Heterodimerization With the Prostacyclin Receptor Triggers Thromboxane Receptor Relocation to Lipid Rafts

Salam Ibrahim, Ann McCartney, Nune Markosyan, Emer M. Smyth

Objective—Prostacyclin and thromboxane mediate opposing cardiovascular actions through receptors termed IP and TP, respectively. When dimerized with IP, the TP shifts to IP-like function. TP localizes to cholesterol-enriched membrane rafts, but TP and IPTP heterodimer localization is not defined. We examined these receptors’ membrane localization and the role of rafts in receptor function.

Methods and Results—Microdomain distribution of IP, TP, and IPTP heterodimers was examined in COS-7 cells by measuring energy transfer from renilla luciferase-fused receptors to fluorescently labeled rafts. IP raft association was confirmed. TP was raft excluded, but redistributed to rafts upon dimerization with IP. Signaling of the IP and IPTP heterodimer, but not TP alone, was suppressed after raft disruption by cholesterol depletion. Cholesterol enrichment also selectively suppressed IP and IPTP function. Native IP and IPTP signaling in smooth muscle cells and macrophages were similarly sensitive to cholesterol manipulation, whereas macrophages from hypercholesterolemic mice displayed suppressed IP and IPTP function.

Conclusion—IP and TP function within distinct microdomains. Raft incorporation of TP in the IPTP heterodimer likely facilitates its signaling shift. We speculate that changes in IP and IPTP signaling after perturbation of membrane cholesterol may contribute to cardiovascular disease associated with hypercholesterolemia. (Arterioscler Thromb Vasc Biol. 2013;33:60-66.)

Key Words: dimerization ■ G-protein–coupled receptor ■ lipid raft ■ prostacyclin ■ thromboxane

Prostacyclin (PGI₂) and thromboxane (TXA₂) are opposing vasoactive mediators generated by the cyclooxygenase (COX) pathway of arachidonic acid metabolism.¹ PGI₂ has established antithrombotic, atheroprotective, and antiproliferative actions in vivo.²-⁵ Conversely, TXA₂ activates platelets and promotes cell proliferation, migration, and adhesion, consistent with its established role in promoting cardiovascular disease (CVD).⁶ The contribution of TXA₂ to CVD is consistent with its established role in promoting cardiovascular disease (CVD).¹ The contribution of TXA₂ to CVD is underscored by the established efficacy of low-dose aspirin, which inhibits platelet TXA₂ biosynthesis, in secondary prevention of stroke and heart attack.⁷

The biological effects of PGI₂ and TXA₂ are transduced through distinct cell surface G protein–coupled receptors (GPCRs) termed the IP and TP, respectively. The IP is coupled to the Gα-adenyl cyclase pathway. In humans, TP exists as 2 splice variants, TPα and TPβ. The former is the dominant isoform expressed in most tissues⁸ and in mature platelets.⁹ Multiple G protein pathways lie downstream of the TP with activation of the Gα-phospholipase C and Gβγ–Rho pathways most relevant to the biological actions of TXA₂.⁶ We reported physical interaction of the IP and TP to form a heterodimer with a consequent shift in TP function—when dimerized to the IP, the TP signals and traffics via IP pathways resulting in an IP-like cAMP response to TP agonists, coincident with suppressed TP–Gq signaling.¹⁰ Importantly, these phenomena were evident in vascular smooth muscle cells, which naturally express both receptors, providing a mechanism through which IP can directly limit TP function and protect against CVD.

