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ADENYLYL CYCLASE 6 OVER-EXPRESSION DECREASES THE PERMEABILITY OF ENDOTHELIAL MONOLAYERS VIA PREFERENTIAL ENHANCEMENT OF PROSTACYCLIN RECEPTOR FUNCTION

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Running title:

AC6 Over-Expression in HUVEC

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Abbreviations: AC, adenylyl cyclase; GPCR, G-protein coupled receptor; HUVEC, human umbilical vein endothelial cells; cAMP, adenosine 3',5'-cyclic adenosine monophosphate; advAC6, adenoviral-AC6; adv*LacZ,* adenoviral-*LacZ*; PG, prostaglandins; $cPGI_2$, carbaprostacyclin.

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Abstract

Over-expression 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 over-expression 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 over-expression 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 (HUVEC) preferentially enhances prostacyclin receptor (vs. other GPCR)-stimulated cAMP synthesis and, in parallel, inhibits thrombin-stimulated increases in endothelial cell barrier function. Using multiple strategies, including prostacyclin receptor-targeted siRNA, we identify that the enhancement of endothelial barrier function by AC6 over-expression is dependent on an autocrine/paracrine feedback pathway involving release of prostacyclin and activation of prostacyclin receptors. AC6 over-expression in endothelial cells may have utility as a means to enhance prostacyclin function and reduce endothelial barrier permeability.

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Adenylyl cyclase (AC), which is activated by G_s-linked G-protein coupled receptors (GPCRs), catalyzes the synthesis of the second messenger, adenosine 3',5'-cyclic adenosine monophosphate (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 previously been used to over-express 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, over-expression 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. Since the endothelium is the first target of a gene therapy delivered via the vasculature, this raises the question as to the impact of AC6 over-expression on endothelial cell physiology. We thus sought to define this impact and to ask whether over-expression of AC6 in endothelial cells has potential therapeutic benefit in its own right.

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Vascular endothelial cells both act as a barrier between the blood and underlying smooth muscle and tissue cells and in addition, release and respond to vasoactive agents, including prostaglandins (PG), e.g. PGE_2, PGI_2, PGD_2 and $PGF_{2\alpha}$, that have specificity for subtypes of PG receptor, $EP_{1\text{-}4}$, 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 (Cullere et al., 2005; Fukuhara et al., 2005; Patterson et al., 2000; Qiao et al., 2003).

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

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Materials and Methods

Reagents

All reagents were purchased from Sigma-Aldrich (St.Louis, MO) with the following exceptions: HUVEC (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 330-343 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, Netherlands); the EP_4 receptor-selective antagonist, 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 (L-161,982) was a generous gift from Dr.R.Young (Merck-Frosst, Canada) (Machwate et al., 2001).

Cell Culture & Treatments

HUVEC 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), EGF (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 passage 2-4.

Adenoviral Treatment

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AdvAC6 was a gift from H. Kirk Hammond (VA San Diego Healthcare System) 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 hr.

Cell Size Assay

Cells were seeded on collagen I-coated 24 well plates and grown to confluency (3-4 days). On the day of experimentation, cells were washed with Hank's HEPES buffer (HHB, HEPES (15 mM), pH 7.4) and equilibrated in HHB for 30 min at 37°C. Cells were visualized with a Leica microscope using a 40x objective. Digital images were captured immediately prior to drug addition and following 3 min incubation at 37°C. Adobe Photoshop 6.0 was used to align images and quantitate cell shrinkage by channel subtraction. Results are intensity differences between pre- and post-stimulus.

Measurement of Endothelial Barrier Permeability

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

Real-time PCR & Immunoblotting

Real-time PCR was performed as described previously (Bundey and Insel, 2003) using published primer sequences for prostanoid receptors: $EP_{1\text{-}4}$ and FP (Anthony et al., 2001); DP, TP and IP (Sarrazin et al., 2001). Primers used for prostacyclin synthase (PTGIS) detection were: 5'-GCAGACGTGTTTTGTTTGGA-3', 5'-

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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 previously described (Ostrom et al., 2001). cAMP accumulation was stimulated by the addition of agonists in the presence of a non-selective phosphodiesterase inhibitor, isobutylmethylxanthine, IBMX (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 pre-incubated for 1 hr with agonists prior to stimulation for 10 min with a subsequent dose of drug and measurement of cAMP content.

Prostaglandins ELISA

PGE_2 & 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 μg/10^6 cells for 48 hr, 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 (since it is a FITC conjugate).

Statistical analysis

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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 ANOVA where $P<0.05$ was considered significant. Experiments were performed in triplicate on three separate occasions unless stated otherwise.

