Published in final edited form as:

J Mol Cell Cardiol. 2009 May ; 46(5): 682–694. doi:10.1016/j.yjmcc.2009.01.006.

Novel signaling pathways promote a paracrine wave of prostacyclin-induced vascular smooth muscle differentiation

Zsolt Kasza^{1,2}, Kristina M. Fetalvero¹, Min Ding¹, Robert J. Wagner², Klara Acs¹, Anthony K. Guzman², Karen L. Douville¹, Richard J. Powell², John Hwa^{1,3}, and Kathleen A. Martin^{1,2}

¹ Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, NH 03755

² Department of Surgery (Vascular Surgery), Dartmouth Medical School, Hanover, NH 03755

³ Medicine (Cardiology), Dartmouth Medical School, Hanover, NH 03755

Abstract

The important athero-protective role of prostacyclin is becoming increasingly evident as recent studies have revealed adverse cardiovascular effects in mice lacking the prostacyclin receptor, in patients taking selective COX-2 inhibitors, and in patients in the presence of a dysfunctional prostacyclin receptor genetic variant. We have recently reported that this protective mechanism includes the promotion of a quiescent differentiated phenotype in human vascular smooth muscle cells (VSMC). Herein, we address the intriguing question of how localized endothelial release of the very unstable eicosanoid, prostacyclin, exerts a profound effect on the vascular media, often 30 cell layers thick. We report a novel PKA-, Akt-1- and ERK1/2-dependent prostacyclin-induced prostacyclin release that appears to play an important role in propagation of the quiescent, differentiated phenotype through adjacent arterial smooth muscle cells in the vascular media. Treating VSMC with the prostacyclin analog iloprost induced differentiation (contractile protein expression and contractile morphology), and also up-regulated COX-2 expression, leading to prostacyclin release by VSMC. This paracrine prostacyclin release, in turn, promoted differentiation and COX-2 induction in neighboring VSMC that were not exposed to iloprost. Using siRNA and pharmacologic inhibitors, we report that this positive feedback mechanism, prostacyclin-induced prostacyclin release, is mediated by cAMP/PKA signaling, ERK1/2 activation, and a novel prostacyclin receptor signaling pathway, inhibition of Akt-1. Furthermore, these pathways appear to be regulated by the prostacyclin receptor independently of one another. We conclude that prevention of de-differentiation and proliferation through a paracrine positive feedback mechanism is a major cardioprotective function of prostacyclin.

Keywords

human vascular smooth muscle cells; iloprost; prostacyclin receptor; Akt-1; differentiation; COX-2; signaling

Corresponding author: Kathleen A. Martin, PhD., Section of Vascular Surgery, Dartmouth Medical School, Dartmouth-Hitchcock Medical Center, 1 Medical Center Drive, Lebanon, NH 03756, (603) 650-7439 telephone, (603) 650-4928 fax, E-mail: Kathleen.A.Martin@Dartmouth.edu.

DISCLOSURES

None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

INTRODUCTION

Prostacyclin, a bicyclic 20 carbon oxygenated fatty acid derived from arachidonic acid, serves as a vasodilator and inhibitor of platelet aggregation and vascular smooth muscle cell (VSMC) proliferation [1,2]. Cyclooxygenases (COXs) are the rate-limiting enzymes in biosynthesis of the prostaglandins. Prostacyclin is the main cyclooxygenase-2 (COX-2) product of vascular endothelium [3,4]. The recent withdrawal of selective COX-2 inhibitors due to increased adverse cardiovascular events highlights the cardioprotective effects of prostacyclin in human patients [5]. Recent genetic deletion studies in mice revealed that the absence of the prostacyclin receptor (IP) promotes intimal hyperplasia, atherosclerosis and hypercoagulability [6,7]. Furthermore, it now appears that the cardioprotective effects of estrogen in premenopausal women are due in part to the induction of prostacyclin [8]. Despite these important roles in cardioprotection, surprisingly little is known about the molecular basis for this protective effect, in part due to the widely held belief that the prostacyclin system was redundant to that of nitric oxide and thus deficiencies would be well compensated for.

The human prostacyclin receptor is expressed predominantly in platelets and VSMC. In order to clarify the structure-function relationship of the human prostacyclin receptor (hIP), our group previously identified and characterized residues required for hIP activation [9,10], as well as critical proline and cysteine residues [11,12] and naturally occurring hIP genetic variants [13]. The hIP is predominately coupled to the Gs α -subunit, which upon receptor activation enhances adenylyl cyclase activity, generating the second messenger cAMP [2,10]. We have previously shown that prostacyclin signaling, in addition to anti-proliferative effects, induces a differentiated, contractile phenotype in human VSMC, characterized by upregulation of contractile protein expression and differentiated morphology [2]. We found that this effect of hIP is dependent on cAMP/PKA activation.

The increasing body of evidence for an important cardioprotective role for prostacyclin raises the question as to how prostacyclin, a short-lived endothelial-derived compound, can profoundly affect the thick, multilayered vascular media. In the current study, we report that prostacyclin receptor agonists stimulate COX-2 induction and subsequent further release of prostacyclin by VSMC. We provide evidence that this generates a positive feedback loop which propagates a pro-differentiation signal in adjacent VSMC layers. We further implicate PKA as well as novel Akt-1 and ERK1/2 pathway signaling in this hIP response.