Plasma membranes are complex, self-organizing structures that dynamically control cell signaling and trafficking. Phase transitions at certain cholesterol thresholds give rise to liquid ordered (L₀) and liquid disordered (Lα) domains.¹¹ Lipid rafts, in the Lα domain, are dynamic nanoscale sterol–sphingolipid–enriched domains that can coalesce through protein–protein and protein–lipid interactions to form larger more stable platforms.¹² Multiple membrane proteins, including GPCRs and their downstream signaling molecules, can localize to rafts effectively compartmentalizing signaling events.¹³,¹⁴ Modulation of microdomains can impact signaling of both raft-associated and raft-excluded proteins as a result of changes in accessibility and proximity of individual signaling components.¹⁵,¹⁶ Raft signaling is linked with diverse pathologies, including CVD, focusing interest on factors that modify membrane microdomain function.¹¹,¹²,¹³,¹⁶ Regulation of membrane cholesterol appears critical to control of rafts and associated proteins,¹⁸ with increasing evidence of a direct effect on signaling and cell function. Depletion of membrane cholesterol is frequently used experimentally to disrupt lipid rafts,¹⁶,¹⁷,¹⁸ however physiological and pathophysiological cholesterol depletion events are common. Indeed, rafts are...
a dominant site for cholesterol exchange between cells and lipoproteins, regardless of the lipoprotein class.\textsuperscript{21} Oxidized low-density lipoproteins, a major cholesterol carrier with established links to atherogenesis, deplete endothelial cells of membrane cholesterol,\textsuperscript{22} whereas high-density lipoproteins, which promote reverse cholesterol transport and are atheroprotective, reduce raft cholesterol in monocytes.\textsuperscript{23} Elevating cholesterol either in vivo or in vitro increases platelet reactivity,\textsuperscript{24,25} while increased raft formation in hypercholesterolemic mice lead to myeloproliferation and leukocytosis.\textsuperscript{26} Thus, precise control of raft cholesterol content is a critical component of cellular signaling in normal and disease settings. The IP localizes to rafts,\textsuperscript{27} but membrane localization of the TP or the IPT heterodimer has not been examined. In this study, we explored the role of lipid rafts and cholesterol in IP and TP biology to determine how membrane microdomain homeostasis contributes to PGI2–TXA3 interplay. We confirmed localization and function of the IP within lipid rafts and determined that the TP is predominantly raft excluded. Interestingly, the membrane microdomain localization and function of TP was dramatically altered when the IP and TP dimerized. Our studies provide novel evidence that tight control of membrane cholesterol is essential for the cardiovascular protective signaling of the IP, and the restraint it places on TP function.

Materials and Methods
Detailed methods are provided in the online-only Data Supplement Materials. Receptors were hemagglutinin tagged and fused to either renilla luciferase (rLuc) or yellow fluorescent protein (YFP), as described.\textsuperscript{10} COS-7 cell transfection was with Fugene-6, as described;\textsuperscript{10} experiments were performed 48 hours later. Receptor dimerization was quantified as bioluminescence resonance energy transfer (BRET) from donor (rLuc-receptor) to acceptor (YFP-receptor), as described.\textsuperscript{18} Microdomain localization was defined by energy transfer from rLuc-fused receptor to 1,1'-Dihexadecyl-3,3,3',3'-tetramethylindocarbocyanine iodide (DiIC16), a fluorescent carbocyanine that labels the L\(_0\) membrane phase.\textsuperscript{10} Second messenger levels were measured using LANCE cAMP and IP-One HTRF assay systems.

Results

Membrane Domain Localization
IP localization to lipid rafts has been reported in transfected and native cells,\textsuperscript{27} consistent with its extensive lipidation.\textsuperscript{29} The absence of lipid modification on the TP\textsubscript{α} predicts L\(_0\) distribution. As these 2 receptors heterodimerize,\textsuperscript{10,28} we first examined their individual microdomain distribution.

Fractionation
Cells transfected with either TP\textsubscript{α} or IP were fractionated under detergent-free conditions to separate light (caveolin containing) and heavy (clathrin containing) fractions. Both IP and the TP\textsubscript{α} were found in the light, caveolin-containing fractions (Figure I in the online-only Data Supplement). Such cosegregation with caveolin is often taken as evidence for raft association.\textsuperscript{14,15} However, determination of raft versus nonraft by cell fractionation is fraught with technical difficulties and highly dependent on conditions used, often leading to misleading readouts.\textsuperscript{31} Indeed, although it is actually raft-excluded in COS-7 cells, the \(\beta_2\)-adrenoceptor (AR) partitions to caveolin-containing fractions in detergent-free preparations.\textsuperscript{16} We moved, therefore, to a more direct measure of receptor microdomain localization, referencing \(\beta_2\)-AR as raft-excluded control.

