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Adenylyl Cyclase 6 Overexpression Decreases the Permeability of Endothelial Monolayers via Preferential Enhancement of Prostacyclin Receptor Function[S]

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ABSTRACT

Overexpression of adenylyl cyclase (AC) has been proposed as a potential gene therapy strategy to increase cAMP formation in cardiomyocytes and cardiac function in vivo. The impact of AC overexpression on endothelial cells, which will be traversed by genes delivered in vivo, has not been examined. Hence, the goal of the current study was to determine the consequence of AC overexpression on vascular endothelial cells in terms of G-protein-coupled receptor (GPCR) signaling and endothelial barrier function. We demonstrate that adenoviral-mediated gene transfer of AC6 in human umbilical vein endothelial cells preferentially enhances prostacyclin receptor (versus other GPCR)-stimulated cAMP synthesis and, in parallel, inhibits thrombin-stimulated increases in endothelial cell barrier function. Using multiple strategies, including prostacyclin receptor-targeted small interfering RNA, we identify that the enhancement of endothelial barrier function by AC6 overexpression is dependent on an autocrine/paracrine feedback pathway involving the release of prostacyclin and activation of prostacyclin receptors. AC6 overexpression in endothelial cells may have use as a means to enhance prostacyclin function and reduce endothelial barrier permeability.

Adenylyl cyclase (AC), which is activated by G_s-linked G-protein-coupled receptors (GPCRs), catalyzes the synthesis of the second-messenger cAMP. cAMP, in turn, activates protein kinase A (Francis and Corbin, 1994), a low molecular weight G-protein exchange factor, Epac (de Rooij et al., 1998; Kawasaki et al., 1998), and cyclic nucleotide-gated channels (Robinson and Siegelbaum, 2003). Nine membrane-bound AC isoforms and one soluble isoform have been identified that differ in their chromosomal locations, tissue expression, and regulation (Hanoune and Defer, 2001; Zippin et al., 2004). AC6, a predominant AC isoform expressed by cardiomyocytes (Wang and Brown, 2004) and vascular endothelial cells (Bundey and Insel, 2003), is believed to play a key physiological role in cAMP production by those cell types.

An adenoviral-AC6 (advAC6) construct has been used previously to overexpress AC6 in cardiomyocytes, thereby increasing their ability to produce cAMP in response to the direct activator of AC, forskolin, and to GPCR agonists (Gao et al., 1998). Furthermore, overexpression of AC6 in murine and porcine heart increases cardiac contractility and cardiomyocyte cAMP-generating capacity (Lai et al., 2000; Roth et al., 2004). Increasing AC6 expression in models of heart failure improves cardiac performance (Lai et al., 2004; Tang et al., 2004). Such increases in AC6 expression have been achieved by using both a transgenic approach and via intracoronary delivery of an advAC6. Because the endothelium is the first target of a gene therapy delivered via the vasculature, this raises the question of the impact of AC6 overexpression on endothelial cell physiology. We thus sought to define this impact and to ask whether overexpression of AC6 in endothelial cells has potential therapeutic benefit in its own right.

Vascular endothelial cells both act as a barrier between the blood and underlying smooth muscle and tissue cells and

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ABBREVIATIONS: AC, adenylyl cyclase; GPCR, G-protein-coupled receptor; HUVEC, human umbilical vein endothelial cell; advAC6, adenoviral-AC6; adv*LacZ,* adenoviral-*LacZ*; PG, prostaglandin; $cPGI_2$, carbaprostacyclin; L-161,982, 4′-[3-butyl-5-oxo-1-(2-trifluoromethyl-phenyl)-1,5-dihydro-[1,2,4]triazol-4-ylmethyl]-biphenyl-2-sulfonic acid (3-methyl-thiophene-2-carbonyl)-amide; siRNA, small interfering RNA; FITC, fluorescein isothiocyanate; PCR, polymerase chain reaction; BW245C, 4-imidazolidineheptanoic acid, 3-((3*R*)-3-cyclohexyl-3-hydroxypropyl)-2,5-dioxo-, (4*S*)-rel-; SKF 38393, 6-phenyl-4-azabicyclo[5.4.0]undeca-7,9,11-triene-9,10-diol; SC-19220, dibenz(*b*,*f*)(1,4)oxazepine-10(11H)-carboxylic acid, 8-chloro-, 2-acetylhydrazide; BWA868, 3-[2-cyclohexyl-2-hydroxyethyl)amino]-2,5-dioxo-1-(phenylmethyl)-4-imidazdine-heptanoic acid.

release and respond to vasoactive agents, including prostaglandins (PGs; e.g., PGE_2, PGI_2, PGD_2, and $PGF_{2\alpha}$) that have specificity for subtypes of PG receptors (EP_{1-4}, IP, DP, and FP, respectively) (Breyer et al., 2001). Certain PG receptor subtypes, $EP_{2,4}$, IP, and DP, are G_s-linked and, upon stimulation, activate AC to increase intracellular cAMP concentration, thereby regulating various aspects of endothelial cell physiology, including barrier function (Patterson et al., 2000; Qiao et al., 2003; Cullere et al., 2005; Fukuhara et al., 2005).

Increased vascular permeability is observed under many pathological conditions, including sepsis, development of atherosclerosis, and predisposition to tumor metastasis, asthma, and related pulmonary disorders (Toborek and Kaiser, 1999; Lush and Kvietys, 2000; Orr et al., 2000); however, few effective therapies are available to enhance barrier function (van Nieuw Amerongen and van Hinsbergh, 2002). Because cAMP levels can regulate this function of vascular endothelial cells, we used human umbilical vein endothelial cells (HUVECs) and tested whether advAC6 treatment alters barrier function of these cells. Our results show that AC6 overexpression enhances prostacyclin response and reduces endothelial barrier permeability.