MATERIALS AND METHODS

Cell culture

Isolation and maintenance of human aortic and coronary artery VSMC were described previously [2]. All signaling experiments were performed in both cell types with similar results. Iloprost was purchased from Amersham Biosciences (Pittsburgh, PA). Myristoylated PKA inhibitor-(14–22) amide (myrPKI) and U0126 (MEK inhibitor), wortmannin (PI 3-kinase inhibitor) were purchased from Calbiochem (San Diego, CA). Prostacyclin receptor (IP) antagonist (CAY10441) and selective COX-2 inhibitor (NS-398) were purchased from Cayman Chemical. 8-Br-cAMP was purchased from Sigma-Aldrich (St. Louis, MO).

Semiquantitative RT-PCR

RNA isolation and RT-PCR for PDH and PKA subunits were as described previously [2]. The following new primers were used for PCR: COX-2, sense 5' aatgagtaccgaaacgtttatg, antisense 5' catctagtccggagcgggaagaac; PGIS, sense 5' ctccatgcctatgccatctc, antisense 5' tggaggttggtatacatggcttct. PCR with COX-2 primers was performed for 25 cycles, 94°C (45s),

55°C (30 sec), and 70°C (1 min) and with PGIS primers 30 cycles: 94°C (45 s), 66°C (30 s), and 70°C (1 min).

Cell lysis and immunoblotting

Cell lysis and immunoblotting were performed as described previously [2]. The following primary antibodies were used: COX-2, PGIS (Cayman Chemical), P-Ser473 Akt (Cell Signaling and Santa Cruz Biotechnology), Akt-1, total Akt, (Cell Signaling), phospho-ERK1/2 (Thr202/Tyr204) (Sigma), total ERK1/2 (gift of John Blenis), beta-tubulin, GAPDH, PKA- α and - β (Santa Cruz Biotechnology). Horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents were used.

Transient transfection

Small interfering RNA (siRNA) was transfected using the Nucleofector method (Amaxa) as described previously [2,15]. For transient plasmid transfection, 2 μ g of plasmid encoding wild-type Akt1 (courtesy of Dr. Jin Q. Cheng), GFP (pmaxGFP, Amaxa), or pcDNA3 vector was transfected into human VSMC using the Nucleofector method as indicated above.

Prostacyclin measurement

Cells were transferred to M199 medium with 2.5% FBS at least 12 hours prior to drug treatment. The media was changed immediately (M199 medium containing 2.5% FBS) prior to iloprost treatment to reduce potential contaminating background levels. 6-keto-prostaglandin F1 α (PGF1 α) (stable metabolite of prostacyclin) was measured using an immunoassay kit (Cayman Chemical, Ann Arbor, MI). No significant immunoreactivity was observed with iloprost itself.

Confocal microscopy

A first monolayer of VSMC was cultured in six well plates. Following iloprost treatment (1h), the cells were washed three times. A second VSMC cell layer cultured on a coverslip was then placed cell side down on the washed activated cell layer. After 24 hours, the cells on the coverslip were washed in PBS and fixed in methanol:acetone (1:1) for 10 minutes at -20°C. Immunohistochemical analysis by confocal microscopy was carried out as described previously [14]. In additional experiments, the first monolayer of VSMC was pretreated with specific COX-2 inhibitor (NS-398) or vehicle (DMSO) for 1 hour prior to iloprost treatment and the second layer was fixed after 8h coculture with layer 1 as above. Alternately, the second cell layer was pretreated with the specific IP-antagonist (CAY 10441) or vehicle (EtOH) for 2 hours prior to coculture with the iloprost activated first cell layer for 8h.

Cell area measurement

Cells were treated as described for confocal microscopy. After 24 hours, the cells on the second layer were washed, fixed with methanol:acetone (1:1), stained with toluidine blue, and mounted on slides. Fourteen or fifteen separate high-power fields from each slide were photographed in a blinded and random process, and cell area determined using NIH Image software. An ANOVA (multiple comparison post hoc Bonferroni test) was used to determine statistically significant differences ($P < 0.05$).

In vitro assay for PKA activity—The kinase assay was carried out as described previously [2]. In brief, cells were treated with vehicle or iloprost for 20 minutes, total cell lysate was incubated in a reaction with fluorescence-labeled peptide substrate kemptide (Promega) for 15 minutes. The phosphorylated (reflecting PKA activity) and nonphosphorylated substrate were separated on a 0.8% agarose gel.

RESULTS

Iloprost increases COX-2 expression in human aortic smooth muscle cells

To test the hypothesis that prostacyclin may exert protective effects through the vascular wall by a paracrine mechanism, we assessed COX-2 expression in human primary VSMC in culture in response to treatment with the more stable prostacyclin analogue iloprost. Treatment with 100nM iloprost revealed an strong upregulation of the COX-2 protein, but no significant change in expression of prostacyclin synthase (PGIS) or COX-1 was detected (Fig. 1A.) Iloprost induced COX-2 upregulation was confirmed at mRNA level with semiquantitative RT-PCR as well (Suppl. Fig 1). Interestingly, COX-2 mRNA and protein levels were sustained after 24 hours, suggesting that iloprost may either additionally stabilize mRNA, or, as will be described, that an autocrine/paracrine signaling loop may maintain mRNA/protein production. As iloprost at higher concentrations can potentially activate other prostaglandin receptors (PGE receptors), we performed experiments to confirm the hIP selectivity of the iloprost effect on COX-2. Iloprost upregulated COX-2 protein expression in a dose-dependent manner, and, notably, as little as 0.5 nM iloprost was sufficient to induce COX-2 (Suppl. Fig 2A). Furthermore, a selective hIP antagonist (CAY10441) abolished the iloprost-induced COX-2 protein upregulation (Fig. 2), and prostacyclin itself or another analogue (cicaprost) also induced COX-2 expression (Suppl. Fig. 2B).

