Dimerization of the Human Receptors for Prostacyclin and Thromboxane Facilitates Thromboxane Receptor-mediated cAMP Generation*

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Prostacyclin (PGI₂) and thromboxane (TxA₂) are biological opposites; PGL₂, a vasodilator and inhibitor of platelet aggregation, limits the deleterious actions of TxA₂, a vasoconstrictor and platelet activator. The molecular mechanisms involved in the counterregulation of PGI₂/TxA₂ signaling are unclear. We examined the interaction of the receptors for PGI₂ (IP) and TxA₂ (TPα). TPα-induced cAMP and TP-induced inositol phosphate generation were unaltered when the receptors were co-expressed in HEK 293 cells (IP/TPα-HEK). TP-cAMP generation, in response to TP agonists or a TP-dependent isoprostane, iPE₂III, was evident in IP/TPα-HEK and in aortic smooth muscle cells, but not in cells expressing either receptor alone, or in IP-deficient aortic smooth muscle cells. Augmentation of TP-induced cAMP generation, with the IP agonist cicaprost, was evident in IP-deficient and in TxA₂-expressing either receptor alone, or in IP/TPα-deficient aortic smooth muscle cells. Both TPα and IP/TPα heterodimers were formed constitutively when the receptors were co-expressed, with no overt changes in ligand binding to the individual receptor sites. However, despite inefficient binding of iPE₂III to either the IP or TPα, expressed alone or in combination, robust cAMP generation was evident in IP/TPα-HEK, suggesting the formation of an alternative receptor site. Thus, IP/TPα dimerization was coincident with TP-cAMP generation, promoting a "PGI₂-like" cellular response to TP activation. This represents a previously unknown mechanism by which IP may limit the cellular effects of TP.

PGI₂ and TxA₂ are the predominant products of cyclooxygenase (COX) metabolism of arachidonic acid formed in the macrovascular endothelium and platelets, respectively (1, 2). These two mediators are biological opposites. PGI₂, a potent vasodilator, inhibitor of platelet aggregation (3) and smooth muscle cell (SMC) growth in vitro (4), demonstrates anti-thrombotic and anti-platelet actions in vivo (5). In contrast, TxA₂ is a potent vasoconstrictor (6), stimulates platelet aggregation (7), amplifies the activity of other platelet agonists (7), and stimulates proliferation of SMC (8). The hypothesis that PGI₂ modulates cardiovascular homeostasis and disease gained support from the association of a selective COX-2 inhibitor, rofecoxib, which depresses PGI₂ levels without affecting platelet TxA₂ biosynthesis, with a higher risk of myocardial infarction in humans, compared with a nonselective COX-1/COX-2 inhibitor naproxen (VIGOR trial) (9). Recent work, using mice genetically deficient in the receptors for PGI₂ (the IP) or TxA₂ (the TP), demonstrated that the proliferative and platelet response to vascular injury was TP-mediated and was limited specifically by PGI₂ (5). In addition, delivery of PGI₂ synthase in vivo prevents proliferation and migration of SMC, key features of restenosis and atherosclerosis (10, 11), whereas the antioxidant and antiplatelet actions of PGI₂ delayed atherogenesis and may underlie the protection from cardiovascular disease afforded by female gender (12). Maintenance of the PGI₂/TxA₂ balance appears to be a critical regulator of vascular disease; however, the molecular mechanisms underlying the counterregulation of PGI₂/TxA₂ signaling have not been fully elucidated.

A single gene encoding a G protein-coupled receptor (GPCR) has been reported for both mediators (13, 14), although in contrast to IP, where splice variants have not been described, two variants of TP, termed TPα and TPβ, have been identified (15). IP is coupled to at least two signaling systems, namely the generation of intracellular cAMP and activation of PLC (16). Both TPα and TPβ are coupled to PLC, whereas the former may activate and the latter may inhibit AC activity (16). There is substantial evidence for reciprocal regulation between IP and TP. TPα, but not TPβ, is a target for IP-mediated, PKA-dependent phosphorylation, resulting in TPα desensitization (17). Similarly, U46619-mediated activation of TP enhances IP-mediated cAMP generation in human platelets (18), where only TPα is expressed (19).

The interaction between IP and TP may not, however, be limited to events occurring secondary to activation of their respective second messenger systems. GPCRs have long been considered to exist and function as independent monomeric units. However, GPCRs from both closely related and distinct subfamilies are capable of interacting physically with one another to form heterodimers (20, 21). Far from being a benign association, GPCR heterodimerization can substantially modify receptor function (20, 21). Signaling may change as a result of altered agonist affinity for the receptors, altered affinity of the receptors for their respective G proteins or signaling via alternate pathways (21). Heterodimerization of the δ- and κ-opioid receptors, for example, creates a "new" receptor binding site that has a reduced affinity for individual δ- or κ-selective ligands but that can ligate cooperatively selective agonists to induce synergistic functional responses (22).