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Results

Over-expression of Functional AC6 in HUVEC

Treatment of HUVEC with adv*LacZ* (10 pfu/cell) for 20 hr 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 10-20 pfu advAC6/cell cAMP formation was increased 6-8 fold compared to control (adv*LacZ*-treated) cells (Figure 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 HUVEC (Table 1) and showed ~ 2-fold greater (isoproterenol, histamine) maximum responses but only minimal changes in EC_{50} values with AdvAC6-treament. By contrast, cAMP generated in response to PGE_2 and IP receptor-selective agonists (beraprost, carbaprostacyclin ($cPGI_2$)) was enhanced in AC6 over-expressing cells to a similar extent as was 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 over-expressing cells. Analysis of the concentration-response curve of PGE_2-stimulated cAMP identified a two-site relationship (Figure 1B), which contrasted with the one-site curve for $cPGI_2$ (Figure 1C). Use of the EP_4 receptor-selective antagonist, L-161,982 (1 μM), revealed that the high

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affinity site for stimulation of cAMP formation by PGE_2 is primarily mediated by the EP_4 receptor (Figure 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 if 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 (Hasse et al., 2003). AdvAC6-treated cells were incubated for 1 hr in the absence or presence $cPGI_2$ (a stable analogue of prostacyclin) (10 μM) or PGE_2 (10 μM) prior to a wash step and subsequent 10 min incubation with receptor agonist so as to define desensitization-insensitive and -sensitive components (Figure 1D). Response to forskolin was unchanged by pre-treatment with $cPGI_2$ or PGE_2, indicating 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. By contrast, response to PGE_2 was reduced in cells pre-treated with PGE_2 (but not $cPGI_2$), implying that PGE_2-stimulated cAMP response of AC6-over-expressing 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 over-expression we thought it important to define the expression of PG receptors present in HUVEC. 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 (Figure 2A). AdvAC6 treatment did

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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 (Figure 2B). We detected multiple bands in HUVEC cells with several antibodies, suggesting that EP_1, EP_3 and IP receptors undergo post-translational modifications. Indeed, the upper (~57 & 61kDa) bands in the IP receptor immunoblot were sensitive to N-glycosidase F treatment (data not shown).

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 (Figure 3, 'x' symbols) diffusion of FITC-dextran was significantly greater (after 20 or 30 min) than in the presence of a cell monolayer (Figure 3, open squares). Although the permeability of AdvAC6-treated cells was similar to that of control cells (Figure 3, solid squares), thrombin (0.2 U/ml)-stimulated increase in permeability was reduced in advAC6-treated cells (Figure 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, 3min): morphological changes included a retraction of cell borders, which appeared as an increase in interendothelial gap formation and could be better visualized by subtraction of post- and pre-stimulation images (see 'change' panels, Figure 4A). Image density analysis allowed quantitation

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of the interendothelial gap formation (Figure 4B). AdvAC6 treatment significantly reduced the thrombin-stimulated gap formation compared to control cells. Treatment with the COX inhibitor indomethacin (100 μM, 1 hr) prior to thrombin-stimulation abolished the inhibitory effect of advAC6 (Figure 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 (Figure 4C) and prostacyclin (Figure 4D).

IP receptor siRNA

Because HUVEC 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 following AC6 over-expression. Immunoblotting revealed that IP receptor siRNA treatment reduced IP receptor expression ~50% (Figure 5A). Real-time PCR analysis verified the specificity of the IP receptor siRNA treatment (Figure 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±20nM; siRNA, 550±28nM) without a substantial change in maximum response (Figure 5C). In contrast, IP receptor siRNA treatment of AC6 over-expressing cells prominently reduced maximal cAMP response to beraprost (Figure 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 hr prior to thrombin-stimulation) (Figure 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 over-expression on cAMP formation and endothelial cell barrier function.

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Discussion

Over-expression of AC6 in HUVEC

Although primary cultures of endothelial cells can be difficult to transfect, we found that use of an adenoviral construct was effective to accomplish transfer and expression of AC6, as demonstrated by X-gal staining of adv*LacZ*-infected HUVEC 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 (Figure 5C). IP receptor expression was reduced ~50% by siRNA; while this treatment increased the IC_{50} for beraprost, the maximal level of cAMP in response to beraprost was unaltered in control HUVEC, 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 knock-down in advAC6-treated cells produced a large reduction in the maximal levels of beraprost-stimulated cAMP, implying that the ~50% IP receptor expression becomes limiting when AC6 is over-expressed. These results, along with those in Figure 1 and Table 1, support the conclusion that expression of AC is the limiting signaling component for receptor and post-receptor 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 2-3 fold or enhancement to a similar extent as was forskolin response, 7-9 fold. IP

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receptor response was preferentially enhanced by AC6 over-expression, an effect that underlies the enhancement of response to PGE_2. Evidence supporting this conclusion includes: the responses enhanced preferentially by advAC6 were, with the exception of forskolin and PGE_2, ligands selective for the IP receptor; 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 likely attributable to the IP receptor since 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 HUVEC.