Prostacyclin-induced prostacyclin release in human aortic smooth muscle cells through COX-2

To determine whether the dramatic selective increase in COX-2 protein led to an increase in prostacyclin synthesis, we measured 6-keto-PGF1 α levels (stable metabolite of prostacyclin) in VSMC treated with vehicle or iloprost. Prostacyclin was clearly induced above control levels at each time point (Fig. 3A). NS-398, a selective COX-2 inhibitor, reduced the iloprost-induced prostacyclin production to baseline levels, supporting the imperative role of COX-2 in this process (Fig. 3B). These data suggested a possible positive feedback model of prostacyclin-induced prostacyclin release.

Prostacyclin-induced prostacyclin release propagates a differentiation response

Based upon the observed robust prostacyclin-induced prostacyclin release, we hypothesized that endothelial cell prostacyclin release stimulates the proximal VSMC layer to further release prostacyclin that is then able to activate the next adjacent VSMC cell layer, generating a cascade mechanism in the whole wall of the blood vessel. To test this hypothesis, we initially treated a plated layer of human VSMC with iloprost. After 1 hour treatment, the cells were washed three times to eliminate residual iloprost and fresh media was added to these “activated” cells (see schematic, Fig. 4A). A second VSMC cell layer was cultured on a coverslip and placed cell side down upon the first “activated” cell layer, ensuring cell-cell contact. After 24 hours, the second layer coverslips were washed, fixed, and analyzed by immunohistochemical staining. Notably, exposure to the iloprost-activated first cell layer was able to induce expression of COX-2 and the differentiation marker calponin in the second layer of cells that were never directly exposed to iloprost (Fig. 4B). We have previously shown that hIP activation promotes VSMC differentiation, as measured by induction of calponin (and other contractile proteins), and by a change in morphology that results in a reduction in two-dimensional cell area [2]. The iloprost-activated cells were able to induce morphologic differentiation in the second layer of VSMC, as the area of the cells of the second cell layer was significantly decreased after exposure to the iloprost-treated first layer, compared to cells exposed to a vehicle treated first layer (Fig. 4C). As an important control to confirm that no residual iloprost remained after washing the first treated layer, the third wash was used to treat a separate coverslip of VSMC. The third wash did not induce COX-2 or calponin expression in these cells (data not shown). Furthermore, transient transfection of the first layer with GFP confirmed that

cells from this layer do not adhere to the second layer (data not shown). Confirming the role of hIP in the paracrine transactivation, pretreatment of the second cell layer with the IP antagonist CAY10441 blocked COX-2 upregulation (Fig. 4D). Activation of COX-2 in the first layer is necessary to generate the paracrine effect, as pretreatment of the first cell layer with NS-398 also blocked the COX-2 upregulation in the second layer (Fig. 4E).

These data confirm that iloprost treated cells are able to induce a differentiated phenotype in an untreated adjacent VSMC layer, as well as to potentially propagate a prodifferentiation signal as evidenced by induction of COX-2 in the second layer. Our data suggest that hIP and COX-2 play a crucial role in this transactivation.

PKA contributes to the iloprost-induced COX-2 upregulation and prostacyclin release

We have previously shown that prostacyclin-induced VSMC differentiation requires activation of cAMP/PKA signaling [2]. Knockdown of the PKA catalytic α and β subunits reduced the iloprost induced COX-2 expression, but this effect did not reach statistical significance ($P=0.07$) (Fig 5A). However, the reduction in COX-2 expression resulted in a functional decrease in 6-keto PGF1 α production ($P=0.01$) (Fig 5B). The PKA inhibitor myrPKI also reduced iloprost-induced COX-2 expression (Suppl. Fig. 3). Finally, 8-Br-cAMP treatment induced COX-2 expression, although to a lesser extent than iloprost alone (Fig. 5E), while this same concentration of 8-Br-cAMP was previously shown to activate PKA and induce contractile protein expression similar to iloprost [2]. Combined, these data confirm that the cAMP/PKA pathway contributes to, but may not be the only pathway involved in promoting prostacyclin-induced COX-2 upregulation.

Novel Akt-1 signaling in the COX-2 regulation

In order to investigate the possible role of other signaling pathways in the iloprost induced COX-2 upregulation, we employed pharmacological inhibitors as a first approach to implicate other candidate pathways. Interestingly, pretreatment with the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin produced a synergistic COX-2 induction compared to iloprost alone. Wortmannin alone was also able to upregulate COX-2 protein expression (Fig. 6A). As this suggested that Akt inhibition may induce COX-2, we next investigated whether iloprost might inhibit Akt activity, as measured by Akt phosphorylation on Ser⁴⁷³. We found that iloprost induced a rapid and robust inhibition of Akt phosphorylation (Fig. 6B). The specific hIP antagonist CAY 10441 rescued iloprost-induced downregulation of Akt activity (Fig. 6C), and even low doses of iloprost were able to inhibit Akt (Suppl. Fig. 4A). In addition, cicaprost or prostacyclin itself also inhibited Akt (Suppl. Fig. 4A), suggesting that this novel signaling is specific to the hIP G-protein-coupled receptor. Because our previous study revealed that Akt-1 inhibits contractile protein expression in VSMC [15], we next determined the role of the Akt-1 isoform in the regulation of the iloprost induced COX-2 expression. Both basal and iloprost-induced COX-2 expression and 6-keto PGF1 α production were dramatically increased following siRNA-mediated selective knockdown of Akt-1 compared to the nontargeting siRNA treated samples (Fig. 6D–6E), suggesting that Akt-1 has an inhibitory effect on COX-2 expression. Finally, overexpression of the Akt-1 isoform dramatically decreased the COX-2 expression at the basal level and also following iloprost treatment (Fig. 6F). These observations confirm that Akt-1 has an inhibitory effect on COX-2 expression, and that iloprost stimulates COX-2 expression through both activation of PKA and inhibition of Akt1.