Materials and Methods

Reagents. All reagents were purchased from Sigma-Aldrich (St. Louis, MO) with the following exceptions: HUVECs (VEC Technologies, Rensselaer, NY); TRIzol reagent, NuPage gels, and Superscript II (Invitrogen, Carlsbad, CA); QuantiTect PCR Mix (QIAGEN, Valencia, CA); WestDura chemiluminescent reagent (Pierce Reagents, Rockford, IL); all PGR agonists, BWA868, and EP_2, EP_4 receptor subtype-specific antibodies (Cayman Chemicals, Michigan, CA); human IP receptor antibody was raised against the peptide "CRRDPRAPSAPVGKE" corresponding to residues 330 to 343 in the C-terminal tail (custom synthesis by Pro-Sci Inc., Poway, CA); IP receptor siRNA SMARTpool reagent (Dharmacon, Lafayette, CO); SignalSilence Control siRNA (Cell Signaling Technologies, Danvers, MA); SAINT-MIX transfection reagent (Synvolux, Groningen, The Netherlands); and the EP_4 receptor-selective antagonist L-161,982 was a generous gift from Dr. R. Young (Merck-Frosst, Kirkland, QC, Canada) (Machwate et al., 2001).

Cell Culture and Treatments. HUVECs were grown in MCDB-131 medium containing 10% fetal calf serum, hydrocortisone (1 μg/ml), bovine neural tissue extract (50 μg/ml), heparin (100 μg/ml), epidermal growth factor (10 ng/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were grown in 75-cm^2 flasks and maintained at 37°C in a 95%/5% humidified air/CO_2 incubator. The cells were split 1:4 every 7 days; experiments were performed on cells at passages 2 to 4.

Adenoviral Treatment. AdvAC6 was a gift from H. Kirk Hammond (Veterans Affairs San Diego Healthcare System, San Diego, CA) and generated as described previously (Gao et al., 1998). Adenoviral-*LacZ* (adv*LacZ*) was used as the control. Adenoviral constructs were used at a concentration of 10 pfu/cell except where stated otherwise, and treatment was for 20 h.

Cell Size Assay. Cells were seeded onto collagen I-coated 24-well plates and grown to confluence(3–4 days). On the day of experimentation, cells were washed with Hanks' HEPES buffer (15 mM HEPES, pH 7.4) and equilibrated in Hanks' HEPES buffer for 30 min at 37°C. Cells were visualized with a Leica microscope using a 40× objective lens. Digital images were captured immediately before drug addition and after 3-min incubation at 37°C. Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA) was used to align images and quantitate cell shrinkage by channel subtraction. Results are intensity differences between pre- and poststimulus.

Measurement of Endothelial Barrier Permeability. Fluorescein isothiocyanate (FITC)-dextran conjugate (molecular mass = 70 kDa) was used to assess macromolecule permeability of HUVEC monolayers grown for 7 days on transwell collagen-treated supports (pore size, 0.4 μm; Corning, Corning, NY). FITC-dextran (1 mg/ml) was applied to the top chamber. After the addition of drugs, fluorescence (λ_{ex} 492 nm, λ_{em} 518 nm) was measured from aliquots taken from the lower chamber at 0 to 30 min.

Real-Time PCR and Immunoblotting. Real-time PCR was performed as described previously (Bundey and Insel, 2003) using published primer sequences for prostanoid receptors: EP_{1-4} and FP (Anthony et al., 2001); DP, TP, and IP (Sarrazin et al., 2001). Primers used for prostacyclin synthase detection were the following: 5′-GCAGACGTGTTTTGTTTGGA-3′ and 5′-TGTGAATGCAGAAGCAGACC-3′. Using serial dilutions of template, primer pairs were validated for use in the estimation of relative abundance by confirmation that their amplification efficiencies were similar (>0.99). Immunoblotting used the NuPage gel system (Invitrogen) following the manufacturer's protocol.

cAMP Radioimmunoassay. cAMP formation was measured as described previously (Ostrom et al., 2001). cAMP accumulation was stimulated by the addition of agonists in the presence of a nonselective phosphodiesterase inhibitor, isobutylmethylxanthine (200 μM); the reaction was terminated after 10 min by aspiration of medium and addition of ice-cold trichloroacetic acid (7.5% solution). In certain experiments involving receptor desensitization, cells were preincubated for 1 h with agonists before stimulation for 10 min with a subsequent dose of drug and measurement of cAMP content.

Prostaglandins Enzyme-Linked Immunosorbent Assay. PGE_2 and 6-keto $PGF_{1\alpha}$ (the stable breakdown product of prostacyclin) were measured using enzyme immunoassays following the manufacturer's protocol (Cayman Chemical).

IP Receptor siRNA. IP receptor siRNA was purchased as a SMARTpool reagent (four individual siRNA targeting different regions of the IP receptor mRNA). siRNA treatment was 0.25 $\mu g/10^6$ cells for 48 h and used in conjunction with SAINT transfection reagent following the manufacturer's protocol. SignalSilence Control siRNA was used to control for the effect of siRNA and affirm efficient transfection (because it is an FITC conjugate).

Statistical Analysis. Differences between two data points was determined by Student's t test, where $P < 0.05$ was considered significant. Differences between groups was determined by analysis of variance where $P<0.05$ was considered significant. Experiments were performed in triplicate on three separate occasions unless stated otherwise.

Results

Overexpression of Functional AC6 in HUVECs. Treatment of HUVECs with adv*LacZ* (10 pfu/cell) for 20 h and subsequent X-gal staining for β-galactosidase expression demonstrated gene transfer (~50% efficiency, supplemental data). AdvAC6 treatment produced a concentration-dependent increase in cAMP formation in response to forskolin (20 μM, 10 min) such that at 10 to 20 pfu, advAC6/cell cAMP formation was increased 6- to 8-fold compared with control (adv*LacZ*-treated) cells (Fig. 1A). Formation of cAMP in the absence of forskolin (i.e., "basal") was similar for control (2.0 ± 0.3 pmol/10^6 cells) and advAC6-treated cells (2.7 ± 0.6 pmol/10^6 cells).