Heterodimers of the angiotensin II AT1 receptor and the
bradykinin B₂ receptor demonstrate increased activation of ATI-coupled G proteins in response to angiotensin II, a phenomenon that may underlie preeclampsia hypertensive (23). Thus, two opposing vascular mediators, angiotensin II, a vaso-constrictor, and bradykinin, a vasodilator, can alter the action of the other via a direct interaction of their receptors (23). In the present study, we examined whether the interaction between two similarly opposing vascular mediators, PGI₂ and TxA₂, is also mediated via receptor interaction. We demonstrate that coexpression of IP and TPα, either endogenously or in an overexpression cell model, facilitated TP-mediated cAMP generation. The absence of the IP, in SMCs cultured from IP knockout (IPKO) mice or in HEK 293 cells, rendered the TP inactive when exposed to TxA₂. This interaction between IP and TPα is not dependent on IP-cAMP signaling, but is coincident with the formation of an IP/TPα heterodimer.

EXPERIMENTAL PROCEDURES

Materials—Cyclic AMP radioimmunoassay kit, enhanced chemiluminescence kit, protein G-Sepharose, and all radiochemicals were purchased from Amersham Biosciences. Cell culture reagents, G418, and Albumax were obtained from Invitrogen. Complete protease inhibitor tablets were obtained from Roche Applied Science. IBOP, SQ 29548, and Albumax were obtained from Invitrogen. Modified Eagle’s medium supplemented with 20% heat-inactivated fetal bovine serum, 50 units/ml penicillin, 5 μg/ml streptomycin, 25 mM HEPES, and 2 mM l-glutamine. Human aortic SMC (hAMSC, BioWhittaker Inc., Walkersville, MD) were cultured in smooth muscle cell basal medium supplemented with fetal bovine serum (5%), human recombinant epidermal growth factor (hEGF; 0.5 ng/ml), insulin (5 μg/ml), human recombinant fibroblast growth factor (hFGF; 2 ng/ml) plus gentamicin (50 μg/ml), and amphotericin-B (50 μg/ml). hASMC of passages 5–9 were used in experiments. Smooth muscle cells were isolated from WT or IPKO mouse aortic explants and grown in Dulbecco’s modified Eagle’s medium/F-12 Ham’s medium supplemented with 20% heat-inactivated fetal bovine serum, 25 units/ml penicillin, 25 μg/ml streptomycin, and 2 mM l-glutamine. mASMC of passages 3–6 were used in experiments. All cells were maintained in a humidified atmosphere of 5% CO₂, 95% air.

For stable transfections, HEK 293 cells were seeded at 1.5 × 10⁶ cells/100-mm dish and transfected the following day with 10 μg of DNA by liposomemediated transfer (DOTAP), as described previously (25, 26). Stable transfectants were selected in the presence of G418 (0.5–1.5 mg/ml) and/or hygromycin (50–75 μg/ml).

Western Blotting—Membranes or whole cell lysates were resolved (30 μg/lane) on NuPAGE (Invitrogen) 10% gels. HA-tagged or Myc-tagged receptors were visualized with anti-HA or anti-Myc (1:1000 dilution) in secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA). Ciprofloxin was obtained from Schering AG under agreement.

Epitope Tagging of hIP and hTP—The 9-amino acid hemagglutinin epitope (HA; YPYDVPDYA) or 10-amino acid Myc epitope (EQKLISEEDL) was inserted between the N-terminal initiator methionine and the second amino acid of the hTPα or hIP to generate HA-hIP, MychIP, or HAhTPα. Generation of HA-hIP was as described previously (24). To generate HAhTPα and MychIP, 5′-oligonucleotides that contained 3′ miscellaneous bases, 6 bases encoding a HindIII site, the 3′ miscellaneous bases immediately 5′ of the initiator methionine, 3 bases encoding a methionine, the epitope tag coding sequence, and 21 bases encoding amino acids 2–8 were generated. 3′-Oligonucleotides were complementary to the receptor coding sequence downstream of a unique restriction site (an EcoN47 site for hIP or a NotI site for hTPα). Using the hTPα or hIP cDNAs as templates, polymerase chain reactions were carried out to generate the 5′-HA-hIP and 5′-Myc-hIP fragments. The resulting products were cloned into PCR 2.1 (Qiagen, CA) and, following verification of the sequence, were excised using HindIII/NotI or HindIII/EcoN47, as appropriate. Using the same enzymes, the 3′ fragment in pcDNA3.1 (or pcDNA3.1 Hygro for MychIP) was generated, and the two receptor pieces were ligated to each other. The integrity of the splice site was verified by sequencing.