The role of ERK1/2 in COX-2 induction

U0126, a specific MEK (ERK1/2 signaling pathway) inhibitor, strongly inhibited the iloprost-induced COX-2 upregulation (Fig. 7A). Moreover, iloprost induced a rapid time-dependent activation of ERK1/2 (Fig. 7B). Consistent with these results, 6-keto PGF1 α production was significantly reduced with U0126 treatment (Fig. 7C). The prostacyclin receptor antagonist

(CAY10441) also diminished the iloprost-induced ERK1/2 activation in a dose-dependent manner (Fig. 7D). Moreover, low doses of iloprost (Suppl. Fig. 4B), cicaprost or prostacyclin (Suppl. Fig. 4B) were also able to activate the ERK1/2 pathway, confirming that the ERK1/2 pathway induction was due to hIP activation. This result suggests that at least three different pathways (PKA, Akt-1 and ERK1/2) are involved in the iloprost-induced COX-2 upregulation.

Independent regulation of PKA-, Akt- and ERK1/2 signaling pathways by activated hIP

Knockdown of the PKA catalytic α and β subunits did not significantly affect iloprost-induced ERK1/2 induction or Akt downregulation (Fig. 8A–C). Similarly, wortmannin or U0126 treatment did not influence iloprost-induced PKA activity (Fig. 8D). Finally treatment with wortmannin did not change iloprost induced ERK1/2 upregulation and U0126 did not modify Akt downregulation (Fig. 8E). Taken together, these results suggest that hIP regulates the cAMP/PKA, ERK1/2 and Akt signaling pathways independently from each other (Fig. 8F).

DISCUSSION

Prostacyclin-induced prostacyclin release: a positive feedback loop

Our new findings suggest a mechanism by which prostacyclin may propagate protective effects through the vessel media (Fig. 8F). Prostacyclin initially arises from COX-2 production in the endothelial layer of blood vessels, and because of its very short half-life, would be expected to primarily affect only the most proximal layer of VSMC. We report that a single layer of iloprost treated VSMC can induce both COX-2 upregulation and differentiation in an adjacent layer of untreated cells. The essential roles of the prostacyclin receptor in the adjacent (“second”) layer and of COX-2 of the iloprost-activated (“first”) layer in the positive feedback loop was confirmed by using IP antagonist and selective COX-2 inhibitor. These results suggest that prostacyclin-induced prostacyclin release is a mechanism by which the prostacyclin signal can be propagated throughout the thickness of the media. In our *in vitro* model, discrete VSMC layers are brought into contact by placing a coverslip onto another cell layer. It is likely that there is heterogeneity in the efficiency of cell-cell contacts, with variable distances between cells. We speculate that the transmission occurs with much greater efficiency in the *in vivo* setting due to the organization of the VSMC layers in the media. It is also possible that both prostacyclin release and hIP receptors may be localized to facilitate propagation of the “wave” of prostacyclin signaling, somewhat analogous to a synaptic cleft in neurotransmission. The time scale, however, would differ from neurotransmission as the COX-2 induction occurs at the mRNA level.

PKA-dependent COX-2 induction by hIP

In the current study, we found that the cAMP/PKA pathway is involved in the iloprost-induced COX-2 and PGF1 α upregulation. Another study supported a role for PKA in hIP-induced COX-2 expression, showing that dibutyryl cAMP or forskolin could induce COX-2 mRNA in VSMC, but to a lesser extent than iloprost [16]. Our work greatly extends these preliminary findings, as the previous work did not confirm the COX-2 induction at the protein level nor functional induction of COX-2-derived prostacyclin. Interestingly, we noted a clear trend toward inhibition of the COX-2 induction when PKA was inhibited with siRNA, but this did not reach statistical significance. While it is possible that the partial inhibition was due to incomplete knockdown of PKA activity, we have previously shown that comparable levels of knockdown completely prevent iloprost-induced VSMC contractile protein expression [2]. These studies led us to conclude that while the cAMP/PKA is the pathway classically associated with the Gs-coupled hIP, other signaling pathways must also contribute to the hIP-induced COX-2 expression.

Novel hIP signaling to Akt1 promotes COX-2 induction

The serine/threonine protein kinase Akt is an important mediator of PI3K signaling and regulates a wide variety of cellular functions (cell survival, angiogenesis, metabolism, growth) [17]. Our data, using pharmacological inhibition, siRNA and overexpression approaches revealed that Akt-1 activity inhibits basal levels of COX-2 expression, and that iloprost inhibition of Akt-1 activity contributes to the iloprost-induced COX-2 upregulation. To our knowledge, a role for Akt in prostacyclin signaling has not been previously reported. This finding is consistent with recent work from our laboratory which identified opposing roles for Akt-1 and Akt-2 in VSMC differentiation: Akt-1 opposes, but Akt-2 promotes VSMC differentiation, as measured by contractile protein expression [15]. We have not investigated a role for Akt-2 in prostacyclin signaling. However, as we have shown iloprost inhibition of Akt phosphorylation using an antibody that recognizes both Akt-1 and Akt-2 phosphorylated at Ser473, and that the effect of wortmannin, which inhibits both Akt-1 and Akt-2, was to induce COX-2, it is unlikely that activation of Akt-2 would play a significant role in the hIP-mediated COX2 induction. The mechanism underlying this novel hIP inhibition of Akt-1 remains under investigation by our group. Interestingly, the activation of G_{α_s} -coupled D2 dopamine receptors leads to formation of an internalization complex that includes β -arrestin, which nucleates a signaling complex that allows the PP2A-mediated dephosphorylation and inhibition of Akt [18]. Several studies have also shown that the Gq-coupled GPCR α_1 -adrenergic receptor [19] and neurotensin receptor 1 [20] can inhibit Akt in other cell types. The lipoxin LXA4 receptor can also inhibit Akt, through an unknown mechanism [21]. There are, however, studies where different Gq-coupled GPCR instead activate PI3K/Akt signaling [22,23]. Overexpressed hIP can couple to Gq [24,25] but in human SK-N-SH neuroblastoma cells which endogenously express the receptor, hIP was unable to activate the Gq signaling pathway [26]. The specific G protein coupling of endogenous hIP in human VSMC has not yet been fully characterized. It may be that the aggregate combination of signals induced by a GPCR determines whether the net effect is activation or repression of Akt. Importantly, none of these studies has determined whether Gq activation preferentially inhibits Akt-1. Our preliminary studies suggest that the iloprost Akt-1 inhibition in VSMC is Gq-independent (unpublished observations).