Impact of AdvAC6 on Receptor-Mediated Increases in cAMP Formation. Agonists for multiple G_s-linked GPCR stimulate cAMP formation in HUVECs (Table 1) and showed ~2-fold greater (isoproterenol, histamine) maximum responses but only minimal changes in EC_{50} values with AdvAC6 treatment. In contrast, cAMP generated in response to

PGE_2 and IP receptor-selective agonists [beraprost, carbaprostacyclin ($cPGI_2$)] was enhanced in AC6 overexpressing cells to a similar extent as was in response to forskolin [i.e., much more than other GPCR agonists, including for EP receptor (11-deoxyPGE_1) or DP receptor (BW245C)] (Table 1). Other EP receptor subtype-selective agonists, butaprost (EP_2) or sulprostone (EP_3), were ineffective at elevating cAMP levels in control or AC6 overexpressing cells. Analysis of the concentration-response curve of PGE_2-stimulated cAMP identified a two-site relationship (Fig. 1B), which contrasted with the one-site curve for $cPGI_2$ (Fig. 1C). Use of the EP_4 receptor-selective antagonist L-161,982 (1 μM) revealed that the high-affinity site for stimulation of cAMP formation by PGE_2 is primarily mediated by the EP_4 receptor (Fig. 1B). BWA868 (1 μM), a DP receptor antagonist, had no effect on PGE_2-stimulated cAMP levels; however, SC-19220 (100 μM), an EP_1-selective antagonist, slightly increased maximal responses elicited by PGE_2 in both control and advAC6-treated cells (data not shown).

To determine whether a portion of the PGE_2-stimulated cAMP response is attributable to IP receptors, we exploited the ability of those receptors to resist acute desensitization

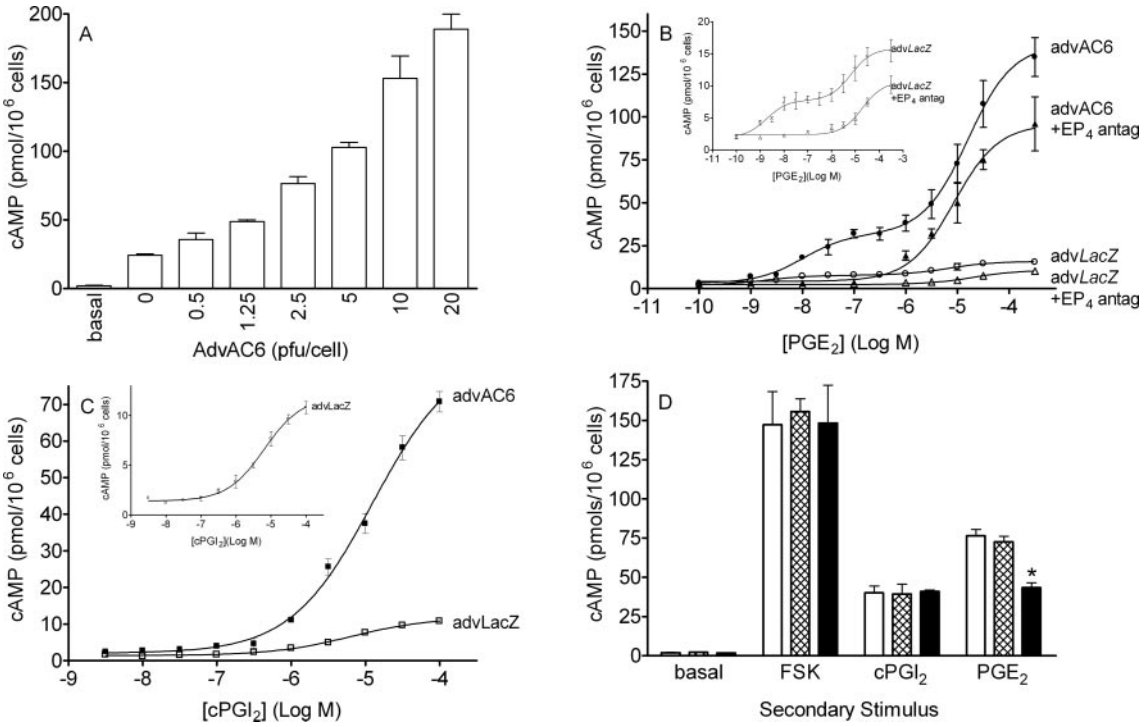

Fig. 1. PGE_2- and $cPGI_2$-stimulated cAMP formation is prominently enhanced by treatment of HUVECs with advAC6. A, AdvAC6 enhances forskolin (20 μM, 10 min)-stimulated cAMP in an adenoviral concentration-dependent manner. B, PGE_2-stimulated cAMP accumulation in control (○) and advAC6-treated (●) cells with a two-site concentration-response relationship. Pretreatment of cells with the EP_4 receptor antagonist L-161,982 (1 μM) inhibited the high-affinity response in both control (△) and advAC6-treated (▲) cells. C, cPGI2-stimulated cAMP accumulation in control (□) and advAC6-treated (■) cells. Inset, control (adv*LacZ*-treated) cell responses displayed on an optimized *y*-axis because they generate much less cAMP than advAC6-treated cells. D, pretreatment of advAC6-treated cells with PGE_2 or $cPGI_2$ identifies a desensitization-resistant component of the PGE_2 response. Cells were incubated for 1 h in the absence (□) or presence of $cPGI_2$ (10 μM, ▨) or PGE_2 (10 μM, ■) before a wash step and subsequent 10-min incubation with a second exposure to receptor agonist (same concentrations as first stimulation). cAMP generated in response to PGE_2 (10 μM) was significantly (*, $P < 0.01$, Student's *t* test, $n = 3$) reduced from unpretreated controls by pretreatment with PGE_2 but not with $cPGI_2$. As described in the text, the desensitization-insensitive and -sensitive components are probably mediated by the IP receptor and an EP receptor, respectively. Agonist incubations were in the presence of isobutylmethylxanthine (200 μM). The data shown are mean ± S.E.M. ($n = 3$).