Membrane Labeling With DiIC16
Cells expressing rLuc-fused IP or TP\textsubscript{α} were loaded with DiIC16, to label the cholesterol-rich L\(_0\) membrane phase in which rafts are found. BRET from rLuc to DiIC16 gives a measure of receptor localization to the L\(_0\) phase.\textsuperscript{16} Distinct DiIC16 energy-transfer curves were seen with IP\textsubscript{rLuc} and TP\textsubscript{Luc} (Figure 1)—IP\textsubscript{rLuc}→DiIC16 energy transfer was readily saturable indicating the receiver’s L\(_0\) association, whereas the shallow and more linear readout for TP\textsubscript{Luc} indicated L\(_0\) exclusion. The \(\beta_2\)-AR\textsubscript{rLuc}→DiIC16 curve was also shallow and approached linearity consistent with its reported L\(_0\) exclusion in this model.\textsuperscript{16} These data indicate that the TP\textsubscript{α} is excluded from the L\(_0\) phase and that IP and TP\textsubscript{α} localize to distinct membrane microdomains, when expressed separately. We reasoned that to heterodimerize, IP and TP\textsubscript{α} must organize to coexist in the same microdomain. IP\textsubscript{rLuc}→DiIC16 energy transfer was not modified by expression of untagged TP\textsubscript{α}. However, untagged IP shifted TP\textsubscript{Luc}→DiIC16 energy transfer to a saturable curve, indistinguishable from the IP\textsubscript{rLuc} alone (Figure 1). Thus, upon heterodimerization, the IP dominated the TP\textsubscript{α} causing its redistribution to L\(_0\) microdomains.

Membrane Cholesterol Depletion

IP and TP Signaling
Acute cholesterol depletion is commonly used to implicate lipid rafts in protein function.\textsuperscript{16,19,20} We explored the contribution of L\(_0\) domains to IP, TP\textsubscript{α}, or IPT\textsubscript{α} function compared with the L\(_0\)-excluded \(\beta_2\)-AR. Generation of cAMP was used as a readout of IP or \(\beta_2\)-AR function, whereas inositol phosphate (InsP) generation was a measure of TP\textsubscript{α} function. TP\textsubscript{α} switches from InsP to cAMP generation when dimerized with the IP\textsuperscript{10}, therefore TP agonist-induced cAMP was
αTP function was not offset consistent with Lo exclusion. We
unaltered by cholesterol depletion (Figure 2C). Thus, in trans-
raft integrity, further supporting their Lo association, whereas
fected COS-7 cells, IP and IPTP coupling to cAMP generation
was elevated (Figure 2D) reportedly after increased Gs
availability upon raft disruption,16 and TP–InsP, which was
unaltered by cholesterol depletion (Figure 2C). Thus, in trans-
fected COS-7 cells, IP and IPTP coupling was dependent on
raft integrity, further supporting their Lα association, whereas
TPα function was not offset consistent with Lα exclusion. We
confirmed that this control mechanism was operational in
cells that natively express these receptors. We used primary
human aortic smooth muscle cells (hAoSMC) and a macro-
phage cell line, RAW 264.7. In both cell types, and similar to
the COS-7 cells, IP and IPTP coupling to cAMP generation
was suppressed by cholesterol depletion (Figure 2E, 2F, 2I,
and 2J), whereas TP–InsP (in hAoSMCs; Figure 2G) and β2-
AR–cAMP signaling (RAW 264.7) were resistant to cholesterol
loading (Figure 4C and 4D). Thus, cholesterol enrichment selectively
impacted the function of raft-associated GPCRs in the COS-7
model. We confirmed the effect of cholesterol enrichment
in hAoSMC and RAW 264.7 macrophages. Results are
percentage of control±SEM from n=3. * P<0.05; ** P<0.005; and ***
P<0.0005 compared with control.

Effect of Cholesterol Enrichment

IP and TP Dimerization

Studies report that GPCR dimerization is a prerequisite for
normal membrane expression and function.34–36 Given our evidence
for differential membrane microdomain localization of IP, TPα, and IPTPα heterodimers, we considered whether raft
disruption would modify differentially their physical associa-
tion. Dimerization was assessed, as we described previ-
ously,26 by measuring BRET from an rLuc-fused receptor to
an acceptor YFP-fused receptor. Cholesterol depletion did not
alter substantially IP homodimerization, TPα homodimeriza-
tion, or IPTPα heterodimerization (Figure 3), indicating that
Lα-included and -excluded dimers remain associated despite
cholesterol depletion. Thus, it appears that homodimerization
and heterodimerization of the IP and TPα is independent of
their microdomain localization.