However different subcellular localization and expression profile of the Akt isoforms [27] could potentially explain a selective effect of iloprost on Akt-1. One other attractive mechanism could be a potential role for the recently described PH domain leucine-rich repeat phosphatases (PHLPPs) which dephosphorylate Akt. PHLPP1 regulates Akt-2 and Akt-3, whereas PHLPP2 regulates Akt-1 and Akt-3 [28,29].

Novel hIP signaling to ERK1/2 promotes COX-2 induction

We report here that the ERK signaling pathway is involved in iloprost-induced COX-2 upregulation. The classical extracellular-signal regulated kinase (ERK) pathway governs fundamental processes such as cell proliferation, differentiation and survival [30]. G_{α_s} can stimulate ERK1/2 via PKA or EPAC (exchange protein directly activated by cAMP) [31], but our data (Fig. 8A and B) suggests that hIP mediates PKA-independent ERK activation, although we have not excluded a potential role for EPAC. Another potential mechanism of ERK activation is G_{α_i} [31]. G protein-independent activation of ERK1/2 can also occur through β -arrestin [32]. Chu et al. [25] reported that overexpressed human IP- and mouse IP-mediated activated the ERK1/2 signaling pathway through a Gq/11/PKC and phosphoinositide-3-kinase signaling pathway which was cAMP-insensitive. Our observation is the first describing ERK1/2 activation by the endogenous hIP in human VSMC, as well as a role for the ERK pathway in COX-2 mediated vasoprotective prostacyclin production, but the exact mechanism of ERK activation is still under investigation by our group.

Clinical implications for selective COX-2 inhibition

Our study helps to elucidate the mechanisms for the increased adverse cardiovascular effects associated with the recent COX-2 inhibition trials. Although COX-2 regulates the production of many eicosanoids, one of its major vascular products is prostacyclin. In our study, the selective COX-2 inhibitor NS-398 prevented the prostacyclin-induced prostacyclin release. Our model therefore predicts that reduction of prostacyclin with selective COX-2 inhibitors in vivo, such as rofecoxib or valdecoxib, would result in attenuation of this positive feedback response, thus predisposing VSMC to the phenotypic modulation (dedifferentiation) that is key in the pathophysiologic progression of atherosclerosis and intimal hyperplasia. The nonselective NSAIDs, through COX-2 inhibition, could also potentially attenuate this positive feedback loop. The inhibition of this protective mechanism within the vessel wall, along with attenuation of the anti-thrombotic effects of prostacyclin on platelets, suggests that selective COX-2 inhibition increases the risk of cardiovascular disease, especially in populations with prior risk factors (e.g. smoking, diabetes, hypertension). Indeed, in recent studies we report that a naturally occurring variant in the human prostacyclin receptor with diminished activity increases cardiovascular risk [33,34]. As we have identified multiple signaling pathways that contribute to the prostacyclin-induced prostacyclin release, we propose that the redundancy in signaling may provide additional targets in the prevention of VSMC proliferation, dedifferentiation and atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Jin Q. Cheng for the Akt1 expression plasmid, John Blenis for the total ERK1/2 antibody, and Eva Rzucidlo for helpful comments.

SOURCES OF FUNDING

This study was supported by grants from NIH NHLBI (HL077612 to RP, HL074190 to JH, and HL091013 to KAM). JH is an Established Investigator of the American Heart Association.

Abbreviations

hIP	human prostacyclin receptor
hVSMC	human vascular smooth muscle cell(s)
COX	cyclooxygenase
PGIS	prostacyclin synthase
PKA	protein kinase A/cAMP-dependent protein kinase
myrPKI	myristoylated PKA inhibitor-(14–22) amide
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

