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

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Abstract

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 following 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 was similarly sensitive to cholesterol manipulation while macrophages from hypercholesterolemic mice displayed suppressed IP and IPTP function.

Conclusions—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 following perturbation of membrane cholesterol may contribute to cardiovascular disease associated with hypercholesterolemia.

Keywords

G protein-coupled receptor; dimerization; lipid raft; thromboxane; prostacyclin

Introduction

Prostacyclin (PGI_2) and thromboxane (TXA_2) are opposing vasoactive mediators generated by the cyclooxygenase (COX) pathway of arachidonic acid metabolism[1]. PGI_2 has established anti-thrombotic, atheroprotective and anti-proliferative actions *in vivo*[2–5]. Conversely, TXA_2 activates platelets and promotes cell proliferation, migration and adhesion, consistent with its established role in promoting cardiovascular disease (CVD)[6]. The contribution of TXA_2 to CVD is underscored by the established efficacy of low dose aspirin, which inhibits platelet TXA_2 biosynthesis, in secondary prevention of stroke and/or heart attack[7].

The biological effects of PGI_2 and TXA_2 are transduced through distinct cell surface G protein-coupled receptors (GPCRs) termed the IP and TP, respectively. The IP is coupled to

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the G_s-adenylyl cyclase pathway. In humans TP exists as two splice variants, TPα and TPβ. The former TPα is the dominant isoform expressed in most tissues, including vascular smooth muscle cells[8] and is the only isoform found in mature platelets[9]. Multiple G protein pathways lie downstream of the TP although activation of the G_q-phospholipase C and $G_{12/13}$-Rho pathways appear most relevant to the biological actions of TXA_2[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-G_q 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 occur at certain cholesterol thresholds giving rise to liquid ordered (L_o) and liquid disordered (L_d) domains[11]. Lipid rafts, in the L_o 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 because 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[21]. Oxidized low density lipoproteins, a major cholesterol carrier with established links to atherogenesis, deplete endothelial cells of membrane cholesterol[22] while high density lipoproteins, which promote reverse cholesterol transport and are atheroprotective, reduce raft cholesterol in monocytes[23]. Elevating cholesterol either *in vivo* or *in vitro* increases platelet reactivity[24, 25] while increased raft formation in hypercholesterolemic mice lead to myeloproliferation and leukocytosis[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[27] but membrane localization of the TP or the IPTP heterodimer, and the functional consequences for PGI_2 and TXA_2 interplay, 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 PGI_2- TXA_2 interplay. We confirmed localization and function of the IP within lipid rafts and determined that the TP is predominantly raft excluded. Interestingly, TP's membrane microdomain localization and function 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.

Methods

Detailed methods are provided in the Supplemental Materials. Receptors were hemagglutinin tagged and fused to either renilla luciferase (rLuc) or yellow fluorescent protein (YFP), as described[28]. COS-7 cell transfection was with Fugene-6, as described[10]; experiments were performed 48hrs later. Receptor dimerization was quantified as bioluminescence resonance energy transfer (BRET) from donor (rLUC-receptor) to acceptor (YFP-receptor), as described[28]. Microdomain localization was defined by energy transfer

from rLUC-fused receptor to DiIC16, a fluorescent carbocyanine, that labels the L_o membrane phase[16]. Second messenger levels were measured using LANCE cAMP and IP-One Tb assay systems.

Results

Membrane domain localization

IP localization to lipid rafts has been reported in transfected and native cells[27], consistent with its extensive lipidation[29]. The absence of lipid modification on the TPα$_{30}$ [31] predicts L_d distribution. Since these two receptors heterodimerize[10, 28], 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 (Fig. S1). Such co-segregation with caveolin is often taken as evidence for raft association[14, 32]. However, determination of raft versus non-raft by cell fractionation, whether in detergent-free conditions or in detergent insoluble isolates, is fraught with technical difficulties and highly dependent on conditions used, often leading to misleading readouts[33]. Indeed, although it is actually raft excluded in COS-7 cells, the β_2- adrenoreceptor (AR) partitions to caveolin-containing fractions in detergent free preparations[16]. We moved, therefore, to a more direct measure of receptor microdomain localization, referencing β_2-AR as raft excluded control.

Membrane labeling with DiIC16—Cells expressing rLUC fused IP or TPα, were loaded with DiIC16. This probe labels the cholesterol rich L_o membrane phase, in which rafts are found, BRET from rLUC to DiIC16 gives a measure of receptor localization to the L_o phase[16]. Distinct DiIC16 energy transfer curves were seen with IPrLUC and TPrLUC (Fig. 1) - IPrLUC→DiIC16 energy transfer was readily saturable indicating the receptor's L_o association while the shallow and more linear readout for TPrLUC indicated L_o exclusion. The β_2-ARrLUC→DiIC16 curve was also shallow and approached linearity consistent with its reported L_o exclusion in this model[16]. These data indicate that the TPα is excluded from the L_o 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 co-exist in the same microdomain. We examined whether co-expression of these receptors altered each other's localization. IPrLUC→DiIC16 energy transfer was not modified by expression of untagged TPα. In the reverse experiment, however, untagged IP shifted TPrLUC→DiIC16 energy transfer to a saturable curve, indistinguishable from the IPrLUC alone (Fig. 1). Thus, upon heterodimerization the IP dominated the TPα causing its redistribution to L_o microdomains.