IP and TP Signaling

Modified raft function and signaling has been reported in
cells that are enriched in vitro or in vivo with cholesterol.37–39
We explored the effect of cholesterol enrichment on IP, TPα,
and IPTPα function. Cholesterol loading suppressed cAMP
 generation in IP- and IPTPα-transfected cells (Figure 4A
and 4B). In contrast, signaling of the Lα-excluded β2-AR
(cAMP) and TP (InsP) were resistant to cholesterol loading
(Figure 4C and 4D). Thus, cholesterol enrichment selectively
impacted the function of raft-associated GPCRs in the COS-7
model. We confirmed the effect of cholesterol enrichment
in hAoSMC and RAW 264.7, demonstrating suppressed
IP and IPTP coupling to cAMP generation (Figure 4E, 4F,
and 4J), with unaltered TP–InsP in hAoSMC (Figure 4G)
and unaltered β2-AR–cAMP signaling in both native
models (Figure 4H and 4K). As hypercholesterolemia is an
established risk factor in CVD,40,41 we considered whether in
vivo elevation of plasma cholesterol would similarly impact
selectively IP and IPTP function. Plasma cholesterol levels in
draw-dense lipoprotein–receptor–deficient (LDLR−/−) mice on
normal chow are 3 to 4 times higher than similarly fed wild-
type (WT) mice,32 coincident with elevated plasma membrane
cholesterol content in several cell types.43–45 We examined ex
vivo IP signaling in peritoneal macrophages isolated from WT
and LDLR<sup>−/−</sup> mice. Similar to in vitro cholesterol enrichment of transfected or native cells, function of both IP and IPTP was suppressed in macrophages from hypercholesterolemic LDLR<sup>−/−</sup> mice (Figure 5).

**IP and TP Dimerization**

Given that cholesterol enrichment selectively impacted the function of Lo-associated receptors, we examined whether their dimerization was also modified. Dimers remained associated after cholesterol enrichment, although, interestingly, a lower BRET<sub>max</sub> was seen for the 2 raft-associated species, IP<sub>I</sub>IP<sub>α</sub> and IPTP<sub>α</sub>, but not the raft-excluded TPαTPα (Figure 3). This differential effect provides further, albeit indirect, evidence that the IP<sub>I</sub>IP<sub>α</sub> and IPTP<sub>α</sub> reside in a distinct membrane microdomain compared with the TPαTPα.

**Receptor Surface Expression Is Unaltered by Cholesterol Enrichment**

Cholesterol enrichment can induce sequestration of raft-associated proteins, raising the possibility that reduced IP or IPTP signaling in transfected and native cells exposed to elevated cholesterol simply reflected loss of surface receptor. We examined cell surface expression, by flow cytometry, in the transfected COS-7 model and found no change in IP or TP<sub>α</sub>, expressed alone or in combination, in control and cholesterol-loaded conditions (Figure IIIA in the online-only Data Supplement). Similarly,

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**Figure 3.** IP and TP dimerization in living cells. Receptor dimerization was examined by measuring bioluminescence resonance energy transfer (BRET) from (A) IP-renilla luciferase (rLuc) to IP-yellow fluorescent protein (YFP), (B) TP<sub>α</sub>Luc to IPYFP, or (C) TP<sub>α</sub>Luc to TPYFP in transfected COS-7 subjected to cholesterol depletion (grey line), loading (solid black line), or no manipulation (dashed line). Data are the percentage of maximum in control cells vs the ratio of YFP-receptor to rLuc-receptor and are mean±SEM of n=3. *P=0.05 compared with control.