PDH

pyruvate dehydrogenase

PI3K

phosphatidylinositol 3-kinase

References

1. Arehart E, Gleim S, Kasza Z, Fetalvero KM, Martin KA, Hwa J. Prostacyclin, atherothrombosis, and cardiovascular disease. *Curr Med Chem* 2007;14:2161–2169. [PubMed: 17691954]
2. Fetalvero KM, Shyu M, Nomikos AP, Chiu YF, Wagner RJ, Powell RJ, Hwa J, Martin KA. The prostacyclin receptor induces human vascular smooth muscle cell differentiation via the protein kinase A pathway. *Am J Physiol Heart Circ Physiol* 2006;290:H1337–1346. [PubMed: 16399867]
3. McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. *Proc Natl Acad Sci U S A* 1999;96:272–277.
4. Topper JN, Cai J, Falb D, Gimbrone MA Jr. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci U S A* 1996;93:10417–10422. [PubMed: 8816815]
5. Fitzgerald GA. Coxibs and cardiovascular disease. *N Engl J Med* 2004;351:1709–11. [PubMed: 15470192]
6. Murata T, Ushikubi F, Matsuoka T, Hirata M, Yamasaki A, Sugimoto Y, Ichikawa A, Aze Y, Tanaka T, Yoshida N, Ueno A, Oh-ishi S, Narumiya S. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 1997;388:678–682. [PubMed: 9262402]
7. Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, FitzGerald GA. Role of prostacyclin in the cardiovascular response to thromboxane A₂. *Science* 2002;5567:539–41. [PubMed: 11964481]
8. Egan KM, Lawson JA, Fries S, Koller B, Rader DJ, Smyth EM, Fitzgerald GA. COX-2-derived prostacyclin confers atheroprotection on female mice. *Science* 2004;306:1954–1957. [PubMed: 15550624]
9. Stitham J, Stojanovic A, Merenick BL, O'Hara KA, Hwa J. The unique ligand-binding pocket for the human prostacyclin receptor. Site-directed mutagenesis and molecular modeling. *J Biol Chem* 2003;278:4250–4257. [PubMed: 12446735]
10. Stitham J, Stojanovic A, Ross LA, Blount AC Jr, Hwa J. Clusters of transmembrane residues are critical for human prostacyclin receptor activation. *Biochemistry* 2004;43:8974–8986. [PubMed: 15248755]
11. Stitham J, Martin KA, Hwa J. The critical role of transmembrane prolines in human prostacyclin receptor activation. *Mol Pharmacol* 2002;61:1202–1210. [PubMed: 11961139]
12. Stitham J, Gleim SR, Douville K, Arehart E, Hwa J. Versatility and differential roles of cysteine residues in human prostacyclin receptor structure and function. *J Biol Chem* 2006;281:37227–37236. [PubMed: 17015447]
13. Stitham J, Stojanovic A, Hwa J. Impaired receptor binding and activation associated with a human prostacyclin receptor polymorphism. *J Biol Chem* 2002;277:15439–15444. [PubMed: 11854299]
14. Martin KA, Rzczidlo EM, Merenick BL, Fingar DC, Brown DJ, Wagner RJ, Powell RJ. The mTOR/p70 S6K1 pathway regulates vascular smooth muscle cell differentiation. *Am J Physiol Cell Physiol* 2004;286(3):C507–17. [PubMed: 14592809]
15. Martin KA, Merenick BL, Ding M, Fetalvero KM, Rzczidlo EM, Kozul CD, Brown DJ, Chiu HY, Shyu M, Drapeau BL, Wagner RJ, Powell RJ. Rapamycin promotes vascular smooth muscle cell differentiation through insulin receptor substrate-1/phosphatidylinositol 3-kinase/Akt2 feedback signaling. *J Biol Chem* 2007;282:36112–36120. [PubMed: 17908691]
16. Debey S, Meyer-Kirchraht J, Schror K. Regulation of cyclooxygenase-2 expression by iloprost in human vascular smooth muscle cells. Role of transcription factors. CREB and ICER *Biochem Pharmacol* 2003;65:979–988.

17. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007;129(7):1261–74. [PubMed: 17604717]
18. Beaulieu JM, Gainetdinov RR, Caron MG. The Akt-GSK-3 signaling cascade in the actions of dopamine. *Trends Pharmacol Sci* 2007;28:166–172. [PubMed: 17349698]
19. Ballou LM, Lin HY, Fan G, Jiang YP, Lin RZ. Activated G alpha q inhibits p110 alpha phosphatidylinositol 3-kinase and Akt. *J Biol Chem* 2003;278:23472–23479. [PubMed: 12704201]
20. Liu F, Yang P, Baez M, Ni B. Neurotensin negatively modulates Akt activity in neurotensin receptor-1-transfected AV12 cells. *J Cell Biochem* 2004;92:603–611. [PubMed: 15156571]
21. Mitchell D, Rodgers K, Hanly J, McMahon B, Brady HR, Martin F, Godson C. Lipoxins inhibit Akt/PKB activation and cell cycle progression in human mesangial cells. *Am J Pathol* 2004;164:937–946. [PubMed: 14982847]
22. Rahman A, True AL, Anwar KN, Ye RD, Voyno-Yasenetskaya TA, Malik AB. Galpha(q) and Gbetagamma regulate PAR-1 signaling of thrombin-induced NF-kappaB activation and ICAM-1 transcription in endothelial cells. *Circ Res* 2002;91:398–405. [PubMed: 12215488]
23. Anger T, Klintworth N, Stumpf C, Daniel WG, Mende U, Garlischs CD. RGS protein specificity towards Gq- and Gi/o-mediated ERK 1/2 and Akt activation, in vitro. *J Biochem Mol Biol* 2007;40:899–910. [PubMed: 18047785]
24. Miggin SM, Kinsella BT. Investigation of the mechanisms of G protein: effector coupling by the human and mouse prostacyclin receptors. Identification of critical species-dependent differences. *J Biol Chem* 2002;277:27053–27064. [PubMed: 12016224]
25. Chu KM, Chow KB, Wong YH, Wise H. Prostacyclin receptor-mediated activation of extracellular signal-regulated kinases 1 and 2. *Cell Sign* 2004;16:477–486.
26. Kam Y, Chow KB, Wise H. Factors affecting prostacyclin receptor agonist efficacy in different cell types. *Cell Sign* 2001;13:841–847.
27. Mendoza MC, Blenis J. PHLPPing it off: phosphatases get in the Akt. *Mol Cell* 2007;25:798–800. [PubMed: 17386258]
28. Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Mol Cell* 2005;18:13–24. [PubMed: 15808505]
29. Brognard J, Sieracki E, Gao T, Newton AC. PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. *Mol Cell* 2007;25:917–931. [PubMed: 17386267]
30. English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb MH. New insights into the control of MAP kinase pathways. *Exp Cell Res* 1999;253:255–270. [PubMed: 10579927]
31. Goldsmith ZG, Dhanasekaran DN. G protein regulation of MAPK networks. *Oncogene* 2007;26:3122–3142. [PubMed: 17496911]
32. DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD, Bunnett NW. Beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2. *J Cell Biol* 2000;148:1267–1281. [PubMed: 10725339]
33. Arehart E, Stitham J, Asselbergs FW, Douville K, MacKenzie T, Fetalvero KM, Gleim S, Kasza Z, Rao Y, Martel L, Segel S, Robb J, Kaplan A, Simons M, Powell RJ, Moore JH, Rimm EB, Martin KA, Hwa J. Acceleration of cardiovascular disease by a dysfunctional prostacyclin receptor mutation: potential implications for cyclooxygenase-2 inhibition. *Circ Res* 2008;102:986–993. [PubMed: 18323528]
34. Patrignani P, Di Febbo C, Tacconelli S, Douville K, Guglielmi MD, Horvath RJ, Ding M, Sierra K, Stitham J, Gleim S, Baccante G, Moretta V, Di Francesco L, Capone ML, Porreca E, Hwa J. Differential association between human prostacyclin receptor polymorphisms and the development of venous thrombosis and intimal hyperplasia: a clinical biomarker study. *Pharmacogenet Genomics* 2008;18:611–620. [PubMed: 18551041]