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 = 5.9 µM; which compares with, for example, 295 nM in rat sensory neurons (Smith et al., 1998). Since $cPGI_2$ (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} in HUVEC following incubation with advAC6. Similarly, the high affinity PGE_2-stimulated cAMP response is complicated by ability of EP_4 receptors to couple through a pertussis toxin-sensitive G_α subunit (Fujino and Regan, 2005), 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 over-expression consistently increases maximal response

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without a consistent shift in EC_{50}. Such results support our hypothesis that increasing AC does not sensitize cells to receptor agonists but instead increases their ability to activate the enzyme and produce cAMP. This is an important concept since therapeutic interventions designed to raise cAMP levels in target cells by targeting components "upstream" of AC in order to alter cellular function, e.g. enhance barrier function in endothelial cells, will likely be limited by the cellular quantity of active AC.

PG receptor profile in HUVEC

Real-time PCR and immunoblotting identified IP receptor as the predominant G_s-linked PG receptor expressed by HUVEC. Since our data provide the first published immunoblot using an human IP receptor anti-sera, 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 following advAC6 treatment, thus excluding their up-regulation as a mechanism for the observed enhancement in receptor signaling by AC6 over-expression.

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 HUVEC 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).

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Unlike most GPCR, 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 to provide 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 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 over-expression to levels similar to that of forskolin-stimulated (i.e. direct) activation of AC.

Endothelial Permeability and AC6 Over-Expression

Pro-inflammatory mediators, such as thrombin, increase vascular permeability *in vivo* (Bogatcheva et al., 2002). cAMP reverses thrombin-induced barrier dysfunction via both the PKA and Epac/Rap pathways (Cullere et al., 2005; Patterson et al., 2000); conversely, inhibition of cAMP generation is required for thrombin-stimulated endothelial cell gap formation (Cioffi et al., 2002). Since thrombin stimulates release of PGE_2 and prostacyclin from endothelial cells (Jaffe et al., 1987) (Figure 4C-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 (Figure 4C-D) without modifying thrombin-stimulated gap formation (Figure 4B) in control cells (hashed bars), suggesting that a PG autocrine feedback is not a major regulator of thrombin-induced permeability

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changes in untreated cells. However, when cells over-express AC6, indomethacin treatment modifies thrombin-stimulated gap formation, thus implicating a PG autocrine/paracrine component in the effects of AC6 over-expression on thrombin-stimulated changes in endothelial permeability. AdvAC6 treatment does not dramatically alter PG secretion (Figure 4C-D), excluding the possibility that the effect of advAC6 on PG signaling is via enhancing formation of agonist. AdvAC6 treatment of HUVEC was effective at reducing thrombin-stimulated increases in endothelial barrier permeability (Figure 3) and gap formation (Figure 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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Footnotes

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Figure Legends

Figure 1. PGE_2- and $cPGI_2$-stimulated cAMP formation is prominently enhanced by treatment of HUVEC 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 (open circles) and advAC6-treated (solid circles) cells with a two-site concentration-response relationship. Pre-treatment of cells with the EP_4 receptor antagonist, L-161,982 (1 μM), inhibited the high affinity response in both control (open triangles) and advAC6-treated (solid triangles) cells. (C), cPGI2-stimulated cAMP accumulation in control (open squares) and advAC6-treated (solid squares) cells. *Inset*, control (adv*LacZ*-treated) cell responses displayed on an optimized Y-axis since they generate much less cAMP than advAC6-treated cells. (D), Pre-treatment 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 hr in the absence (open bars) or presence of $cPGI_2$ (10 μM, hashed bars) or PGE_2 (10 μM, filled bars) prior to 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 unpre-treated controls by pre-treatment with PGE_2 but not $cPGI_2$. As described in the text, the desensitization-insensitive and –sensitive components are likely mediated by the IP receptor and an EP receptor, respectively. Agonist incubations were in the presence of IBMX (200 μM). The data shown are mean±s.e.m. (n=3).

Figure 2. PG receptor expression in HUVEC determined by (A), real-time PCR and (B), immunoblot. 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 comparable results to those from the real-time PCR data, receptor specific anti-sera identifying bands at the expected molecular weight 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.

Figure 3. Effect of advAC6 on endothelial cell monolayer permeability. AdvAC6-treated cells (solid squares) show similar 'basal' endothelial permeability compared to control cells (open squares). Thrombin (0.2 U/ml) (added at T=0 min) increases endothelial permeability in control cells (open circles) and this increase is inhibited by advAC6 (solid circles). 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, 30 min. Inserts seeded with no cells (x) showed significantly increased FITC-dextran diffusion compared to inserts seeded with cells, confirming effective barrier function of HUVEC. Data points are mean±s.e.m. (n=3). *= $P<0.05$, one-way ANOVA (adv*LacZ* vs. adv*LacZ* + thrombin); #= $P<0.001$, Student's *t* test (advAC6 + thrombin vs. adv*LacZ* + thrombin).