**Figure 4.** Effect of cholesterol enrichment on receptor signaling. COS-7 cells were transfected with (A) IP alone, (B) IP+TP, (C) TP alone, or (D) β<sub>2</sub>-adrenoreceptor (AR) and, after 48 hours, subjected to no treatment (control) or cholesterol loading (cholesterol-methyl-β-cyclodextrin complex 80 μg/mL, 1 hour). IP and IPTP<sub>α</sub> signaling were determined as cAMP generation in response to cicaprost (IP agonist) or U46619 (TP agonist), respectively; TP signaling was determined as U46619-induced inositol phosphate (InsP) generation, and β<sub>2</sub>-AR signaling as metaproterenol (β<sub>2</sub>-AR). Native receptor signaling is shown in (E, F, G and H), human aortic smooth muscle cells (hAoSMC), and (I, J and K) RAW 246.7, macrophages. Results are percentage of control±SEM from n=3. * P<0.05; and ** P<0.005 compared with control.
native IP surface expression was not different in peritoneal macrophages from hypercholesterolemic mice (either 8 months on a high [42%]-fat diet, Figure IIIB in the online-only Data Supplement, or 6-month-old LDLR−/− mice on normal chow, Figure IIIC in the online-only Data Supplement) compared with normal controls. Thus, suppression of receptor signaling after exposure to high cholesterol, either in vitro or in vivo, does not appear related to loss of cell surface receptor expression.

**Discussion**

We reported previously a shift of TP signaling from Gq/InsP to a Gq/cAMP IP-like signaling that occurs when IP and TP heterodimerize.10 The molecular pathways that control the formation and function of IP and TP homodimers or heterodimers in coexpressing cells and the relationship to CVD remain, however, poorly understood. Clustering of GPCRs and their downstream signaling proteins in specialized plasma membrane microdomains has emerged as a key feature of cell- and context-specific receptor function.13,14 Little is known about how membrane microdomains influence the formation and function of GPCR dimers. This is particularly interesting when considering heterodimers of receptors like the IP and TP that are distinct in their signaling, cell trafficking, and regulation, when examined individually.1 In this study, we examined the relative localization of IP and TP to Lα membrane phase, and the relationship between these receptors’ respective localization, and their dimerization and function.

Lipidation of proteins promotes their association with lipid rafts.10 The IP, which is both palmitoylated and isoprenylated,29 is raft localized in this and other studies.37 TPα, which is not lipidated,50 was predominantly Lα excluded. Interestingly, on IP coexpression, redistribution of TPα to the Lα phase was evident. IP localization was, however, unaltered by coexpression of the TPα, consistent with a dominant effect the IP on TPα membrane microdomain localization. We sought to confirm these findings using a standard methodology of membrane fractionation under detergent-free conditions. However, by this methodology, both IP and TPα partitioned with caveolin-rich fractions (a standard raft marker), with no evidence of their differential localization. Significant concerns have been raised about the reliability of cell fractionation for determination of membrane microdomain localization.33,50 Of particular relevance to our work, detergent-free cell fractionation lacked the resolution to identify exclusion of the β2-AR from rafts, whereas energy transfer to DilC16, the fluorescent Lα label that we used, provide clear discrimination receptors that are Lα associated versus Lα excluded.16

Clustering of receptors and their downstream signaling proteins in raft domains appears critical for receptor function.13,14 We next explored whether raft integrity was differentially important for IP and TPα function and dimerization using cholesterol depletion of membrane cholesterol, an established method to disrupt rafts.16,19,20 As we reported previously, a TXA2 analog, U46619, induced a robust cAMP response in IPTPα-transfected cells that was similar to the PGI2 analog, cicaprost.10 The response to either agonist was suppressed after cholesterol depletion of transfected COS-7 cells, as well as in hAoSMC and RAW 246.7 cells, that natively coexpress the IP and TP. Thus, raft integrity appeared essential to transduction of a Gq-cAMP signal via the IP or the IPTP heterodimer across transfected and native cell models. It may be that cholesterol depletion impacted Gq or adenylyl cyclase function, rather than IP or IPTP function per se. However, the increase in cAMP generation through the raft-excluded β2 AR that we, and others,16 observe argues against a general effect of cholesterol depletion on the Gq-cAMP pathway. In contrast to the IPTP heterodimer, discrete TP signaling to Gq was not affected by cholesterol depletion of TP-transfected COS-7 or hAoSMC. Our data indicate, therefore, that raft disruption by cholesterol depletion can differentially modify signaling of Lα-excluded versus Lα-associated receptors, with the former being unaltered or enhanced and the latter being suppressed. Such differential effects of cholesterol depletion consistent with distinct microdomain localization has also been reported for the μ-opioid receptor (both raft and nonraft localized) and δ-opioid receptor (raft excluded).15 Gq is typically localized in rafts, and its release from rafts after their disruption16 likely leads to loss of function for raft-associated Gq-coupled receptors, such as the IP and IPTPα heterodimer. Gq, in contrast, is reported to function in both raft and nonraft domains,33 consistent with the insensitivity of the TPα-Gq-InsP signal to cholesterol depletion.

