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. IP 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 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 surface G protein–coupled receptors (GPCRs) termed the IP and TP, respectively. The IP is coupled to the G-adenyllyl cyclase pathway. In humans, TP exists as 2 splice variants, TPa and TPβ. The former is the dominant isoform expressed in most tissues and in mature platelets.⁸ Multiple G protein pathways lie downstream of the TP with activation of the Gα-phospholipase C and G12/13-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 (Lη) domains.¹¹ Lipid rafts, in the L₀ 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.¹¹,¹²,¹⁷,¹⁸ 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,¹⁶,¹⁹,²⁰ 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. Oxidized low-density lipoproteins, a major cholesterol carrier with established links to atherogenesis, deplete endothelial cells of membrane cholesterol, whereas high-density lipoproteins, which promote reverse cholesterol transport and are atheroprotective, reduce raft cholesterol in monocytes. Elevating cholesterol either in vivo or in vitro increases platelet reactivity, while increased raft formation in hypercholesterolemic mice lead to myeloproliferation and leukocytosis. Thus, precise control of raft cholesterol content is a critical component of cellular signaling in normal and disease settings. The IP localizes to rafts, 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 PGJ₂–TXA₂ 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. COS-7 cell transfection was with Fugene-6, as described. 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.

Microdomain localization was defined by energy transfer from rLuc-fused receptor to 1,1′-Dihexadecyl-3,3,3′,3′′-tetramethylinodocarbocyanine iodide (DiIC₁₆), a fluorescent carbocyanine that labels the L₀ membrane phase. 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, consistent with its extensive lipidation. The absence of lipid modification on the TPα predicts L₀ distribution. As these 2 receptors heterodimerize, we first examined their individual microdomain distribution.

Fractionation

Cells transfected with either TPα or IP were fractionated under detergent-free conditions to separate light (caveolin containing) and heavy (clathrin containing) fractions. Both IP and the TPα 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. 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. Indeed, although it is actually raft-excluded in COS-7 cells, the β₂-adrenoreceptor (AR) partitions to caveolin-containing fractions in detergent-free preparations. We moved, therefore, to a more direct measure of receptor microdomain localization, referencing β₂-AR as raft-excluded control.

Membrane Labeling With DiIC₁₆

Cells expressing rLuc-fused IP or TPα were loaded with DiIC₁₆, to label the cholesterol-rich L₀ membrane phase in which rafts are found. BRET from rLuc to DiIC₁₆ gives a measure of receptor localization to the L₀ phase. Distinct DiIC₁₆ energy-transfer curves were seen with IPrLuc and TPrLuc (Figure I)—IPrLuc→DiIC₁₆ energy transfer was readily saturable indicating the receptor’s L₀ association, whereas the shallower and more linear readout for TPrLuc indicated L₀ exclusion. The β₂-ARrLuc→DiIC₁₆ curve was also shallow and approached linearity consistent with its reported L₀ exclusion in this model. These data indicate that the TPα is excluded from the L₀ phase and that IP and TPα localize to distinct membrane microdomains, when expressed separately. We reasoned that to heterodimerize, IP and TPα must organize to coexist in the same microdomain. IPrLuc→DiIC₁₆ energy transfer was not modified by expression of untagged TPα. However, untagged IP shifted TPrLuc→DiIC₁₆ energy transfer to a saturable curve, indistinguishable from the IPrLuc alone (Figure I). Thus, upon heterodimerization, the IP dominated the TPα causing its redistribution to L₀ microdomains.

Membrane Cholesterol Depletion

IP and TP Signaling

Acute cholesterol depletion is commonly used to implicate lipid rafts in protein function. We explored the contribution of L₀ domains to IP, TPα, or IPTpα function compared with the L₀-excluded β₂-AR. Generation of cAMP was used as a readout of IP or β₂-AR function, whereas inositol phosphate (InsP) generation was a measure of TPα function. TPα switches from InsP to cAMP generation when dimerized with the IP, therefore TP agonist-induced cAMP was...
used to assess IPTPα function. Agonists were used at concentrations that maximally activate the receptor in each of the cell models. Cholesterol depletion substantially reduced IP (Figure 2A) and IPTPα (Figure 2B) signaling. This contrasts with the raft-excluded \( \beta_2 \)-AR, in which cAMP generation was elevated (Figure 2D) reportedly after increased Gs availability upon raft disruption,\(^{15}\) and TP-InsP, which was unaltered by cholesterol depletion (Figure 2C). Thus, in transfected COS-7 cells, IP and IPTPα coupling to cAMP generation was dependent on raft integrity, further supporting their Lo association, whereas TPα function was not offset consistent with Lo 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 macrophage 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 hAoSMC; Figure 2G) and \( \beta_2 \)-AR-cAMP signaling (RAW 264.7 and hAoSMCs; Figure 2H and 2K) was unaltered. A TP–InsP signal was not detected in control or cholesterol-depleted RAW 264.7 cells, suggesting that, in these cells, the entire population of TP may be heterodimerized with the IP and therefore coupled to Gs–cAMP.