Figure 4. Effect of advAC6 and indomethacin on interendothelial cell gap formation. (A), Images of HUVEC pre-stimulation (0 min) and 3 min following stimulation with thrombin (0.2 U/ml). Computational image subtraction was used to determine the

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interendothelial gap formation (3rd column, "change"). (B), Quantification of image changes by thrombin. Thrombin-stimulated gap formation in control cells (open bars) is inhibited by advAC6 (striped bars). *=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 (hashed bars) but blocked the inhibitory effect of advAC6 (solid bars). (C), PGE_2 and, (D), 6-keto PGF1α content of cell media from control (open bars) or thrombin (0.2 U/ml, 30 min)-stimulated cells (closed bars) confirmed the reduction of PG efflux by indomethacin. *=thrombin significantly changed PG secretion compared to untreated controls, $P<0.05$, Student's *t* test, n=3.

Figure 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 anti-sera 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 to those without siRNA treatment (circles) if pre-treated 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. AdvLacZ (open bars) or advAC6 (closed bars)-treated cells were treated with buffer (control), IP receptor-targeted siRNA (24 hr), SignalSilence siRNA (Cell Signaling Technology) (control

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siRNA) or indomethacin (100 μM, 1 hr) prior to 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 ($P<0.05$, Student's *t* test, n=3).

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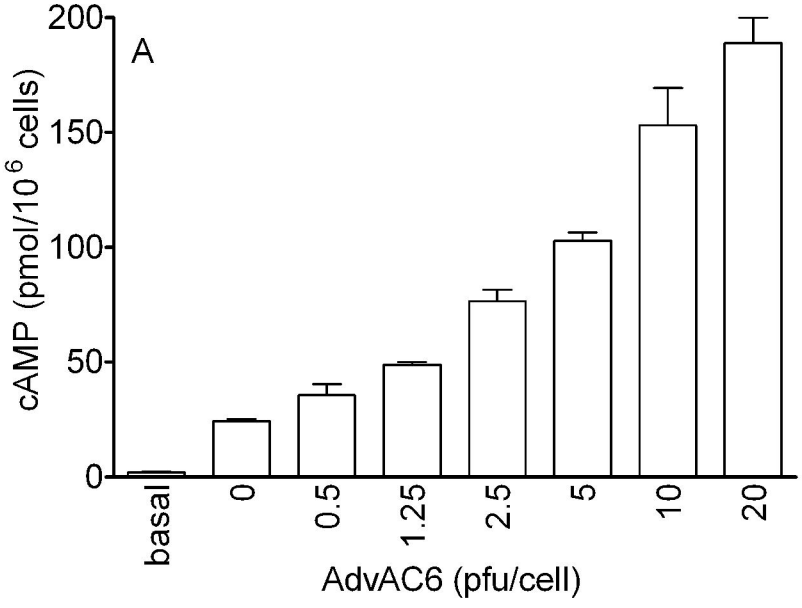
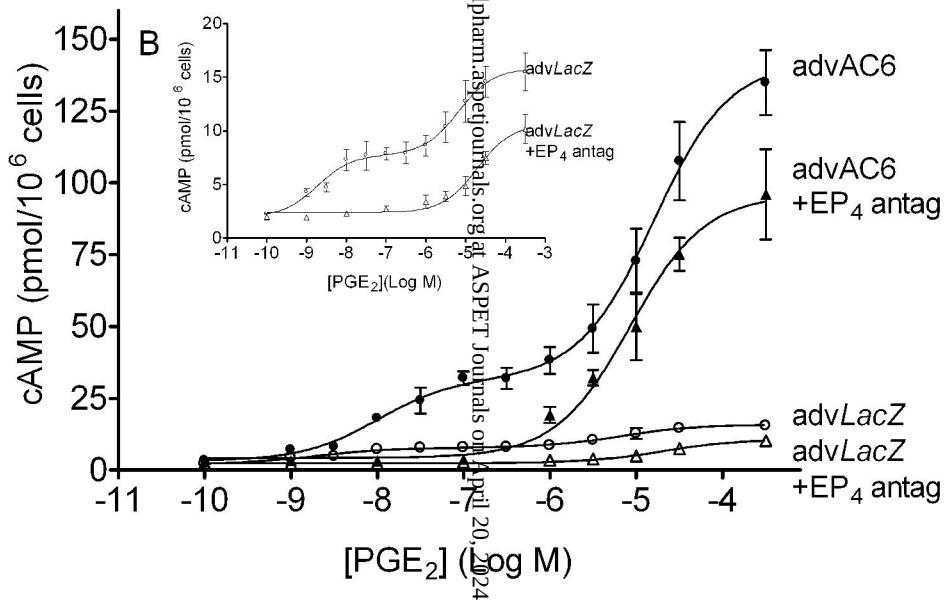
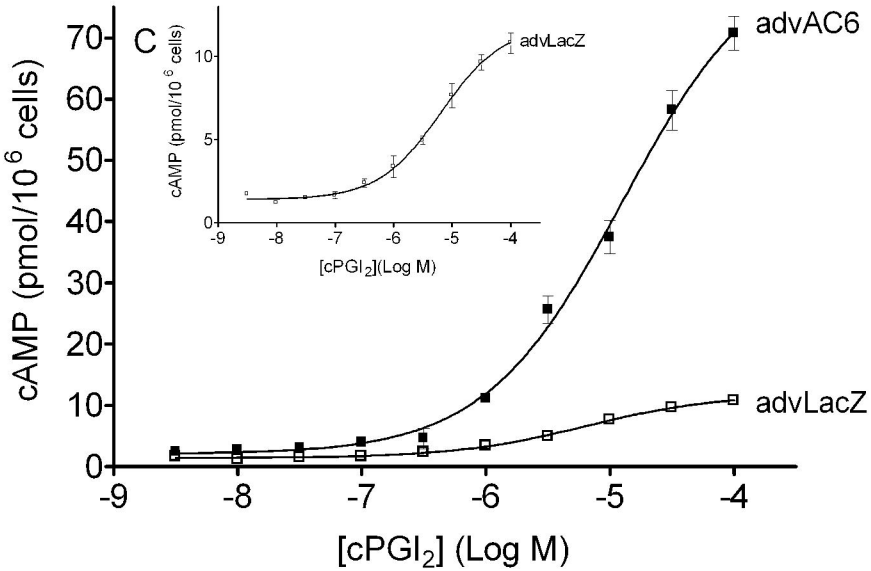
Table 1. EC_{50} values and maximal (Max) responses of cAMP formation in control and advAC6 (10pfu/cell)-treated cells. Values are mean±s.e.m., n≥3.