To our knowledge, no study has examined the role of rafts or other membrane microdomains in GPCRs dimerization. X-ray crystallographic studies indicate, however, that cholesterol may help to form or stabilize GPCR dimers and that rafts may be critical to these assemblies.52 In our model, acute cholesterol depletion did not markedly alter BRET for IP homodimerization, TP homodimerization, or IPTPα heterodimerization. This is consistent with a leading model of GPCR dimerization—that dimers are brought to the plasma membrane as a preformed complex assembled in the endoplasmic reticulum.34–38 Indeed, our previous work supports formation of IP- and TP-containing dimers in the endoplasmic reticulum.
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Receptor Dimerization Modifies Raft Localization

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...and is consistent with the current findings that acute disruption of membrane rafts does not modify the dimerization process itself, but rather the subsequent function of the receptor complex. Interestingly, when cells were instead subjected to cholesterol enrichment, the BRET$_{\text{max}}$ for IIPI, and IPTP, but not TPTT, was blunted. This may reflect reduced association of the IP and TP to form the heterodimeric species. However, this is unlikely given that the BRET$_{\text{eq}}$, which reflects affinity, was not altered. It is instead likely that the process of dimer assembly in the endoplasmic reticulum proceeds normally in cholesterol-loaded cells, but that elevated cholesterol in the L$_2$ phase suppresses energy transfer between the partners once they reach raft sites. The functional impact of cholesterol loading was clearly evident—signaling via both the IP or IPTP, but not TP or $\beta_2$-AR, was reduced after cholesterol enrichment, consistent with reports that cholesterol loading can modify raft function.

Plasma cholesterol levels are directly related to cell membrane cholesterol content. Elevated plasma cholesterol, or cholesterol loading of cells in vitro, is linked with increased leukocyte adhesion and inflammation, as well as augmented platelet reactivity and thrombosis. Processes that are restrained by the PGI$_2$–IP–cAMP pathway. We considered that cholesterol loading of cells in culture may mimic increased plasma cholesterol levels associated with a high-fat diet and genetic abnormalities. The significant impairment of both the IP and IPTP cAMP signal seen in macrophages from hypercholesterolemic mice supports the notion that elevated cholesterol can modify cell surface receptor function in vivo, and supports the relevance of our in vitro studies to CVD associated with high cholesterol. The mechanism(s) through which cholesterol enrichment modifies cell and receptor function remain ill defined. Cell surface levels of IP and TP were unchanged by cholesterol loading, whether they were expressed alone or in combination, thus it is likely that cholesterol loading functionally modified the IP- and IPTP-receptor complexes. Enrichment of platelet membranes with cholesterol was reported to decreased membrane fluidity, and increased rigidity of cholesterol-rich membranes can modulate raft-associated signaling. It may be that the fluid dynamics of the microdomains become less favorable to transduction of the signal from receptor to G-protein and effector. This possibility is currently under investigation.

The IP is well established to reduce disease severity in a range of CVD models. Further, IP limits the prothrombotic and proliferative actions of the TP in vivo. We determined that formation of an IPTP heterodimer contributes to this IP-mediated restraint of TP function. The physiological and pathophysiological importance of GPCR dimerization is emerging. We reported a dominant negative influence of a naturally occurring IP mutant (IP$_{622C}$) on its wild-type counterpart, through dimerization, as a mechanistic basis for the exaggerated loss of platelet responsiveness to PG$_I_2$, analogs in platelets from individuals heterozygous for the mutant. These, and other studies, indicate that understanding GPCR homodimerization and heterodimerization is critical to determining the function of receptor pathways in vivo, and to their successful therapeutic targeting in human disease.