Cell Culture and Transfection—HEK 293 cells (American Type Tissue Culture Collection, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 50 units/ml penicillin, 5 μg/ml streptomycin, 25 mM HEPES, and 2 mM l-glutamine. Human aortic SMC (hAMSC, BioWhittaker Inc., Walkersville, MD) were cultured in smooth muscle cell basal medium supplemented with fetal bovine serum (5%), human recombinant epidermal growth factor (hEGF; 0.5 ng/ml), insulin (5 μg/ml), human recombinant fibroblast growth factor (hFGF; 2 ng/ml) plus gentamicin (50 μg/ml), and amphotericin-B (50 μg/ml). hASMC of passages 5–9 were used in experiments. Smooth muscle cells were isolated from WT or IPKO mouse aortic explants and grown in Dulbecco’s modified Eagle’s medium/F-12 Ham’s medium supplemented with 20% heat-inactivated fetal bovine serum, 25 units/ml penicillin, 25 μg/ml streptomycin, and 2 mM l-glutamine. mASMC of passages 3–6 were used in experiments. All cells were maintained in a humidified atmosphere of 5% CO₂, 95% air.
IP facilitates TPα-induced cAMP generation

**RESULTS**

**Generation of HEK 293 Cell Lines Expressing HA-hTPα or Coexpressing Both HA-hTPα and MycIP**—We have described previously the generation of a HEK 293 cell line stably expressing HA-tagged hIP (hIP-HEK; see Ref. 24). Cell lines stably expressing HAhTPα (TPα-HEK), or coexpressing both MycIP and HAhTPα (IP/TPα-HEK), were generated in the present study in order to examine the effect of IP/TPα coexpression on TPα signaling.

Lysates from each cell line were resolved by SDS-PAGE and immunoblotted with an anti-HA or anti-Myc antibody to establish that the receptors were being appropriately expressed. HAhTPα was observed as a broad complex of between 48 and 60 kDa in lysates derived from TPα-HEK or IP/TPα-HEK cells (Fig. 1). MycIP was observed as a 44–60-kDa complex in IP/TPα-HEK cell lysates (Fig. 1). This corresponds to the molecular weight of HAhTPα previously observed in hIP-HEK cells (24). The broad molecular weight range of both hIP and TPα is a result of receptor glycosylation (19, 24).

Stimulation of hIP-HEK (see Ref. 24) or IP/TPα-HEK with the prostacyclin analogue cicaprost for 5 min induced a concentration-dependent increase in intracellular cAMP (EC_{50} = 0.05 ± 0.02 nM, n = 4; Fig. 2) and inositol phosphate production (EC_{50} = 97.1 ± 33.3 nM, n = 4; Fig. 2), indicating that co-expression of hTPα did not alter hIP-mediated activation of two signaling systems when overexpressed in HEK 293 cells (24). Stimulation of TPα-HEK with the specific TPα agonist U46619 resulted in increased inositol phosphate production (EC_{50} = 174.4 ± 65.2 nM, n = 3; Fig. 3), which was not altered significantly by co-expression of hIP (EC_{50} = 231.7 ± 40.8 nM, n = 3; Fig. 3). Much evidence supports ligation of the TP by isoprostanes, free radical-catalyzed products of arachidonic acid. Indeed, two TP-dependent iso-

**Fig. 2.** IP-mediated signaling in cells coexpressing MycIP and HAhTPα. IP/TPα-HEK were treated with increasing concentrations of cicaprost (5 min) and cAMP (closed circles) or inositol phosphates (open circles), quantified as indicated under “Experimental Procedures.” Data are presented as mean fold over basal ± S.E. from four experiments.

**Fig. 3.** TPα-mediated signaling in cells coexpressing MycIP and HAhTPα. TPα-HEK (A) or IP/TPα-HEK (B) were treated with U46619 (closed squares) or iPE_{III} (closed triangles) for 10 min, and inositol phosphates were quantified as described under “Experimental Procedures.” Data are presented as mean fold over basal ± S.E. from three or four experiments.
prostanes (27), iPE2III (Fig. 3), and iPF2\(_2\)/H9251 stimulated inositol phosphate generation in TP\(_2\)/H9251-HEK and IP/TP\(_2\)/H9251-HEK, albeit with significantly higher EC\(_{50}\) values (3.56 ± 1.12 and 4.43 ± 0.63 \(\mu M\), respectively) compared with U46619. Thus, similar to other studies, the addition of HA or Myc tags to the N terminus of IP and TP did not alter the expression or signal transduction properties of the receptor. Furthermore, co-expression of the receptors did not alter their discrete signal transduction properties.

TP\(_{\alpha}\)-mediated cAMP Formation—Treatment with the TP agonists IBOP or U46619 (100 nM, 10 min) induced a robust increase in cAMP levels in cells coexpressing HA\(\alpha\)TP\(_\alpha\) and MycIP, but not in cells individually expressing the receptors or in mixed cultures of individually expressing cells (Fig. 4A). Pretreatment with the TP antagonist SQ 29548 partially reduced signaling by the TP agonists (Fig. 4B). Interestingly, iPE2\(_{II}\), but not iPF2\(_{II}\), also initiated an increase in cAMP in IP/TP\(_{\alpha}\)-HEK cells (Fig. 4A). In contrast to the TP agonists, the activity of iPE2\(_{II}\) was insensitive to SQ 29548 (Fig. 4B), suggesting that this event was TP-independent. However, signaling was not observed in the absence of hTP\(_\alpha\), demonstrating that inhibition with SQ 29548 is not sufficient to determine TP dependence (Figs. 4A and 5). Generation of cAMP in response to treatment of IP/TP\(_{\alpha}\)-HEK with IBOP, U46619, or iPE2\(_{II}\) proved concentration-dependent, and only minor cAMP increments were observed at the highest concentration of TP agonist in cells expressing TP\(_{\alpha}\) alone (Fig. 5). These results indicate that cAMP formation in response to TP activation by TP agonists or iPE2\(_{II}\) is dependent on the presence of both TP\(_{\alpha}\) and IP.

