L) or indomethacin (10 μmol/L), as described in “Methods.” A representative Western blot is shown, together with the cumulative densitometric data of at least three separate experiments. *P<.05 versus low flow.

**Figure 7.** Effect of increased flow on cocultured and monocultured SMC Gsα (a), Gia3 (b), and Gaq (c) protein expression. A representative Western blot is shown, together with the cumulative densitometric data of at least three separate experiments. Indo indicates indomethacin.

**Effect of Increased Flow on Cocultured EC and SMC Adenylyl Cyclase Activity, Pertussis Toxin–Catalyzed ADP-Dependent Ribosylation of Gia3 Substrates, and Agonist-Stimulated GTPase Activity**

To determine whether flow-induced differential changes in endothelial and vascular SMC G-protein expression correlated with changes in (1) the signaling capacity of these cells and/or (2) G-protein functionality, we measured adenylyl cyclase activity, pertussis toxin–catalyzed NAD-dependent ADP ribosylation of Gia3 substrates, and agonist-stimulated GTPase activity, respectively, in both cell types. Basal adenylyl cyclase activity, as well as that stimulated by GTPγS (a nonhydrolyzable guanine nucleotide
Flow-Mediated G-Protein Regulation

anologue) and forskolin (a diterpine derivative), was measured in ECs and SMCs cocultured under either low- or high-flow conditions. Adenylyl cyclase activities in membranes prepared from cocultured ECs and SMCs were linear with respect to increasing concentrations of protein (5 to 100 μg) (data not shown).

In cocultured ECs, basal adenylyl cyclase activity was significantly greater in membranes prepared from ECs exposed to high-flow conditions compared with those under low-flow conditions (15.57±0.77 versus 6.8±0.27 pmol cAMP per milligram protein per minute). Similarly, GTPyS (10 μmol/L)- and forskolin (50 μmol/L)-stimulated adenylyl cyclase activity was significantly greater in membranes prepared from ECs exposed to high-flow conditions (Fig 8).

Similarly, in cocultured SMCs, basal adenylyl cyclase activity was significantly greater in SMC membranes prepared from cocultured SMCs exposed to high-flow conditions compared with those under low-flow conditions (31.0±3.4 versus 15.3±2.0 pmol cAMP per milligram per minute). Moreover, GTPyS and forskolin-stimulated adenylyl cyclase activity was significantly greater in SMC membranes prepared from SMCs exposed to high-flow conditions (Fig 8).

We further examined the functionality of the flow-induced changes in G-protein expression in cocultured cells exposed to high flow by determining pertussis toxin–catalyzed ADP-dependent ribosylation of Giα substrates. Pertussis toxin catalyzed the incorporation of [32P]ADP-ribose into one major peptide band (40 kD) in both low-flow and high-flow cultured cells, a protein that comigrated with Giα proteins immunodetected using specific antibodies for Giα proteins (data not shown). The ribosylation was linear over a range of 10 to 100 μg of membrane protein (data not shown). Pertussis toxin–catalyzed ribosylation of Giα substrates was significantly increased in cocultured EC membranes but significantly decreased in SMC membranes prepared from cells exposed to high-flow conditions, respectively (Fig 9).

Receptor G-protein coupling in both ECs and SMCs exposed to low- and high-flow conditions was determined by measuring agonist-stimulated GTPase activity in membranes prepared from these cells. The release of [32P] from [γ32P]GTP by cocultured EC and SMC membranes was determined in the absence and presence of norepinephrine (10 μmol/L). There was no significant difference in basal EC GTPase activity between low- and high-flow groups: 26.16±1.2 versus 23.1±2.0 pmol [32P] per milligram protein per minute (n=3). Agonist-stimulated GTPase activity was significantly increased in high-flow EC membranes compared with the low-flow group (Fig 10). In contrast, in SMC membranes, basal GTPase activity was significantly greater in the high-flow group; 8.24±3.9 versus 17.83±2.1 pmol [32P] per milligram protein per minute (n=3). However, in cocultured SMCs there was a significant decrease in agonist-stimulated GTPase activity in high-flow membranes (Fig 10).

Discussion

A role for heterotrimeric G-proteins in the pathways proposed for flow-induced changes in signal transduction in ECs has recently emerged.10,31,32 Shear stress and cyclic strain-induced activation of these G-proteins results in several flow-initiated endothelial responses that regulate vascular tone, including release of the vasodilators NO and PGI2 and vasoconstrictors such as endothelin.14-16 Ohno et al19 demonstrated that shear stress stimulates endothelial NO release via a signal-transduction pathway that is coupled to a pertussis toxin–sensitive G-protein. Flow-induced PGI2 is also mediated in part by a pertussis toxin–sensitive G-protein.19 More recently, several studies have demonstrated that fluid shear stress acutely stimulates nitrogen–activated protein kinase activity in bovine aortic ECs via a novel pathway that is in part dependent on a G-protein, protein kinase C activity, and a herbimycin-sensitive tyrosine kinase.33,34 Thus, while G-proteins have been implicated in the acute response to changes in flow, it remained unclear whether chronic exposure of ECs to increased flow could regulate specific EC signaling, in particular,
NO and PGI₂ formation and G-protein expression and activity in ECs. Moreover, it was unclear whether changes in endothelial function due to flow could further influence cellular signaling in the underlying vascular smooth muscle.