	EC_{50} (μM)		Max Response (pmol/10^6 cells)		*Max response ratio*
Drug	control	advAC6	control	advAC6	*(advAC6/control)*
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

Figure 1







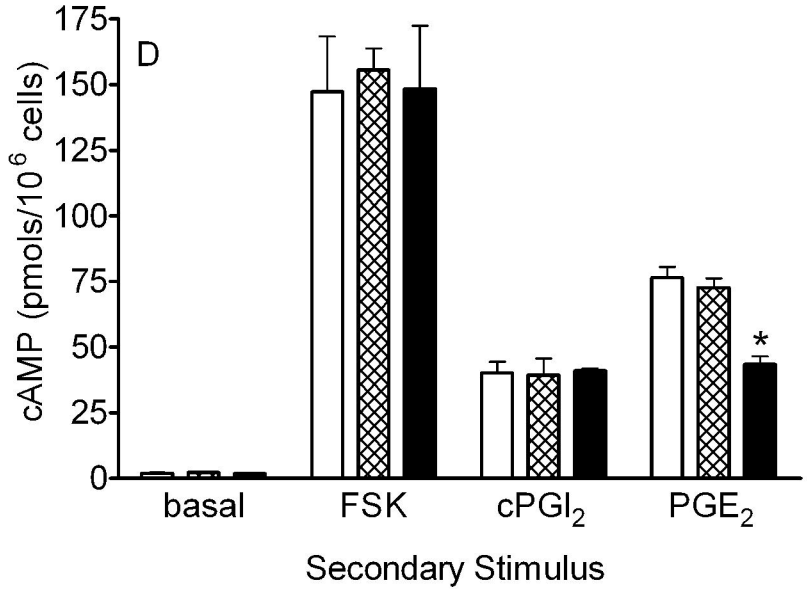


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Figure 2

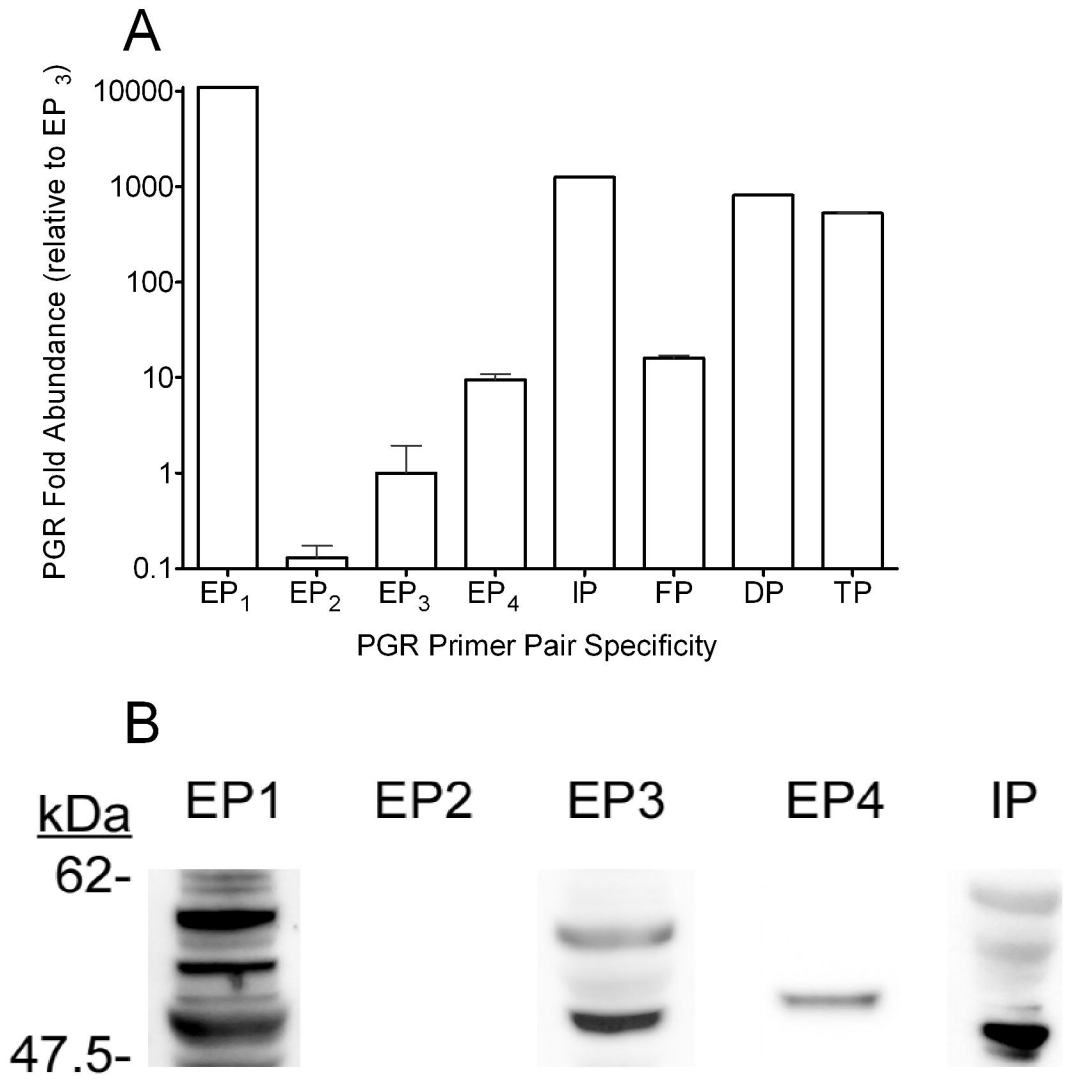

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Figure 3