We examined the biological relevance of this relationship in a cell model that endogenously expresses both IP and TP. cAMP production in response to IBOP or iPE2\(_{II}\) was quantified in aortic smooth muscle cells isolated from humans or WT mice. Increased cAMP levels were observed in hASMC or WT mASMC following a 10 min treatment with IBOP or iPE2\(_{II}\) (Fig. 6). SQ 29548 was partially effective against IBOP and ineffective against iPE2\(_{II}\) (Fig. 6, E and F), in agreement with the HEK 293 cell data (Fig. 4B). IBOP or iPE2\(_{II}\) treatment of ASMC isolated from IPKO mice resulted in minimal cAMP generation (Fig. 6, C and D). Thus, the absence of the IP in ASMC cultured from IPKO mice or in HEK 293 cells (Fig. 5) uncoupled the TP from activation of AC.
Effect of IP Coactivation on TP-mediated cAMP Generation—
Activation of IP in IP/TP co-expressing cells, with a submaximal concentration of cicaprost (0.02 nM, 5 min), resulted in a synergistic enhancement of cAMP generation in response to the TP agonist U46619 and to iPE2III (Fig. 7). Furthermore, activation of IP synergistically enhanced iPE2III-induced cAMP generation in both hASMC and mASMC (Fig. 8, B and D). In contrast, whereas IP activation in IP/TP-HEK cells synergistically enhanced iPE2III-induced cAMP generation in response to 100–500 nM IBOP, this enhancement became additive at 1000 nM IBOP. IP activation was similarly additive with IBOP (1000 and 5000 nM) in both hASMC and mASMC. The absence of IP in mASMC (cultured from IPKO mice) ablated both synergistic and additive effects (Fig. 8, E and F). Thus, activation of the IP enhanced TP-mediated cAMP generation beyond that seen when IP was physically present but not activated.

TP-mediated cAMP Generation, Role of IP-derived cAMP—
We next sought to determine the relative necessity of the physical presence of IP versus the increased cAMP tone in IP-expressing cells. ASMC from IPKO mice were pretreated with 8-Br-cAMP (500 nM, 10 min), elevating cellular cAMP to

**IP Facilitates TPα-induced cAMP Generation**

Effect of SQ 29548 on TP-induced cAMP generation in ASMC. hASMC (A and B) or WT mASMC (open bar) and IPKO mASMC (closed bar) (C and D) were treated with IBOP (A and C) or iPE2III (B and D) for 10 min. E and F, hASMC were pretreated with (open bar) or without (closed bar) SQ 29548 (10 μM, 30 min) followed by IBOP (E) or iPE2III (F) for 10 min. cAMP was quantified as described under “Experimental Procedures.” Data are presented as the mean pmol of cAMP/well ± S.E. from 3–5 experiments, each performed in duplicate.

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![Image](image.png)

**Fig. 6. Effect of SQ 29548 on TP-induced cAMP generation in ASMC.**

hASMC (A and B) or WT mASMC (open bar) and IPKO mASMC (closed bar) (C and D) were treated with IBOP (A and C) or iPE2III (B and D) for 10 min. E and F, hASMC were pretreated with (open bar) or without (closed bar) SQ 29548 (10 μM, 30 min) followed by IBOP (E) or iPE2III (F) for 10 min. cAMP was quantified as described under “Experimental Procedures.” Data are presented as the mean pmol of cAMP/well ± S.E. from 3–5 experiments, each performed in duplicate.

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**Effect of IP Coactivation on TP-mediated cAMP Generation—**

Activation of IP in IP/TPα co-expressing cells, with a submaximal concentration of cicaprost (0.02 nM, 5 min), resulted in a synergistic enhancement of cAMP generation in response to the TP agonist U46619 and to iPE2III (Fig. 7). Furthermore, activation of IP synergistically enhanced iPE2III-induced cAMP generation in both hASMC and mASMC (Fig. 8, B and D). In contrast, whereas IP activation in IP/TPα-HEK cells synergistically enhanced cAMP formation in response to 100–500 nM IBOP, this enhancement became additive at 1000 nM IBOP. IP activation was similarly additive with IBOP (1000 and 5000 nM) in both hASMC and mASMC. The absence of IP in mASMC (cultured from IPKO mice) ablated both synergistic and additive effects (Fig. 8, E and F). Thus, activation of the IP enhanced TP-mediated cAMP generation beyond that seen when IP was physically present but not activated.