In summary, our data show that IP localizes and functions in membrane rafts, whereas TP is raft excluded but redistributed to rafts when dimerized with the IP. IP function depends on raft integrity, as does the modified IP-like function of the TP in an IPTP heterodimer. Thus, lipid rafts may be a system used by the cell to partition the signaling and function of the IP and TP based on their homodimerization or heterodimerization. Further, we determined in transfected, primary, and ex vivo models that membrane cholesterol homeostasis is critical for normal receptor function least for the receptors that we examined. Coupling of the IP and IPTP to the GS–cAMP signaling cascade is important for the beneficial actions of PGI$_2$ in CVD, as well as the restraint placed by the IP on TP’s deleterious cardiovascular actions. Our studies indicate that elevated plasma cholesterol can significantly suppress this critical signaling pathway across a range of cell types. We speculate that these perturbations in signaling may contribute to altered cardiovascular function in hypercholesterolemic humans.

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Disclosures

None.

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Supplemental Material

Supplemental Methods

 Constructs and Cell Culture – Human IP or TPα, N-terminally tagged with a hemagglutinin tag (3xHA), were from Missouri S&T cDNA Resource Center (Rolla, MO). IP and TPα fused at their C-termini to renilla luciferase (rLUC) or yellow fluorescent protein (YFP) were described previously. COS-7, Raw 264.7, and human aortic smooth muscle cells (Lonza, Basel, Switzerland) were maintained as described. Transfections were with Fu gene 6 (Roche, Indianapolis, IN) as described. Where indicated, cells were cholesterol depleted (2-hydroxypropyl-β-cyclodextrin; 20mM, 1h), or loaded (cholesterol-methyl-β-cyclodextrin complex; 80 µg/ml, 1hr). Peritoneal macrophages were isolated from female mice as previously described.

 Subcellular fractionation – COS-7 cells were transfected with the IP or TPα tagged at the N-terminus with 3 copies of the hemagglutinin epitope tag (3xHA). Cells were harvested, 48hrs later, without the use of enzymes and homogenized in sodium carbonate (500 mM, pH 11) using a polytron tissue grinder (15s), followed by 3x20s sonication on ice. Homogenates were adjusted to 35% iodixanol using OptiprepTM, a 60% iodixanol solution (Sigma, St Louis, MO), placed in the bottom of Ultra-Clear centrifuge tubes (Beckman, Indianapolis, IN) and overlayered with 30% Optiprep and 0% (buffer alone). Samples were centrifuged at 200,000xg for 2h using an SW40Ti rotor (Beckman, Indianapolis, IN). 11x1ml fractions will be taken from the top of the gradient and subjected to NUPAGE electrophoretic separation.

 Bioluminescence Resonance Energy Transfer – Receptor dimerization was examined by measuring bioluminescence resonance energy transfer (BRET) from a donor (rLUC-fused) receptor to an acceptor (YFP-fused) receptor following addition of an rLUC substrate (coelenterazine H; 5μM; Molecular Probes, Grand Island, NY). Cells were transfected with a fixed amount of donor together with increasing amounts of acceptor. BRET measurements were performed, as we described previously, 48hrs later. Cells were harvested without the use of enzymes and redistributed into 96-well plates (black, clear bottom, 100,000 cells/well). Donor and acceptor emissions were gathered sequentially from each well, at 485nm and 555nm, respectively, using a luminescence multi-plate reader (VICTOR3, Perkin Elmer, Waltham, MA) at 37°C. BRET is calculated as the ratio of Em555 over Em485 nm corrected for cells expressing donor alone. Each experimental condition included 4-6 replicates.

 Membrane labeling for localization: membrane localization of IP and TPα was examined using a modified BRET assay. COS-7 cells were transfected with a fixed amount of rLUC-receptor. Cells were fluorescently labeled with increasing concentrations of a redorange fluorescent, lipophilic carbocyanine DiIC16 (Molecular Probes, Grand Island, NY) that labels the L0 membrane phase, at 37°C for 2 min. Cells were washed with PBS, treated with coelenterazine H as above and energy transfer to DiIC16 measured at 530nm.
Membrane cholesterol measurement: COS-7 cells were harvested without the use of enzymes and homogenized in 10 mM Tris buffer using a polytron tissue grinder (15s), followed by 3x20s sonication on ice. After discarding the post-nuclear fraction, the lysate was centrifuged at 50,000 rpm at 4°C for 1h using an Optima TLX ultracentrifuge (Beckman). Membrane cholesterol was measured by Cholesterol E enzymatic colorimetric assay (Wako Diagnostics).