TABLE 1

EC_{50} values and maximal (Max) responses of cAMP formation in control and advAC6 (10 pfu/cell)-treated cells

Values are presented as mean ± S.E.M. ($n \geq 3$).

Drug	EC_{50}		Max Response		Max Response Ratio
	Control	advAC6	Control	advAC6	
	μM		*pmol / 10^6 cells*		
Forskolin	10.1 ± 0.3	4.4 ± 0.2	28.0 ± 2.1	213 ± 17	7.6
Histamine	13.6 ± 0.8	11.2 ± 0.4	5.3 ± 0.7	12.0 ± 1.2	2.3
Isoproterenol	0.32 ± 0.06	0.7 ± 0.1	8.1 ± 1.0	18.2 ± 1.8	2.2
SKF-38393		1.5 ± 0.1		3.7 ± 0.2	
PGE_2 high affinity	0.002 ± 0.0001	0.01 ± 0.001	16.2 ± 1.4	142 ± 12	8.9
PGE_2 low affinity	0.5 ± 0.04	16.0 ± 0.3			
Beraprost	0.1 ± 0.02	4.4 ± 0.2	5.8 ± 0.7	46.0 ± 6.4	7.9
$cPGI_2$	5.9 ± 0.1	12.5 ± 0.4	11.8 ± 0.8	84.3 ± 1.1	7.1
11-Deoxy PGE_1	0.08 ± 0.01	0.13 ± 0.01	5.0 ± 0.4	11.0 ± 0.8	2.2
BW245C	5.0 ± 0.3	2.9 ± 0.1	4.5 ± 0.4	6.0 ± 0.3	1.3

(Hasse et al., 2003). AdvAC6-treated cells were incubated for 1 h in the absence or presence $cPGI_2$ (a stable analog of prostacyclin) (10 μM) or PGE_2 (10 μM) before a wash step and subsequent 10-min incubation with receptor agonist so as to define desensitization-insensitive and -sensitive components (Fig. 1D). Response to forskolin was unchanged by pretreatment with $cPGI_2$ or PGE_2, indicating the absence of desensitization of AC activity (Sobolewski et al., 2004). Response to $cPGI_2$ was not desensitized by prior exposure to receptor agonists, confirming the insensitivity of the IP receptor to acute desensitization. In contrast, response to PGE_2 was reduced in cells pretreated with PGE_2 (but not $cPGI_2$), implying that PGE_2-stimulated cAMP response of AC6-overexpressing cells consists of both desensitization-insensitive (IP receptor) and desensitization-sensitive (EP receptor) components.

PG Receptor Expression. Because of the prominent enhancement in PG receptor-stimulated cAMP by AC6 overexpression, we believed it was important to define the expression of PG receptors present in HUVECs. Real-time PCR revealed that the relative abundance of PG receptor expression is $EP_1 > IP > DP > TP > FP > EP_4 > EP_3 > EP_2$ (Fig. 2A). AdvAC6 treatment did not alter PG receptor mRNA or $G\alpha_i$ protein levels but slightly reduced $G\alpha_s$ protein expression (to ~80% of untreated; data not shown). Immunoblotting experiments using EP-subtype specific antibodies and a custom IP receptor antibody detected PG receptor protein expression for all except the EP_2 receptor (Fig. 2B). We detected multiple bands in HUVECs with several antibodies, suggesting that EP_1, EP_3, and IP receptors undergo post-translational modifications. Indeed, the upper (~57 and 61 kDa) bands in the IP receptor immunoblot were sensitive to *N*-glycosidase F treatment (data not shown).

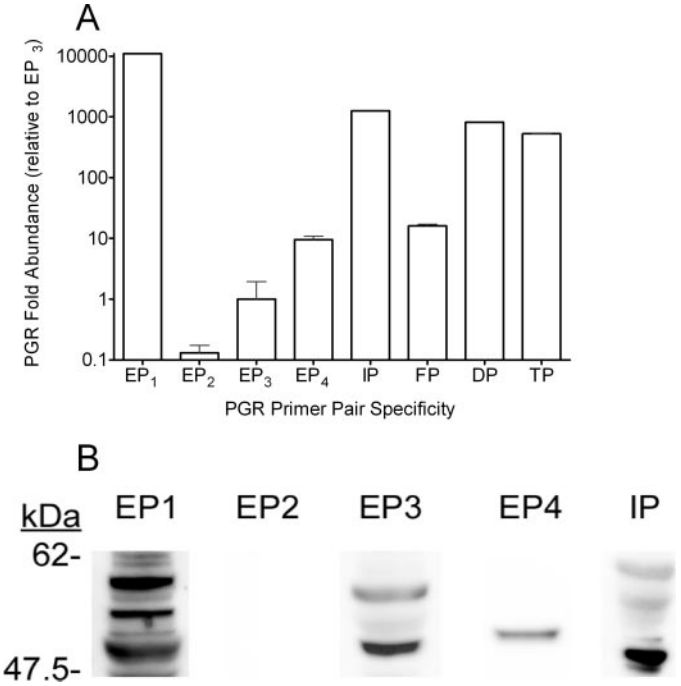

Fig. 2. PG receptor expression in HUVECs determined by real-time PCR (A) and immunoblot (B). PG receptor gene expression is expressed relative to EP_3 receptor; EP_1, IP, and DP receptors were the most abundant gene transcript levels of those tested. Immunoblot yielded results comparable with those from the real-time PCR data, with receptor-specific antisera identifying bands at the expected molecular mass of PG receptors (~50 kDa) with the exception of EP_2 receptor, which was undetectable. Immunoblots shown are representative of experiments repeated at least twice.