**TP-mediated cAMP Generation, Role of IP-derived cAMP—**

Interaction between IP and TP signaling pathways has been described previously; prostacyclin-induced desensitization of hTPα is mediated by PKA phosphorylation of the TPα C-terminal tail. To determine whether PKA is also involved in IP-dependent, TPα-induced cAMP generation, IP/TPα-HEK cells were pretreated with the PKA inhibitor H89 (10 μM, 30 min) prior to cotreatment with cicaprost followed by U46619, IBOP, or iPE2III, as described above. Inhibition of PKA resulted in a slight increase in the overall cellular cAMP levels but had no impact on the synergistic interaction resulting from IP/TPα co-activation (Fig. 9). The increase in basal cAMP levels in response to H89 is unsurprising, since PKA is capable of activating a number of cAMP phosphodiesterases (28). Similarly, pretreatment with H89 did not inhibit cAMP production in response to IBOP or iPE2III (1000 or 5000 nM, 10 min) or the potentiation of TPα-induced cAMP by cicaprost (0.5 nM, 5 min) in hASMC (Fig. 10). These data indicate that PKA does not play a role in IP-dependent, TPα-mediated cAMP generation.

**IP-dependent Modulation of TP Signaling, Role of IP-derived cAMP—**

We next sought to determine the relative necessity of the physical presence of IP versus the increased cAMP tone in IP-expressing cells. ASMC from IPKO mice were pretreated with 8-Br-cAMP (500 nM, 10 min), elevating cellular cAMP to
levels similar to those present in cicaprost-treated WT cells (Fig. 11). However, correction of the cAMP deficit in this manner did not restore cAMP generation in response to IBOP or iPE2III (1000 or 5000 nM, 10 min, Fig. 11). Similarly, when HEK 293 cells expressing TPα alone were treated with 8-Br-cAMP (100 nM, 10 min) to boost the intracellular cAMP to approximatively the same level as IP/TPα-HEK cells treated with cicaprost, this did not restore TP-mediated cAMP generation in response to U46619, IBOP, or iPE2III (Fig. 12). Thus, TP-mediated cAMP generation did not occur secondary to IP-induced cAMP formation was instead dependent on, and enhanced by, the physical presence and activation of the IP.

**Formation of IP/TPα Heterodimers**—TP-mediated cAMP generation was dependent on the physical presence of both TPα and IP. Many studies have demonstrated that G-protein-coupled receptor heterodimer formation, typically a constitutive process occurring when both partners are co-expressed, often results in alterations in the signaling of constituent receptors (20). Thus, we sought to determine whether coexpression of MychIP and HAHTPα resulted in heterodimer formation. MychIP was immunoprecipitated from IP/TPα-HEK cell lysates. Immunoblotting with anti-HA revealed the presence of HAHTPα in lysates derived from cells co-expressing HAHTPα and MychIP but not in lysates from cells expressing HAHTPα (Fig. 13). Similarly, immunoprecipitation of HAHTPα resulted in the co-immunoprecipitation of MychIP (Fig. 13). In either case, the co-immunoprecipitated partner appeared primarily in the monomeric rather than in the oligomeric form (Fig. 13). This is not unexpected, since reduced conditions were used to disrupt protein complexes that were stabilized, prior to immunoprecipitation, with a cross-linker (dithiobis(succinimidylpropionate)). Furthermore, these conditions disrupt disulfide linkages, important for the formation of many GPCR dimers (22), possibly contributing further to the lower molecular weight. The presence of MychIP in HAHTPα immunoprecipitates and *vice versa* indicated that coexpression of these receptors results in the formation of an IP/TPα heterodimer.

**Receptor-Ligand Interactions**—It has been demonstrated previously that receptor heterodimerization can alter the affinity of the individual receptors for their specific ligands (22, 29, 30). We examined if changes in ligand binding in the IP/TPα heterodimer could underlie the changes we observed in TPα signaling. Saturation binding using the TP-specific antagonist [3H]SQ 29548 (Fig. 14) revealed the presence of a single high affinity binding site in membranes from IP/TPα-HEK (K_d = 34.4 ± 6.7 nM; B_max = 4.8 ± 0.3 pmol/mg; n = 4). Co-expression of hIP did not alter significantly the affinity (K_d = 29.2 ± 6.1 nM, n = 5) but did reduce the number of [3H]SQ 29548 binding sites (B_max = 1.5 ± 0.1 pmol/mg; n = 5). High and low affinity binding sites for the IP agonist [3H]iloprost were observed in membranes derived from IP/TPα-HEK cells, similar to those reported previously for IP-HEK (24) (data not shown).

Similarly, coexpression of hIP and hTPα did not alter the affinity of TPα for the specific TP ligands SQ 29548, IBOP, and U46619 in displacement analysis (Table I). As expected, iPE2III (31) did not bind to the TPα, and this was unaltered when IP was co-expressed. Interestingly, despite its ability to induce robust cAMP signaling in IP/TPα coexpressors (Fig. 5), iPE2III bound very weakly to TPα, whether IP was co-expressed or not (Table I). The affinity of hIP for cicaprost was not altered by coexpression with TPα, and no binding of TP-specific ligands or isoprostanes to hIP was observed. Thus, coexpression of IP and TPα reduced the number of TP binding sites but did not result in obvious alterations in binding affinities for the individual receptor sites. In addition, iPE2III activity was dependent on the presence of both TPα and IP, although this ligand did not bind efficiently to either of the individual receptor sites. This is consistent with the generation of a modified binding site generated through the physical association of TPα with IP.