ECs cocultured with SMCs significantly increased their expression of a pertussis toxin–sensitive Giₐ₁ protein after exposure to high flow. The flow-induced changes in EC G-protein expression correlated with changes in (1) the signaling capacity of these cells (ie, enhanced NO and PGI₂ production) and (2) the functionality of the G-protein, since agonist-stimulated GTPase activity and pertussis toxin–catalyzed ribosylation of Giα proteins were significantly increased in these cells. As a consequence of these observations, decreased basal adenylyl cyclase activity might be anticipated if these proteins are coupled in part to this enzyme. In contrast, enhanced basal and G-protein–stimulated adenylyl cyclase activity was observed, an effect previously reported for monocultures of ECs exposed to cyclic stretch. Enhanced basal and stimulated adenylyl cyclase activity was observed, an effect previously reported for monocultures of ECs exposed to cyclic stretch. These data are consistent with a flow-mediated increase in EC adenylyl cyclase activity that appears independent of changes in either Gα or Giα protein expression. Adenylyl cyclase isoforms are regulated at many different levels including α- and βγ-subunits, any of which could contribute to the enhanced activity in ECs exposed to high flow. Therefore, the enhanced basal and stimulated activity observed in ECs may reflect enhanced stimulatory Gα protein activity, βγ-subunit activity, and/or enhanced intrinsic activity of the adenylyl cyclase enzyme itself in ECs exposed to high flow. Whether these changes or other pathways that modulate adenylyl cyclase activity occur in ECs exposed to high flow requires further investigation.

The role of increased EC Giₐ₁ protein expression and subsequent cellular signaling remains unclear, but it may be involved in the sustained flow-induced production of NO and/or PGI₂. While previous studies have reported that shear stress–induced changes in NO and PGI₂ production are both pertussis toxin sensitive and insensitive, depending on the length of exposure to flow, preliminary experiments from our laboratory using cocultured ECs suggest that pertussis toxin inhibits flow-induced eNOS activity after a 2-hour exposure to high flow. Alternatively, the changes in Giₐ₁ protein expression and activity may underlie an enhanced sensitivity of the sheared EC to endothelial agonist (ATP, thrombin, endothelin, and α₁-adrenergic) receptor Gi–protein–coupled activation of NOS and COX activity. It is unclear if these proteins are coupled in part to this enzyme. This occurs in mesenteric resistance vessels exposed to chronic increases in blood flow in vivo in that eNOS activity is enhanced, and endothelial-dependent Gi–protein–induced relaxation is greater in vitro. In the present study, norepinephrine, which activates eNOS by binding to α₁-adrenergic receptors on ECs, stimulated GTPase activity (an index of receptor G-protein coupling), to a greater extent in ECs exposed to high flow, confirming an enhanced signaling capacity of these cells.

Few studies have examined the chronic effects of flow-induced changes in ECs on signaling within the underlying vascular smooth muscle. Our study is the first in vitro study to demonstrate that flow induces signaling changes in ECs that subsequently impact on the expression of Gi proteins in cocultured vascular SMCs. The EC dependency of this effect was evident in monocultured smooth muscle, where G-protein expression was unaltered. The flow-induced change in SMC Giₐ₁–2 protein expression correlated with a significant decrease in pertussis toxin–catalyzed ADP ribosylation of Giα substrates and agonist-stimulated GTPase activity and enhanced basal and stimulated adenylyl cyclase activity. Since flow increased the production of NO and PGI₂ from ECs, there was the possibility of the involvement of either endothelium–derived vasoactive substance in mediating the changes in vascular smooth muscle Giₐ₁–2 expression. Indeed, exogenous NO has recently been shown to decrease the expression of both Gα and pertussis toxin–sensitive Gi-proteins in peritoneal macrophages, vascular SMCs, and neuroblastoma cells, respectively. Inhibition of COX activity with indomethacin resulted in a significant decrease in 6-keto-PGF₁α levels in the conditioned media concomitant with a complete reversal of the flow-induced endothelium–dependent regulation of vascular smooth muscle Giₐ₁–2 protein expression. In contrast, eNOS inhibition with L-NAME failed to reverse the decrease in vascular smooth muscle Giₐ₁–2 protein expression despite decreasing eNOS activity and therefore subsequent NO production from ECs. Whether PGI₂ or another endothelium–derived COX product is responsible for the observed decrease in smooth muscle Giₐ₁–2 protein expression remains uncertain.

The consequences of the endothelium–dependent, indomethacin–sensitive decrease in SMC Giₐ₁–2 protein expression and subsequent altered cellular signaling remains unclear. Decreased Giₐ₁–2 protein expression on vascular SMCs may serve to adapt local vascular tone to the
modifications of increased flow. In fact, the role of pertussis toxin–sensitive G-proteins in signaling pathways that regulate vascular tone have been well documented. Continuous release of NO and PGI$_2$ from ECs exposed chronically to high flow may act to alter neurogenic and myogenic vasoconstriction directly or indirectly by modulating pressor hormone G-protein signaling. Furthermore, altered G$i_1$-$\alpha$ protein expression on SMCs due to sustained flow may contribute to the control of vascular cell proliferation. Several studies have demonstrated the importance of pertussis toxin–sensitive G-proteins in signaling pathways that mediate vascular remodeling and mitogenesis, including the mitogen-activated protein kinase pathways. The present study demonstrates that there exists potentially important mechanisms of vessel diameter modulation involving endothelial and SMC G$i$-protein expression regulation in response to chronic changes in flow. These changes in G$i$-protein expression occur when flow is increased well within the physiological range of shear stress that is found along the vascular tree. Moreover, they correlate well with characterized physiologic responses; eNOS activity and PGI$_2$ release in ECs, and reflect significant changes in the signaling capacity of both ECs and SMCs. In conclusion, changes in vascular cell G$i$-protein expression after chronic increases in flow may represent an important pathway by which vascular cells adapt to changes in blood flow.

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