Effect of AdvAC6 on Endothelial Permeability. We examined barrier function, a cAMP-regulated property of endothelial cells, by monitoring FITC-dextran diffusion across Transwell inserts and observed the formation of an effective barrier in HUVEC cultures: in the absence of cells (Fig. 3, × symbols) diffusion of FITC-dextran was significantly greater (after 20 or 30 min) than in the presence of a cell monolayer (Fig. 3, □). Although the permeability of AdvAC6-treated cells was similar to that of control cells (Fig. 3, ■), thrombin (0.2 U/ml)-stimulated increase in permeability was reduced in advAC6-treated cells (Fig. 3, circles).

Effect of AdvAC6 on Interendothelial Gap Formation. Morphological analyses provided a further means to evaluate endothelial barrier function in response to thrombin stimulation (0.2 U/ml, 3 min): morphological changes included a retraction of cell borders, which appeared as an increase in interendothelial gap formation and could be better visualized by the subtraction of post- and prestimulation images (see "change," Fig. 4A). Image density analysis allowed the quantitation of the interendothelial gap formation (Fig. 4B). AdvAC6 treatment significantly reduced the thrombin-stimulated gap formation compared with control cells. Treatment with the cyclooxygenase inhibitor indomethacin (100 μM, 1 h) before thrombin-stimulation abolished the inhibitory effect of advAC6 (Fig. 4B). Measurement of PGE_2 and 6-keto $PGF_1\alpha$, a stable breakdown product of prostacyclin, in the media of control and advAC6-treated cells confirmed that indomethacin reduced extracellular content of basal and thrombin-stimulated PGE_2 (Fig. 4C) and prostacyclin (Fig. 4D).

IP Receptor siRNA. Because HUVECs produce prostacyclin and express IP receptors, we used a knockdown strategy to help define the role of autocrine/paracrine release of and response to prostacyclin on cellular responses after AC6 overexpression. Immunoblotting revealed that IP receptor siRNA

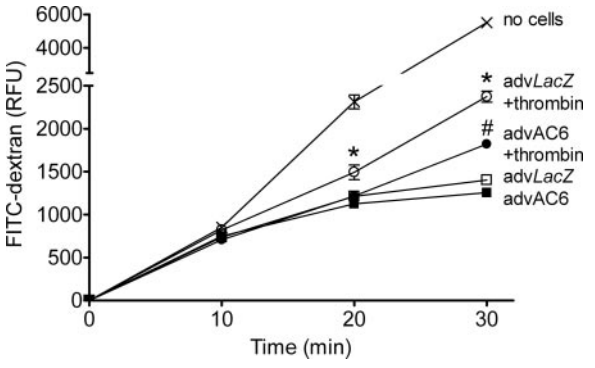

Fig. 3. Effect of advAC6 on endothelial cell monolayer permeability. AdvAC6-treated cells (■) show similar "basal" endothelial permeability compared with control cells (□). Thrombin (0.2 U/ml) (added at T = 0 min) increases endothelial permeability in control cells (○), and this increase is inhibited by advAC6 (●). Diffusion of FITC-dextran (70 kDa) across HUVEC monolayers grown on Transwell inserts (0.4 μm pore size) was monitored by fluorescent readings at 10, 20, and 30 min. Inserts seeded with no cells (×) showed significantly increased FITC-dextran diffusion compared with inserts seeded with cells, confirming effective barrier function of HUVECs. Data points are mean ± S.E.M. (n = 3). *, $P<0.05$, one-way analysis of variance (adv*LacZ* versus adv*LacZ* + thrombin); #, $P<0.001$, Student's t test (advAC6 + thrombin versus adv*LacZ* + thrombin).

treatment reduced IP receptor expression ~50% (Fig. 5A). Real-time PCR analysis verified the specificity of the IP receptor siRNA treatment (Fig. 5B): IP receptor mRNA was reduced ~7-fold with siRNA treatment, whereas gene transcripts of related components (prostacyclin synthase and other PG receptors) were changed <2-fold. IP receptor siRNA treatment of control cells significantly ($P < 0.05$, Student's t test, $n = 3$) increased EC_{50} for beraprost-stimulated cAMP (control, 100 ± 20 nM; siRNA, 550 ± 28 nM) without a substantial change in maximum response (Fig. 5C). In contrast, IP receptor siRNA treatment of AC6 overexpressing cells prominently reduced maximal cAMP response to beraprost (Fig. 5C; advAC6 alone, 46.0 ± 6.4 pmol/10^6 cells; advAC6 plus IP receptor siRNA, 10.2 ± 0.6 pmol/10^6 cells). The inhibitory effect of advAC6 on thrombin-stimulated increases in endothelial permeability was reduced by either IP receptor siRNA treatment or indomethacin treatment (100 μM for 1 h before thrombin stimulation) (Fig. 5D), results consistent with the idea that autocrine/paracrine production of prostacyclin and activation of IP receptors are responsible for the observed effects of AC6 overexpression on cAMP formation and endothelial cell barrier function.