**Effect of SQ 29548 on TP-induced cAMP Formation**—When the TP site was blocked with SQ 29548, IP-induced TP-mediated cAMP generation in IP/TPα-HEK cells was partially reduced (Fig. 15). In contrast, iPE2III-induced cAMP generation, in IP-activated cells, was also prevented with SQ 29548, although this isoprostane did not displace efficiently [3H]SQ 29548 binding (Table I). Thus, although iPE2III ligated an altered binding site in the IP/TPα complex, subsequent signaling events were TP-mediated, in the HEK 293 cell model. In a similar fashion, IBOP-induced cAMP generation in cicaprost-stimulated hASMC was reduced by antagonism of the TP (Fig. 15). However, in contrast, the iPE2III response was unaltered in hASMC treated with SQ 29548. This inconsistency between the HEK 293 cell model and the hASMC, with regard to the activity of iPE2III, suggests a greater level of complexity in the native cell model.
DISCUSSION

PGI₂ and TxA₂ are important regulators of vascular homeostasis, and their respective levels dictate the response to vascular injury. Recently, IP was shown to limit specifically the deleterious effects of TP activation during the response to vascular injury (5). Given the importance of the PGI₂/TxA₂ balance for vascular function, their coincident biosynthesis in vascular disease (32, 33), and the frequent co-expression of IP and TP in vascular cells (16), we sought to examine their relationship at the molecular level. We concentrated our efforts on the hTP, since this isoform is expressed more ubiquitously and abundantly compared with hTP (34).

Studies have demonstrated that, whereas both TPα and TPβ are similarly coupled to Gq and activation of PLC, they oppositely regulate adenylate cyclase; TPα increases cAMP formation via Gi, whereas TPβ couples to Gs and inhibition of cAMP formation in CHO cells overexpressing the individual receptors (35). Similar to previous studies using TPα transfected HEK 293 cells (36), we observed an approximate 2-fold increase in cAMP upon treatment of TPα-HEK cells with the TP agonists U46619 or IBOP. Strikingly, when IP was co-expressed, the same treatments elicited a 15-fold (U46619) and 30-fold (IBOP) increase in cAMP generation (Fig. 4). No induction of cAMP generation was observed in HEK 293 cells expressing IP alone or in mixed cultures of singly expressing cells, following treatment with IBOP or U46619 under identical conditions, demonstrating that this effect was not mediated by nonspecific agonist interaction (Fig. 4). Thus, the physical presence of IP dramatically enhanced cAMP generation in response to TPα activation in HEK 293 cells. We addressed the biological relevance of this phenomenon using primary smooth muscle cells that express both IP and TPα endogenously.

Fig. 8. Effect of IP coactivation on TP-induced cAMP generation in ASMC. hASMC (A and B), WT mASMC (C and D), and IPKO mASMC (E and F) were treated with (open bar) or without (closed bar) cicaprost for 10 min and subsequently cotreated with IBOP (A, C, and E) or PEIII (B, D, and F) for 10 min. The theoretical additive effect of IP and TP activation was also calculated (gray bar; IP activation alone + TP activation alone = basal). cAMP was quantified as described under “Experimental Procedures.” Data are presented as the mean pmol of cAMP/well ± S.E. from 3–6 experiments, each performed in duplicate. *, p < 0.05 relative to theoretical additive. ***, p < 0.001 relative to theoretical additive.
cAMP formation via the TP requires the presence of the IP.

Intriguingly, this phenomenon extended to the isoprostane, iPE2III. Isoprostanes are free radical catalyzed products of arachidonic acid that are increased in syndromes of vascular disease and act via TP \((27)\). Isoprostane levels are elevated within developing atherosclerotic lesions in mice, and normalization of isoprostane levels correlates with disease regression \((37)\). Furthermore, antagonism of TP, but not inhibition of TxA2 synthesis with aspirin, reduced atherosclerosis in mice, suggesting that mediators other than TxA2, possibly isoprostanes, act at the TP to propagate the disease \((38)\). Thus, the isoprostanes are both a marker and a mediator of disease.

We used a well characterized TP antagonist, SQ 29548, to block the TP receptor. The activity of IBOP was partially inhibited by SQ 29548 in IP/TPα-HEK and hASMC (Figs. 4A and 6E). However, iPE2III activity was unaffected in both cell models. At first glance, this suggests that iPE2III is not acting through the TP. However, iPE2III did not signal in the absence of the TP in both our study (Fig. 5) and in mice \((27)\), demonstrating its TP-dependent action. Indeed, our data suggest that sensitivity to SQ 29548 does not reliably define TP dependence.