Figure 1. Iloprost induces the expression of COX-2

Timecourse analysis of COX-2, COX-1 and PGIS protein expression in human VSMC. Cells were incubated with 100nM iloprost or PBS vehicle for the indicated times. Beta-tubulin was used as a loading control. Results are representative of 2 separate experiments.

Figure 2. Human prostacyclin receptor antagonist inhibits iloprost-induced COX-2 protein expression in human VSMC

Human VSMC were pretreated for 30 min with the indicated concentration of a selective prostacyclin receptor antagonist (CAY10441-dark gray bars) or vehicle (ethanol), then treated with 100nM iloprost or vehicle as indicated for 6 hours. GAPDH serves as the loading control. One blot representative of 3 independent experiments is shown. Data are means of the three experiments $\pm$ SE.

Figure 3. Iloprost treatment induces prostacyclin production through COX-2. A

Timecourse analysis of 6-keto-PGF_{1α} production (pg/50μl media) in human VSMC. Cells were incubated with 100 nM iloprost (black bars) or vehicle (PBS – gray bars) for the indicated time and media were collected and assayed for 6-keto-PGF_{1α} production. Data are means of 6 independent experiments ± SE. **B:** Human VSMC were pretreated for 30 minutes with 10 μM specific COX-2 inhibitor (NS-398-dark gray bar) or vehicle (DMSO), then treated with 100 nM iloprost (black bar) or vehicle (PBS-gray bar) for 6 hours. Media was collected and assayed for 6-keto-PGF_{1α} production (pg/50μl media). Data are means of 5 independent experiments ± SE.

COX-2 staining (red)
in second cell layer (24h)

First cell layer
treated with (1h):

vehicle

iloprost

Calponin staining (red)
in second cell layer (24h)

First cell layer
treated with (1h):

vehicle

iloprost

secondary antibody only

Figure 4. Paracrine actions of prostacyclin signaling. A

Experiment schematic: Human VSMC were treated with iloprost (100 nM) or vehicle (PBS) for 1h (**B**, **C**, **E**) or 2h (**D**), then washed three times. The second layer of VSMC was cultured on a coverslip and placed cell side down on the first iloprost-activated cell layer. After 8h (**D**, **E**) or 24h (**B**, **C**), cells on the coverslips were washed and fixed for immunohistochemical confocal microscopy analysis. Blue, DAPI (nuclei); Red, COX-2 or calponin, as indicated. **B**: Second layer of VSMC after exposure to the first layer. Results are representative of 2 separate experiments. **C**: Two-dimensional cell area of the second layer of VSMC after exposure to the first layer was determined. Data shown are means of 4 separate experiments $\pm$ SE. **D**: The second layer of VSMC was pretreated with 5 μ M IP antagonist for 2h followed by exposure to the first, iloprost-activated cell layer for 8h. 4 fields per treatment are shown. Results are representative of 2 separate experiments. **E**: The first cell layer was pretreated with the COX-2 inhibitor NS-398 (10 μ M) for 1h, then iloprost for 1h, before coculture with the second layer for 8h. 4 fields per treatment are shown. Results are representative of 2 separate experiments.

Figure 5. PKA contributes to COX-2 induction by iloprost. A

Human VSMC were transfected with nonsilencing siRNA (siCONTROL; 1.1 μ g) or siRNA targeted against PKA catalytic subunits- α and - β (PKA-C α + PKA-C β ; 0.55 μ g each) for 48h. Cells were then treated with iloprost (100 nM-black bars) or vehicle (PBS-gray bars) for 6 hours. A representative blot is shown. Data are means + SE of 7 independent experiments. **B:** PKA catalytic subunits were knocked down as described in **5A** and 6-keto-PGF_{1 α} production was measured. Data are means of 6 independent experiments. **C:** Human VSMC were treated with 500 nmol/l 8-Bromo-cAMP (8-Br-cAMP) for the indicated time. Western-blot analysis was performed using antibodies against COX-2 and beta-tubulin (loading control). Results are representative of 2 separate experiments.