Discussion

Overexpression of AC6 in HUVECs. Although primary cultures of endothelial cells can be difficult to transfect, we found that the use of an adenoviral construct was effective to accomplish the transfer and expression of AC6, as demonstrated by X-gal staining of adv*LacZ*-infected HUVECs and by enhanced forskolin-stimulated cAMP formation in advAC6-treated cells. The advAC6 produced a concentration-dependent increase in forskolin-stimulated but not basal cAMP formation, indicating that stimulation of cAMP formation is limited by the amount of AC6 in endothelial cells. Further insight into the stoichiometry of GPCR signaling components comes from analysis of the experiments using IP receptor-targeted siRNA (Fig. 5C). IP receptor expression was reduced ~50% by siRNA; whereas this treatment increased the IC_{50} for beraprost, the maximal level of cAMP in response to beraprost was unaltered in control HUVECs, indicating that the expression level of a signaling component other than the IP receptor determines the maximal ability of the cells to generate cAMP. In contrast, IP receptor knockdown in advAC6-treated cells produced a large reduction in

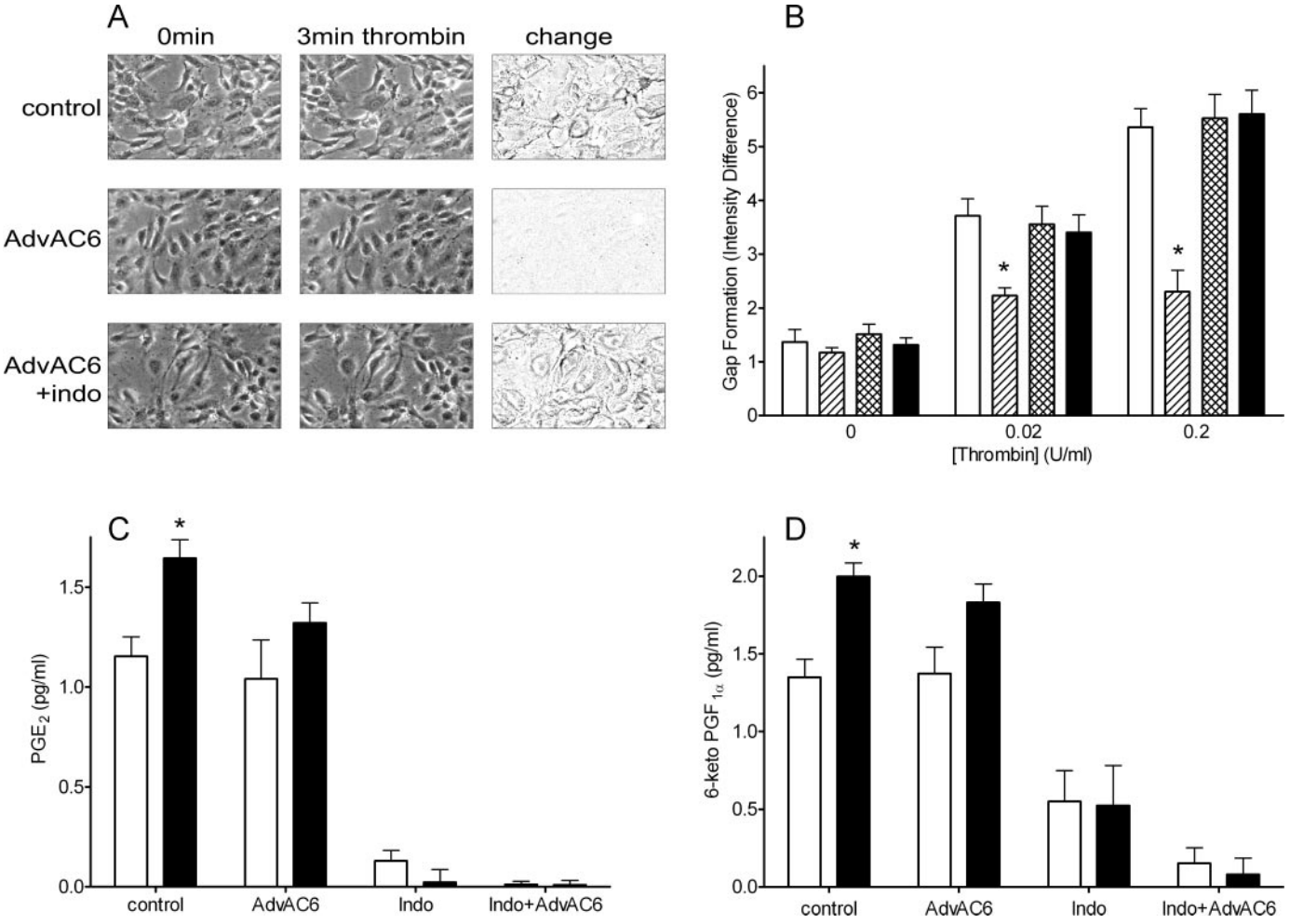

Fig. 4. Effect of advAC6 and indomethacin on interendothelial cell gap formation. A, images of HUVECs before stimulation (0 min) and 3 min after stimulation with thrombin (0.2 U/ml). Computational image subtraction was used to determine the interendothelial gap formation (third column, "change"). B, quantification of image changes by thrombin. Thrombin-stimulated gap formation in control cells (□) is inhibited by advAC6 (▨). Significant change from control cells, ∗, $P < 0.05$, Student's t test, $n = 3$. Indomethacin (100 μM) had no effect on control cell gap formation (▩) but blocked the inhibitory effect of advAC6 (■). PGE_2 (C) and 6-keto PGF1α (D) content of cell media from control (□) or thrombin (0.2 U/ml, 30 min)-stimulated cells (■) confirmed the reduction of PG efflux by indomethacin. Thrombin significantly changed PG secretion compared with untreated controls, ∗, $P < 0.05$, Student's t test, $n = 3$.

the maximal levels of beraprost-stimulated cAMP, implying that the ~50% IP receptor expression becomes limiting when AC6 is overexpressed. These results, along with those in Fig. 1 and Table 1, support the conclusion that expression of AC is the limiting signaling component for receptor and postreceptor stimulation of cAMP formation (Ostrom et al., 2000).