Recently, the importance of IP as a specific limit on the deleterious effects of TP following vascular injury was demonstrated \((5)\). Deletion of the IP exacerbated TP-dependent SMC proliferation following arterial injury in mice \((5)\). In this setting, TxA2 and isoprostane biosynthesis is increased as a result of platelet activation and increased oxidant stress. The ability of TP agonists and iPE2III to induce TP-dependent cAMP formation only when IP is physically present may represent a previously unappreciated mechanism by which IP can regulate TP activity. Thus, IP may co-opt TP to generate cAMP, increasing the cell’s PGI2-like response.

Having established a relationship between IP- and TP-induced cAMP formation, we next wanted to examine whether activation of IP might modify this effect. Stimulation of the IP receptor resulted in a synergistic potentiation of TP-mediated cAMP generation in IP/TPα-coexpressing HEK 293 cells treated with U46619 or iPE2III (Figs. 7A and 8B). Synergistic enhancement of iPE2III-induced cAMP generation, upon IP activation, was also observed in mASMC and hASMC, demonstrating that this effect was not an artifact of receptor overexpression (Fig. 8). In contrast, whereas low concentrations of IBOP were syn-

![Fig. 9](image_url)

**Fig. 9.** Effect of PKA inhibition on TPα-induced cAMP production in IP/TPα-HEK. IP/TPα-HEK were pretreated with (open symbols) or without (closed symbols) the PKA inhibitor H89 (10 μM, 30 min) followed by cicaprost (0.02 nM, 5 min) and subsequently cotreated with increasing concentrations of U46619 (A), iPE2III (B), or IBOP (C) for 10 min. cAMP was quantified as described under “Experimental Procedures.” Data are presented as the mean pmol of cAMP per well ± S.E. from three or four experiments, each performed in duplicate.

![Fig. 10](image_url)

**Fig. 10.** Effect of PKA inhibition on TPα-induced cAMP production in hASMC. hASMC were pretreated with (open bars) or without (closed bars) H89 (10 μM, 30 min) prior to treatment with or without cicaprost (0.5 nM, 10 min) followed by cotreatment with IBOP (A) or iPE2III (B) (1000 or 5000 nM, 10 min). cAMP was quantified as described under “Experimental Procedures.” Data are presented as the mean pmol of cAMP per well ± S.E. from three or four experiments, each performed in duplicate.
ergistically potentiated by IP activation, the interaction became additive at 1000 nM IBOP (Fig. 7). Similarly, IP activation was additive with IBOP-induced cAMP formation in both mASMC and hASMC (Fig. 8). A previous study in DAMI and CHRF cell lines, representing middle and late stage megakaryocyte maturation, also observed a synergistic induction of cAMP formation following coactivation of IP and TP (39). Thus, the net generation of cAMP in response to TP activation was maximized when IP was present and activated.

Receptor phosphorylation is an important mechanism via which the signaling of one receptor can modify that of another. Indeed, TPα/H9251 desensitization, in response to IP activation, occurs via PKA-mediated phosphorylation of serine 329 on the C-terminal tail of TPα (17). In addition, GPCR phosphorylation by PKA is capable of altering the affinity of receptors for their respective G-protein (40). PKA-mediated phosphorylation of the βγ-adrenergic receptor decreases the affinity of the receptor for Gγ while increasing its affinity for Gβ (40). We hypothesized that in the presence of IP, PKA activity and the subsequent phosphorylation of TPα might result in increased affinity of TPα for Gγ. However, H89, a selective inhibitor of PKA, did not alter the synergistic interaction between IP and TP in IP/TPα-HEK cells (Fig. 9) or in hASMC (Fig. 10). Furthermore, receptor independent activation of PKA by 8-Br-cAMP did not induce the TP-cAMP response. Thus, unlike IP-mediated TPα desensitization, IP-mediated potentiation of TPα-cAMP formation is independent of PKA phosphorylation.

We next examined whether increased cellular cAMP, which occurred as a consequence of IP expression and activation, facilitated IP-dependent, TP-mediated cAMP generation. Cellular cAMP levels in TPα-HEK cells, in which TP did not couple efficiently to adenyly cyclase (Fig. 5), were elevated using 8-Br-cAMP to approximately the same level as those present in cicaprost-treated IP/TPα-HEK cells. However, TP-induced cAMP generation was not reconstituted (Fig. 12). Similarly, ASMC isolated from IPKO mice were treated with 8-Br-cAMP, elevating cellular cAMP to the levels found in cicaprost-treated WT ASMC. Despite the correction of the cAMP deficiency in IPKO mASMC, IBOP- or iPE2III-induced cAMP formation was not restored (Fig. 11). It appears, therefore, that although activation of IP enhanced TP-induced cAMP, this was independent of signal transduction events resulting directly from IP activation. It may be argued that 8-Br-cAMP, a cell-permeable analogue of cAMP, does not perfectly reproduce the level of complexity, with respect to spatial and temporal distribution, inherent in IP-expressing cells. However, 8-Br-cAMP has been shown to activate the same signal transduction pathways as...
IP/TP heterodimers by co-immunoprecipitation. IP/TPα-HEK or TPα-HEK cell lysates were subjected to co-immunoprecipitation using an anti-Myc or anti-HA antibody as described under "Experimental Procedures." Immunoprecipitates were resolved by 10% reducing SDS-PAGE and co-immunoprecipitated HAhTPα or MychIP was detected using an anti-HA or an anti-Myc antibody, respectively. Co-immunoprecipitation was only observed when MychIP and HAhTPα were coexpressed. Molecular masses are in kDa. The arrows indicate co-immunoprecipitated monomer and oligomers. Western blots are representative of three independent experiments.