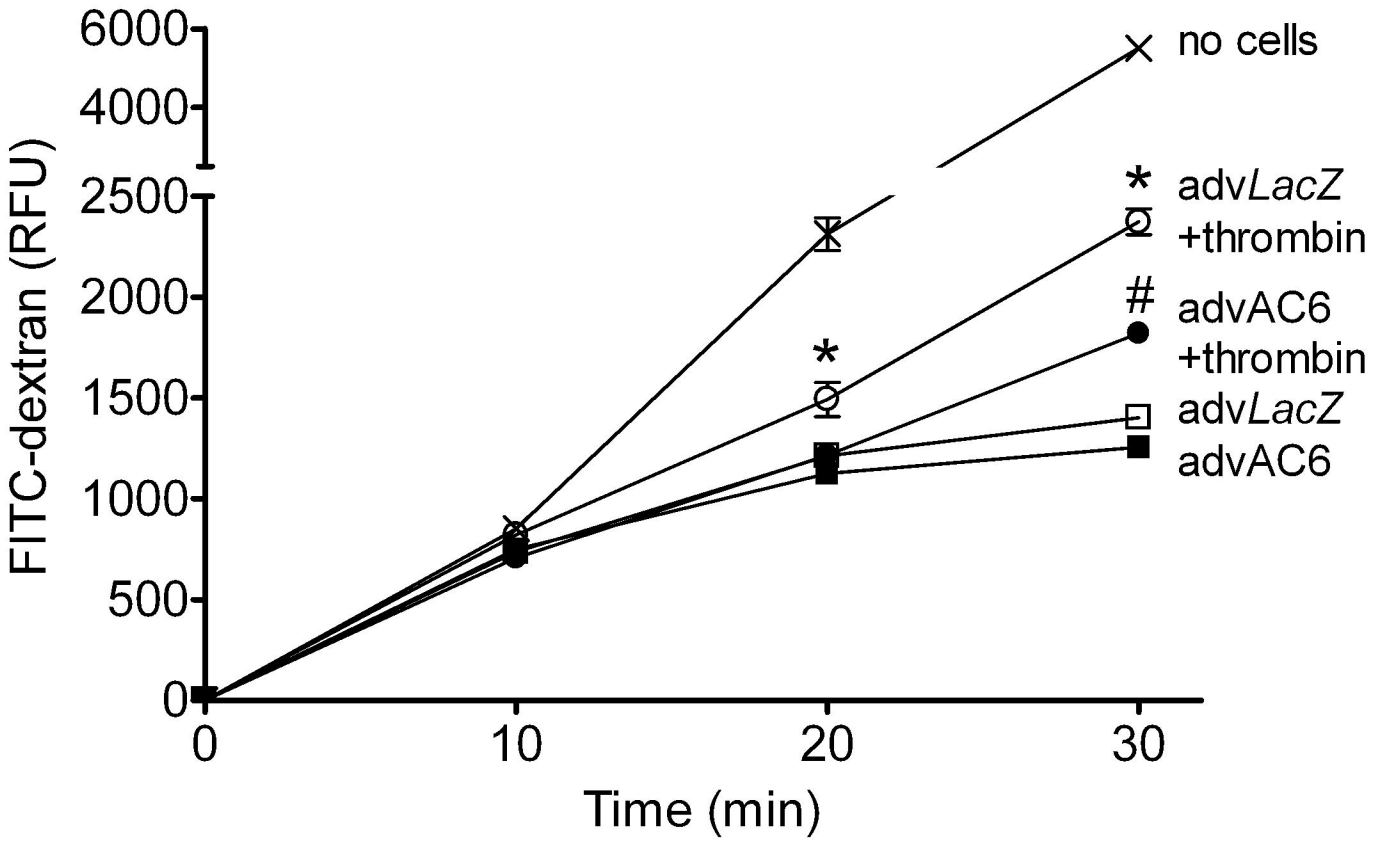
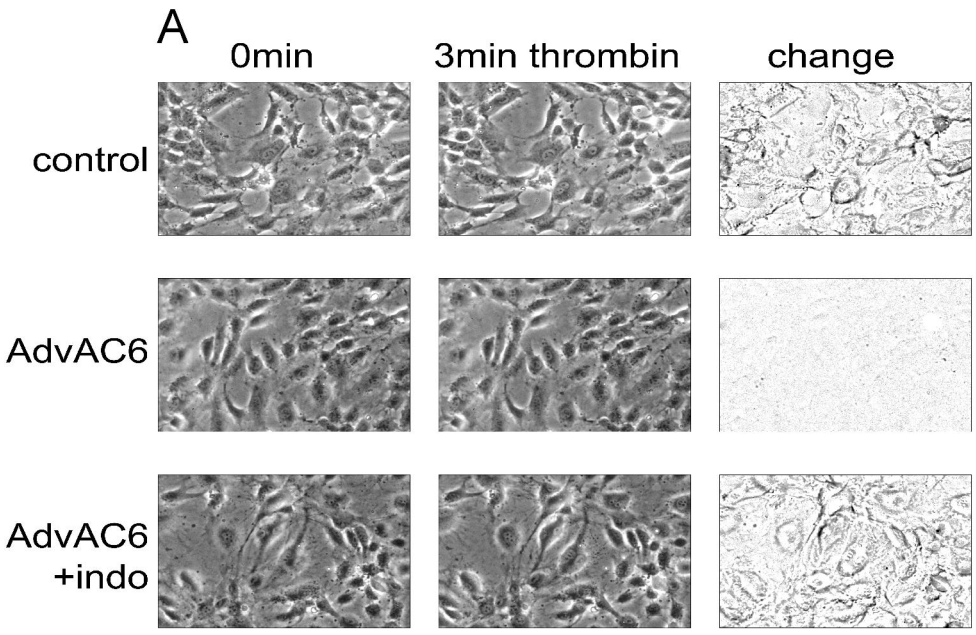
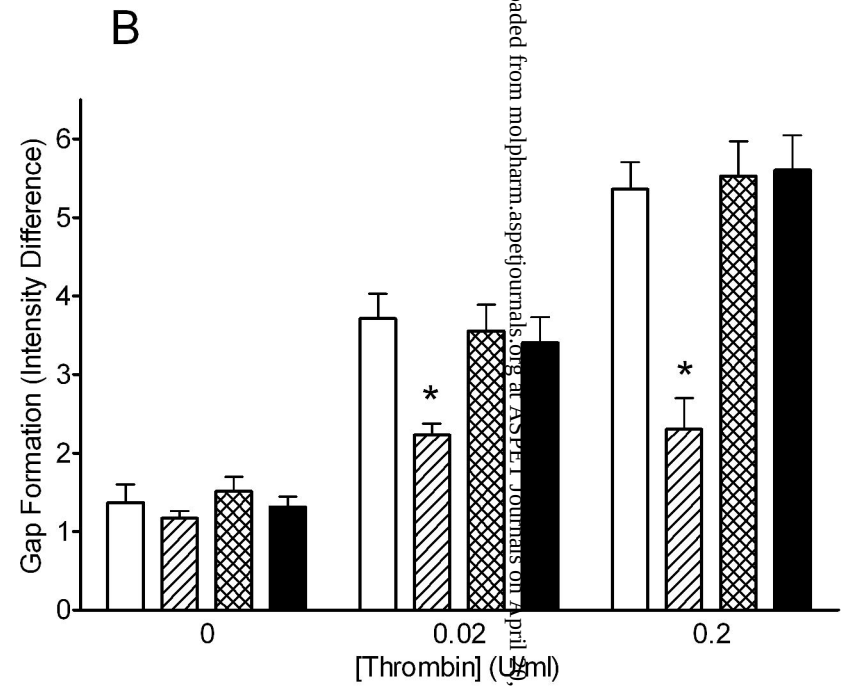
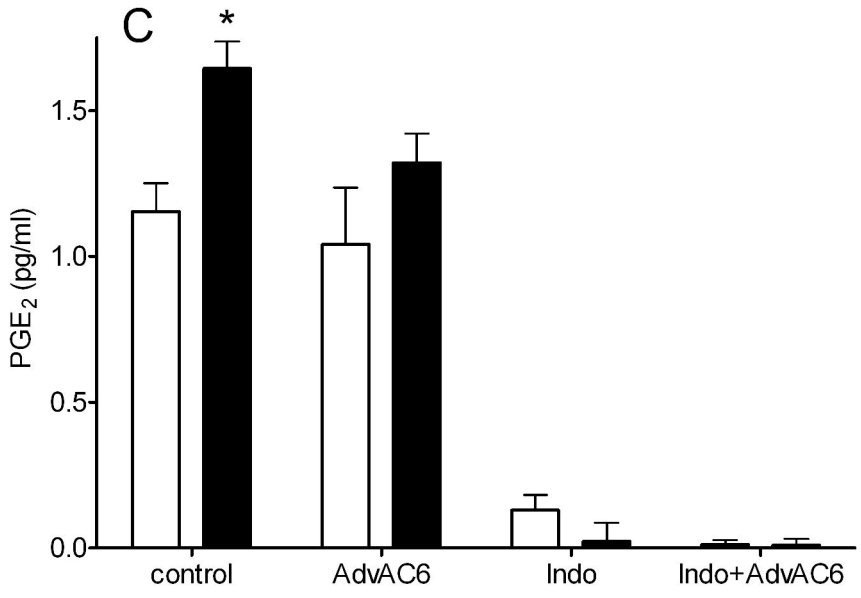