Analysis of the amplitude of the enhancement in maximal cAMP formation by advAC6 treatment for receptor agonists revealed two types of drug response: those enhanced 2- to 3-fold; or enhancement to an extent similar to the forskolin response, 7- to 9-fold. IP receptor response was preferentially enhanced by AC6 overexpression, an effect that underlies the enhancement of response to PGE_2. Evidence supporting this conclusion includes the following: the responses enhanced preferentially by advAC6 were, with the exception of forskolin and PGE_2, ligands selective for the IP receptor; and the concentration-response relationship of PGE_2-stimulated cAMP formation could be dissected into high-affinity (EP_4 receptor antagonist-sensitive) and low-affinity sites, the latter being responsible for the majority of the advAC6 enhancement. This low-affinity site is probably attributable to the IP receptor because a major fraction of the PGE_2 response was IP receptor siRNA-sensitive and other $G\alpha_s$-linked PG receptors ligands with selectivity for EP_2, EP_3, or DP receptor (butaprost, sulprostone, or BWA868, respectively) were ineffective at modulating cAMP levels in HUVECs.

The complex nature of PG receptor signaling and lack of highly specific ligands is evident upon comparison of published EC_{50} values for PG receptor agonists. For example, in the current study, the EC_{50} value for $cPGI_2$-stimulated cAMP was 5.9 μM, which is comparable with, for example, 295 nM in rat sensory neurons (Smith et al., 1998). Because $cPGI_2$ (and beraprost) are also EP_3 receptor agonists (K_d = 31 nM) (Kiriyama et al., 1997), some isoforms of which have been shown to inhibit AC activity via G_i (Fabre et al., 2001), interaction with G_i might contribute to the increase in apparent EC_{50} values in HUVECs after incubation with advAC6. Likewise, the high-affinity PGE_2-stimulated cAMP response is complicated by the ability of EP_4 receptors to couple through a pertussis toxin-sensitive $G\alpha$ subunit (Fujino and Regan, 2006), thus giving rise to the possibility that both G_s and G_i pathways are activated by PGE_2. Despite the difficulty in precisely interpreting EC_{50} values, AC overexpression consistently increases maximal response without a consistent shift in EC_{50} value. Such results support our hypothesis that increasing AC does not sensitize cells to recep-