**IP and TP Dimerization**

Studies report that GPCR dimerization is a prerequisite for normal membrane expression and function.\(^{14-16} \) Given our evidence for differential membrane microdomain localization of IP, TPα, and IPTPα heterodimers, we considered whether raft disruption would modify differentially their physical association. Dimerization was assessed, as we described previously,\(^{16} \) by measuring BRET from an rLuc-fused receptor to an acceptor YFP-fused receptor. Cholesterol depletion did not alter substantially IP homodimerization, TPα homodimerization, or IPTPα heterodimerization (Figure 3), indicating that \( L_\gamma \)-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.

**Effect of Cholesterol Enrichment**

**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 \( L_\gamma \)-excluded \( \beta_2 \)-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, 4I, and 4J), with unaltered TP–InsP in hAoSMC (Figure 4G) and unaltered \( \beta_2 \)-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 low-density 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 mice.
and LDLR⁻/⁻ 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⁻/⁻ mice (Figure 5).

**IP and TP Dimerization**

Given that cholesterol enrichment selectively impacted the function of L₄-associated receptors, we examined whether their dimerization was also modified. Dimers remained associated after cholesterol enrichment, although, interestingly, a lower BRETmax was seen for the 2 raft-associated species, IPIP and IPTPα, but not the raft-excluded TPαTPα (Figure 3). This differential effect provides further, albeit indirect, evidence that the IPIP and IPTPα reside in a distinct membrane micro-domain 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 TPα, expressed alone or in combination, in control and cholesterol-loaded conditions (Figure IIIA in the online-only Data Supplement). Similarly,
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 Gs/cAMP to a Gq/InsP 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 Lo, membrane phase, and the relationship between these receptors’ respective localization, and their dimerization and function.

Lipidation of proteins promotes their association with lipid rafts.29 The IP, which is both palmitoylated and isoprenylated,30 is raft localized in this and other studies.31,32 TPα, which is not lipidated,30,33 was predominantly Lo excluded. Interestingly, on IP coexpression, redistribution of TPα to the Lo 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 Lo label that we used, provide clear discrimination receptors that are Lo associated versus Lo 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 β2AR 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 Lo-excluded versus Lp-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,16 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.34–36 Indeed, our previous work supports formation of IP- and TP-containing dimers in the endoplasmic reticulum.

Figure 5. IP- and IPTP-mediated signaling in mouse peritoneal macrophages. Peritoneal macrophages isolated from wild type (WT) or hypercholesterolemic low-density lipoprotein-receptor-deficient (LDLR−/−) mice (6–8 months of age) were stimulated with (A) cicaprost or (B) U46619, and cAMP generation quantified. Results are fold over basal±SEM; n=6 to 7; *P<0.05 compared with WT.
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 IP\text{IP} and IP\text{TP}, but not TP\text{TP}, 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{\gamma} 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, phase depresses energy transfer between the partners once they reach raft sites. The functional impact of cholesterol loading was easily evident—signaling via both the IP or TP, but not TP or \beta_{2}-AR, was reduced after cholesterol enrichment, consistent with reports that cholesterol loading can modify raft function.\text{\textsuperscript{28-29}}

Plasma cholesterol levels are directly related to cell membrane cholesterol content.\text{\textsuperscript{43-45}} 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.\text{\textsuperscript{24,25,54}} processes that are restrained by the PGL\text{\textsubscript{1}}–IP\text{–cAMP} pathway.\text{\textsuperscript{53}} 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 surface 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 ITPT-receptor complexes. Enrichment of platelet membranes with cholesterol was reported to decreased membrane fluidity,\text{\textsuperscript{46,47}} and increased rigidity of cholesterol-rich membranes can modulate raft-associated signaling.\text{\textsuperscript{48,50}} 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.\text{\textsuperscript{55}} Further, IP limits the prothrombotic and proliferative actions of the TP in vivo.\text{\textsuperscript{35}} We determined that formation of an ITP heterodimer contributes to this IP-mediated restraint of TP function.\text{\textsuperscript{10,28}} The physiological and pathophysiological importance of GPCR dimerization is emerging.\text{\textsuperscript{27-29}} We reported a dominant negative influence of a naturally occurring IP mutant (IP\text{R212C}) on its wild-type counterpart, through dimerization, as a mechanistic basis for the exaggerated loss of platelet responsiveness to PGI\textsubscript{2} analogs in platelets from individuals heterozygous for the mutation.\text{\textsuperscript{28}} These, and other studies,\text{\textsuperscript{30-42}} 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\text{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 ITPT 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 ITPP to the G\textsubscript{s}–cAMP signaling cascade is important for the beneficial actions of PGI\textsubscript{2} in CVD, as well as the restraint placed by the IP on TP’s deleterious cardiovascular actions.\text{\textsuperscript{10,28,55}} 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\(^1\). COS-7, Raw 264.7 (American Type Tissue Culture Collection, Rockville, MD) and human aortic smooth muscle cells (Lonza, Basel, Switzerland) were maintained as described\(^2\). Transfections were with FuGene 6 (Roche, Indianapolis, IN) as described\(^2\). 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\(^3\).

*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 Optiprep\(^{TM}\), 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 (rLUCfused) 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\(^1\), 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 red-orange fluorescent, lipophilic carbocyanine DiIC16 (Molecular Probes, Grand Island, NY) that labels the Lo membrane phase\(^4\), 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 PGI2 analog cicaprost, or TxA2 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 TxA2 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 described3. 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.
Supplemental Figures

Fig. I

**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.