Figure 6. Inhibition of Akt signaling pathway induces COX-2 expression in human VSMC. A Human VSMC were treated with the indicated concentrations of wortmannin for 6 hours (Lanes 3–6). Human VSMC were pretreated with the indicated concentration of wortmannin for 30 minutes followed by the treatment of 100 nM iloprost for an additional 6h (Lanes 7–10.). 0h

timepoint (Lane 1), vehicle (PBS, 6h; Lane 2) and iloprost alone (100 nM, 6h; Lane 11)-treated cells were used as controls. Western-blot analysis was carried out using antibodies against COX-2 and beta-tubulin (loading control). Results are representative of 6 separate experiments. **B:** Human VSMC were treated with iloprost (100nM) for the indicated times. Western-blot was performed using antibodies against phospho-Ser473 Akt (P473-Akt), total-Akt, and GAPDH (loading control). A representative result from independent 3 experiments is shown. **C:** Human VSMC were pretreated for 30 min with the indicated concentration of a selective prostacyclin receptor antagonist (CAY10441-dark gray bar) or vehicle (ethanol) then treated with 100 nM iloprost or vehicle where indicated for 10 minutes. Total Akt was used as the loading control. One representative blot of 3 independent experiments is shown. Data are means of the three independent experiments $\pm$ SE. **D:** Human VSMC were transfected with nonsilencing siRNA (siCONTROL; 2.5 μ g) or siRNA targeting Akt-1 (2.5 μ g). 48h posttransfection, cells were treated with iloprost (100 nM) or vehicle (PBS) for an additional 6h. Western-blot analysis was performed using antibodies against COX-2, Akt-1 and GAPDH. One blot representative of three separate experiments is shown. Data are means of the three experiments $\pm$ SE. **E:** Human VSMC were transfected with siCONTROL or Akt-1 siRNA and treated with iloprost (100 nM) or vehicle (PBS) exactly as in Fig. 5B. Conditioned media were collected and 6-keto-PGF1 α (pg/50 μ l) was measured. Results were normalized to cell number. Data are means $\pm$ SE of six different experiments. All measurements were carried out in either duplicate or triplicate. **F:** Human VSMC were transfected with control vector (pcDNA3) or wild-type Akt-1 plasmid (2 μ g) for 48 hours, then treated with iloprost (100nM) or vehicle (PBS) for an additional 6h. Western-blot analysis was carried out using antibodies against COX-2, Akt-1 and GAPDH.

Iloprost activates ERK at early timepoints

Figure 7. ERK signaling pathway is involved in the iloprost-induced COX-2 upregulation. **A:** Human VSMC were pretreated with MEK/ERK pathway inhibitor (10 μM; U0126) for 30 minutes followed by treatment with 100 nM iloprost or vehicle for 6 hours. Lysates were immunoblotted with antibodies to COX-2 or β-tubulin. Results are representative of 3 independent experiments. **B:** Human VSMC were treated with iloprost (100 nM) for the indicated time. Immunoblotting was performed using antibodies against phospho-ERK1/2 (T202/Y204) (P-ERK1/2) and total-ERK1/2 (loading control). Result is representative of 3 independent experiments. **C:** Human VSMC were pretreated with 10 μM U0126 (dark gray bar) or vehicle for 30 minutes, followed by treatment with iloprost (100nM, black bar) or vehicle (PBS, gray bar) for 6 hours. Conditioned media were collected and 6-keto-PGF_{1α} production was measured. Data are means ± SE of three experiments. **D:** Human VSMC were pretreated for 30 min with the indicated concentration of a selective prostacyclin receptor antagonist (CAY10441, dark gray bars) or vehicle (ethanol), then treated with 100 nM iloprost or vehicle (PBS) for 10 minutes where indicated. Total ERK1/2 was used as the loading control. Data are means of 5 independent experiments ± SE, one representative blot is shown.

Figure 8. Independent regulation of PKA-, ERK- and Akt signaling pathways by the hIP. **A** PKA knockdown was performed as described in **5A**, then cells were treated for 10 minutes with vehicle or iloprost (100nM). Western-blot was performed using antibodies against P473-Akt, total-Akt, P-ERK1/2 and total-ERK1/2. One blot representative of 3 separate experiments is shown. **B–C**: Quantitative representation of the experiments described in **8A**. Data are means of 3 separate experiments. **D**: Human VSMCs were pretreated with 100 nM wortmannin or 10 μ M U0126 for 30 minutes then treated with 100nM iloprost for 15 minutes. Non-radioactive PKA-assay was performed using total cell lysate (10 μ g). One representative gel is shown. Densitometry data are means + SE of 3 separate experiments. **E**: Human VSMC were treated with vehicle or iloprost for 10 minutes (lanes 1–2), with wortmannin or U0126 for 30 minutes (lanes 3–4), pretreated with wortmannin or U0126 (lanes 5–6) and vehicle (DMSO/MetOH; lanes 7–8) for 30 minutes then treated with iloprost for 10 minutes prior to western blotting for P473-Akt, total-Akt, P-ERK1/2 and total-ERK1/2. One blot representative of 3 separate experiments is shown. **F**: Diagram of the innermost cell layers of the vascular wall, showing

endothelial cells and the two most proximal layers of VSMC (I and II). Prostacyclin (PGI₂) is normally produced by the endothelial cell layer leading to a PKA-dependent promotion of a quiescent and differentiated contractile (healthy) phenotype in proximal VSMC. We now report that prostacyclin activation of VSMC can additionally induce COX-2 expression, activity and prostacyclin release through cAMP/PKA, ERK, and Akt-1 signaling pathways. The prostacyclin production from this positive feedback mechanism serves to activate the same signaling pathways in adjacent vascular smooth muscle cell layers (VSMC II), leading to propagation of the quiescent differentiated phenotype.