cAMP Assay: cAMP production was measured using the LANCE cAMP assay (PerkinElmer, Waltham, MA) according to manufacturer instructions. Briefly, cells dispensed in 384-well white optiplate (12,000 cells/well) were treated with vehicle (control), the PGl_2 analog cicaprost, or TxA_2 analog U46619, for 1h at room temperature in the presence of 3-isobutyl-1-methylxanthine (IBMX) to inhibit cAMP degradation by phosphodiesterases. The LANCE signal was quantified by measuring time resolved fluorescence resonance energy transfer (TR-FRET) between cAMP-biotin-streptavidin associated Europium and Alexa Fluor 647 conjugated to an anti-cAMP antibody measured at 665 nm using an Envision 2103 Multilabel Reader (PerkinElmer, Waltham, MA).

Inositol Phosphate Measurement – Cellular inositol phosphate (InsP) generation was quantified by IP-One Tb assay (CisBio, Bedford, MA) according to manufacturer instructions. Cells dispensed in 384-well white optiplate (12,000 cells/well) were treated with vehicle (control) or TxA_2 analog U46619, for 1h at 37°C. The signal was quantified by measuring TR-FRET (665nm, as above) between d2 labeled InsP1 and Lumi4-terbium cryptate conjugated anti-InsP1 antibody.

Surface expression of receptors – COS-7 cells transfected with 3xHA-IP or 3xHA-TPα were labeled with Alexa Fluor 488-anti-HA antibody (Molecular Probes, Grand Island, NY). Native IP was labeled with rabbit anti-IP antibody (Millipore) and with Alexa Fluor 488-goat-anti-rabbit (Molecular Probes). Surface fluorescence was measured by FACSCalibur flow cytometer (BD Biosciences, Billerica, MA).

Animal Experiments – Peritoneal macrophages were isolated from female mice between 6 and 12 months of age as previously described. Briefly, 10ml ice-cold PBS were injected to the mouse peritoneal cavity, harvested few minutes later, and centrifuged (1000g, 4°C, 10min). Cells were resuspended and incubated in Dulbecco’s Modified Eagle’s Medium overnight at 37°C to allow cells to adhere.

Statistical Analysis: Data were analyzed using GraphPad Prism. Comparisons were made using a one-sample t-test or by ANOVA with suitable post-hoc multiple comparison testing.
Fig.I. IP and TP membrane distribution by fractionation. COS-7 cells were transfected with 3xHA-tagged IP (left panel) or 3xHA-tagged TP (right panel). 48 hours later cells were homogenized in detergent-free conditions and subjected to iodixanol gradient fractionation. Fractions (1ml) collected from the top (1, light) to bottom (11, heavy) of the gradient were probed by immunoblotting for the HA-tagged receptor, caveolin-1 or clathrin, commonly used raft and non-raft markers. Blots are representative of at least n=3
**Fig.II.** Membrane cholesterol. COS-7 cells were subjected to no treatment (control), cholesterol depletion (2-hydroxypropyl-β-cyclodextrin; 20mM, 1hr), or Cholesterol loading (cholesterol-methyl-β-cyclodextrin complex; 80 µg/ml, 1hr). Membrane cholesterol was measured and data expressed as % change of control ± sem from n=3. **p<0.005**
Fig. III. Receptor surface expression. (A) COS-7 cells were transfected with 3xHAIP alone, 3xHATP alone, or IP plus 3xHATP, then subjected to no treatment (control) or cholesterol loading (cholesterol-methyl-β-cyclodextrin complex; 80 µg/ml, 1hr). Cells were labelled with Alexa Fluor 488-anti-HA antibody. (B, C) Peritoneal macrophages were harvested from normo- or hyper-cholesterolemic mice. In (B) mice were fed normal chow or a high fat diet (42% fat) for 8 months; in (C) mice were wild type (WT) or low density lipoprotein receptor deficient (LDLR-/-; ~6 months of age). IP was labeled with anti-IP antibody and an Alexa Fluor 488-conjugated secondary antibody. Surface receptor expression measured in all cells by flow cytometry. Data are (A) % change of control mean fluorescence ± sem from n=4, or (B, C) mean fluorescence ± sem. In (B) n=3-5, in (C) n=6.