Figure 4







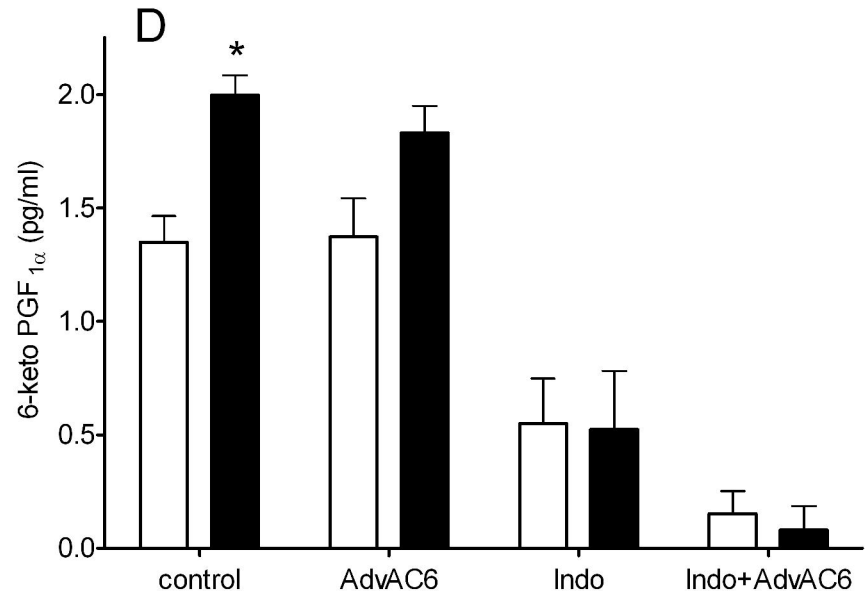


Figure 5