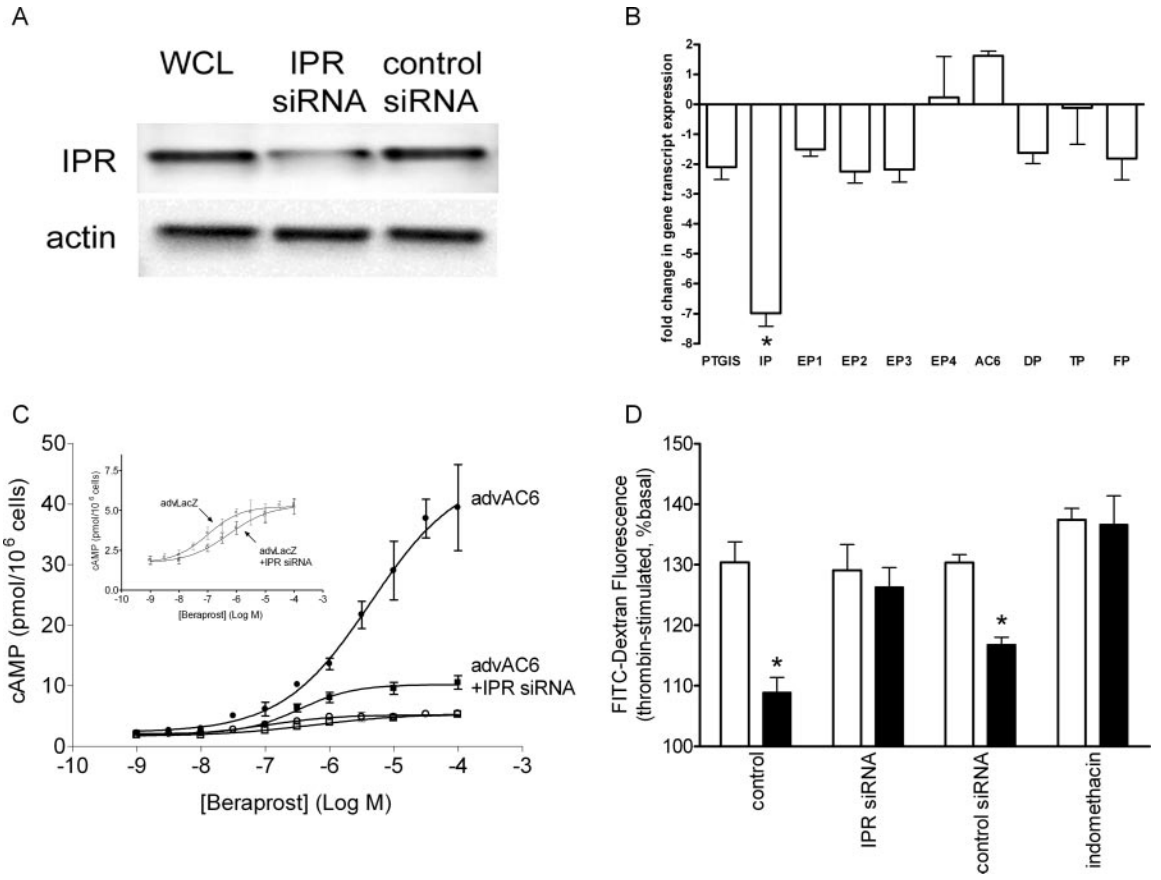

Fig. 5. siRNA knockdown of IP receptor expression reduces the inhibitory effect of advAC6 on thrombin-stimulated increases in endothelial permeability. A, immunoblot using IP receptor antisera demonstrates reduced receptor expression by treatment with IP receptor-targeted siRNA. B, real-time PCR measurement of IP receptor and related gene transcript levels indicates the siRNA treatment displays specificity for knockdown of IP receptor mRNA. C, IP receptor targeted-siRNA treated cells (squares) display reduced maximal cAMP generation in response to beraprost compared with those without siRNA treatment (circles) if pretreated with advAC6 (closed symbols) but not adv*LacZ* (control, open symbols). D, IP receptor-targeted siRNA or indomethacin treatment attenuates the inhibitory effect of advAC6 on thrombin-stimulated increases in endothelial monolayer permeability. Adv*LacZ* (□) or advAC6 (■)-treated cells were treated with buffer (control), IP receptor-targeted siRNA (24 h), SignalSilence siRNA (Cell Signaling Technology) (control siRNA), or indomethacin (100 μM, 1 h) before thrombin (0.2 U/ml, 30 min)-stimulated FITC-dextran flux across the endothelial monolayer. Thrombin-stimulated FITC-dextran fluorescence diffusion across the barrier was significantly different between adv*LacZ* and advAC6-treated cells at *, $P < 0.05$, Student's t test, $n = 3$.

tor agonists but instead increases their ability to activate the enzyme and produce cAMP. This is an important concept because therapeutic interventions designed to increase cAMP levels in target cells by targeting components "upstream" of AC to alter cellular function (e.g., enhance barrier function in endothelial cells) will probably be limited by the cellular quantity of active AC.

PG Receptor Profile in HUVECs. Real-time PCR and immunoblotting identified IP receptor as the predominant G_s-linked PG receptor expressed by HUVECs. Because our data provide the first published immunoblot using an human IP receptor antisera, it is interesting to note that the receptor is detected as several bands, lending support to the proposed existence of isoprenylated species (Miggin et al., 2003). PG receptor or G_s-protein expression did not increase after advAC6 treatment, thus excluding their up-regulation as a mechanism for the observed enhancement in receptor signaling by AC6 overexpression.

AC6 is inhibited by increases in intracellular calcium concentration ($[Ca^{2+}]_i$) (Yoshimura and Cooper, 1992). Therefore, a potential mechanism for the selective enhancement of particular receptor-mediated responses by advAC6 treatment is preferential enhancement of responses that do not concurrently elevate $[Ca^{2+}]_i$. This mechanism is unlikely for PGE_2-stimulated cAMP because EP_1 receptor, which increases $[Ca^{2+}]_i$, is abundantly expressed by HUVECs and would be activated by PGE_2. Indeed, maximal PGE_2-stimulated cAMP in both control and advAC6-treated cells increased in the presence of SC-19220, an EP_1 receptor antagonist (data not shown).

Unlike most GPCRs, the IP receptor does not undergo rapid desensitization (Hasse et al., 2003). We exploited this property to dissect the PGE_2 response into desensitization-insensitive and -sensitive components as a means of providing evidence that IP receptor contributes to PGE_2 (10 μM)-stimulated cAMP formation. We speculate that the inability of IP receptor to undergo rapid desensitization may be teleologically attributable to the short half-life (<30 s) of prostacyclin. Indeed, a recent study suggests that desensitization of IP response occurs at the level of AC5/6 (Sobolewski et al., 2004) rather than at the receptor itself. Hence, this lack of desensitization by stimulation of this GPCR, in contrast with most others, may contribute to the preferential enhancement of IP receptor-promoted cAMP formation by AC6 overexpression to levels similar to that of forskolin-stimulated (i.e., direct) activation of AC.

Endothelial Permeability and AC6 Overexpression. Proinflammatory mediators, such as thrombin, increase vascular permeability in vivo (Bogatcheva et al., 2002). cAMP reverses thrombin-induced barrier dysfunction via both the protein kinase A and Epac/Rap pathways (Patterson et al., 2000; Cullere et al., 2005); conversely, inhibition of cAMP generation is required for thrombin-stimulated endothelial cell gap formation (Cioffi et al., 2002). Because thrombin stimulates the release of PGE_2 and prostacyclin from endothelial cells (Jaffe et al., 1987) (Fig. 4, C and D), we tested whether advAC6 could reduce thrombin-induced barrier dysfunction by enhancing negative feedback of a prostacyclin autocrine/paracrine pathway.

Indomethacin treatment reduced PG secretion (Fig. 4, C and D) without modifying thrombin-stimulated gap formation (Fig. 4B) in control cells (▨), suggesting that a PG autocrine feedback is not a major regulator of thrombin-induced permeability changes in untreated cells. However, when cells overexpress AC6, indomethacin treatment modifies thrombin-stimulated gap formation, thus implicating a PG autocrine/paracrine component in the effects of AC6 overexpression on thrombin-stimulated changes in endothelial permeability. AdvAC6 treatment does not dramatically alter PG secretion (Fig. 4, C and D), excluding the possibility that the effect of advAC6 on PG signaling is via enhancing the formation of agonist. AdvAC6 treatment of HUVECs was effective at reducing thrombin-stimulated increases in endothelial barrier permeability (Fig. 3) and gap formation (Fig. 4), and, as indicated by complementary data (attenuation by blockade of PG synthesis with indomethacin and reduction of IP receptor expression using siRNA), the increase in barrier function occurs via activation of IP receptors.

IP receptor stimulation can reverse thrombin-stimulated increases in permeability (Imai-Sasaki et al., 1995) and is useful in the treatment of pulmonary hypertension, a disease associated with endothelial dysfunction (Miyata et al., 1996). Based on the current results, we propose that increased expression of AC6 in endothelial cells, such as with advAC6, may provide a novel approach to enhance prostacyclin signaling and endothelial barrier function.

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