We were struck by the fact that the physical presence of IP, in overexpressing and native cell models, strengthens the argument that a novel IP-dependent event might heterodimerize with consequent alterations in TPα signaling, the dependence of TPα-mediated cAMP formation on the physical presence of IP, in overexpressing and native cell models, strongly suggests biologically relevant heterodimerization between these two receptors. In addition, data indicating the formation of an alternative ligand binding site (see below), a frequent consequence of GPCR dimerization (22, 29, 30), is consistent with the formation of an IP/TPα complex, as is the synergistic augmentation of TP-cAMP generation following preactivation of IP (22).

The mechanism through which IP/TPα heterodimers couple to cAMP formation is unclear. Dimerization may alter the conformation of TPα, thereby increasing its affinity for Gs. Indeed, formation of a heterodimer between the angiotensin AT1 receptor and the bradykinin receptor results in increased activation of AT1-associated G-proteins (23). Alternatively, TPα may "borrow" the signal transduction machinery of its dimeric partner IP, thereby allowing it to more efficiently signal via Gs. This is not without precedent; an elegant study by Rocheville et al. (44) demonstrated that heterodimerization of the dopamine D1 receptor, with a C-terminal mutant of the somatostatin sst5 receptor, which was unable to couple to AC, restored the ability of the mutant sst5 receptors to activate the cyclase.

It is possible that the formation of an IP/TPα heterodimer generates a new receptor with signaling and binding characteristics distinct from the individual partners. Indeed, a number of studies investigating the signaling properties of newly discovered GPCR heterodimers describe an altered receptor-ligand-effector profile (22, 29, 30). Using SQ 29548 to label the TPα and iloprost to label the IP, we found no alterations in the binding affinities of specific IP or TP ligands to their individual receptor sites, suggesting preservation of the binding characteristics (Table I). It is interesting that expression of TP increased the number of TP binding sites (Fig. 13), suggesting that, similar to other GPCRs (20, 45, 46), the interaction of these receptors may impact on membrane expression and receptor trafficking, considerations that are now under investigation.

Despite the apparent status quo for ligand binding, concentration...
**Effect of IP/TPα coexpression on ligand binding to IP and TPα**

|          | TPα-HEK | IP/TPα-HEK |
|----------|---------|------------|
| SQ 29548 | 2.5 ± 0.2 × 10⁻⁷ | 2.6 ± 0.6 × 10⁻⁷ |
| U46619   | 8.7 ± 2.9 × 10⁻⁷ | 1.2 ± 0.4 × 10⁻⁶ |
| BOP      | 2.9 ± 0.8 × 10⁻⁶ | 2.9 ± 1.2 × 10⁻⁶ |
| iPE₂III  | > 5 × 10⁻⁶    | > 5 × 10⁻⁶    |
| iP₂₆III  | ND       | ND          |
| Cicaprost| ND       | ND          |

**Effect of IP/TPα coexpression on ligand binding to IP and TPα**

Data were presented as the mean pmol of cAMP/well for 10 min. cAMP was quantified as described under “Experimental Procedures.” The ability of the TP agonists SQ 29548, U46619, and BOP; the isoprostanes iPE₂III and iP₂₆III; and the IP agonist cicaprost to bind to IP and TPα was examined. Data were presented as mean IC₅₀ values ± S.E. from three or four experiments each performed in duplicate. ND, not detectable.

In summary, our findings reveal a previously unknown level of interaction between IP and TPα. The presence of IP facilitates TPα-mediated generation of cAMP in a manner independent of IP-induced cAMP formation, and subsequent PKA activation, but coincident with the formation of an IP/TPα heterodimer. Thus, a novel mechanism for the specific limit IP imposes on the deleterious effects of TPα emerges, namely that IP promotes a “PGI₂-like” cellular response to TxA₂ and isoprostanes. Indeed, a recent study in apolipoprotein E knockout mice demonstrated that COX-2 inhibition had no effect on atherosclerotic lesion formation despite selective reduction in PGI₂ generation (52), whereas IPKO mice exhibit increased lesion formation when compared with their wild type counterparts (12). Thus, in agreement with the present study, it appears that the physical presence of IP plays a distinct role in limiting the injurious actions of TxA₂ in vivo. Biosynthesis of PGI₂, TxA₂, and isoprostanes are increased during vascular disease. Our study demonstrates that the association of IP with TPα would maximize the “PGI₂-like” response to TP activation under these conditions, thus limiting the deleterious effects of TxA₂ and coincident TP ligands.

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