Membrane cholesterol depletion

IP and TP signaling—Acute cholesterol depletion is commonly used to implicate lipid rafts in protein function[16, 19, 20]. We explored the contribution of L_o domains to IP, TPα or IPTPα function compared with the L_o-excluded β_2-AR. Generation of cAMP was used as a read-out of IP or β_2-AR function, while InsP generation was a measure of TPα function. TPα switches from InsP to cAMP generation when dimerized with the IP[10], 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 (Fig. 2A) and IPTPα (Fig. 2B) signaling. This contrasts with the raft-excluded β_2-AR, in which cAMP generation was elevated (Fig. 2D) reportedly following increased G_s availability upon raft disruption[16], and TP- InsP, which was

unaltered by cholesterol depletion (Fig. 2C). Thus, in transfected COS- 7 cells, IP and IPTPα function was dependent on raft integrity, further supporting their L_o association, while TP function was not offset by raft disruption, consistent with L_o 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 (Fig. 2E, F, I and J), while TP-InsP (in hAoSMCs; Fig. 2G) and β_2-AR-cAMP signaling (RAW 264.7 cells and hAoSMCs; Fig. 2H, K) 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 the Gs-cAMP cascade.

IP and TP dimerization—Studies report that GPCR dimerization is a prerequisite for normal membrane expression and function[34–36]. Given that 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[28] 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 (Fig. 3), indicating that L_o-included and -excluded dimers remain associated despite cholesterol depletion. Thus it appears that homo- and hetero- dimerization of the IP and TPα is independent of their microdomain localization.

Effect of Cholesterol Enrichment

IP and TP signaling—The impact of elevating plasma membrane cholesterol on cellular signaling is not well defined although 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 (Fig. 4A, B). In contrast, signaling of the L_o-excluded β_2-AR (cAMP) and TP (InsP) were resistant to cholesterol loading (Fig. 4C, D). Thus, cholesterol enrichment selectively impacted the function of raft-associated GPCRs in the COS-7 model. We confirmed the effect of cholesterol enrichment in both native cells models, hAoSMC and RAW 264.7 cells, demonstrating suppressed IP and IPTP coupling to cAMP generation (Fig. 4E, F, I and J) with unaltered TP-InsP in hAoSMC (Fig. 4G) and β_2-AR-cAMP signaling in both native models (Fig. 4H, K). Since hypercholesterolemia is an established risk factor in cardiovascular disease[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 3–4 times higher than similarly fed wild type (WT) mice[42], coincident with elevated plasma membrane cholesterol content in several cell types[43–45]. We examined *ex vivo* IP coupling to cAMP generation in peritoneal macrophages isolated from WT 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 (Fig. 5).

IP and TP dimerization—Given that cholesterol enrichment selectively impacted the function of L_o-associated receptors, we examined whether elevated membrane cholesterol modified their dimerization. Dimers remained associated following cholesterol enrichment although, interestingly, a lower $BRET_{max}$ was seen for the two raft associated species, IPIP and IPTPα), but not the raft excluded TPαTPα (Fig. 3). This differential effect provides further, albeit indirect, evidence that the IPIP and IPTPα reside in a distinct membrane microdomain compared to the TPαTPα.

Receptor surface expression is unaltered by cholesterol enrichement

Cholesterol enrichment can induce sequestration of raft-associated proteins[46–48] 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 (Fig. S3A). Similarly, native IP surface expression was not different in peritoneal macrophages from hyper-cholesterolemic mice (either 8 months on a high (42%) fat diet, Fig. S3B or 6 month old LDLR−/− mice on normal chow Fig S3C) compared to normal controls. Thus, suppression of receptor signaling following 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 G_q/InsP to a G_s/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 homo or heterodimers in co-expressing cells, and the relationship to cardiovascular disease 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 L_o membrane phase, and the relationship between these receptors' respective localization and their dimerization and function.

Lipidation of proteins promotes their association with lipid rafts[49]. The IP, which is both palmitoylated and isoprenylated[29] and is raft localized in this and other[27] studies. TPα, which is, in contrast, not lipidated[30, 31], was predominantly L_o excluded. Interestingly, upon IP co-expression, redistribution TPα to the L_o phase was evident. IP localization was, however, unaltered by co-expression 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 β_2-AR from rafts while energy transfer to DiIC16, the fluorescent L_o-label that we used, provide clear discrimination receptors that are L_o associated versus L_o 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 TXA_2 analog U46619 induced a robust cAMP response in IPTPα transfected cells that was similar to the PGI_2 analog cicaprost[10]. The response to either agonist was suppressed following cholesterol depletion of transfected COS-7 cells, as well as in hAoSMC and RAW 246.7, cells that natively co-express the IP and TP. Thus, raft integrity appeared essential to transduction of a G_s-cAMP signal via the IP or the IPTP heterodimer across transfected and native cell models. It may be that cholesterol depletion impacted G_s or adenylyl cyclase function rather than IP or IPTP function *per se*. However, the increase in

cAMP generation through the raft-excluded β_2AR that we, and others[16], observe argues against a general effect of cholesterol depletion on the G_s-cAMP pathway. In contrast to the IPTP heterodimer, discrete TP signaling to G_q 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 L_o-excluded versus L_o-associated receptors with the former being unaltered or enhanced and the latter being suppressed. Such differential effect of cholesterol depletion consistent with distinct microdomain localization has also been reported for the μ-opioid receptor (both raft and non-raft localized) and δ-opioid receptor (raft excluded)[15]. G_s is typically localized in rafts and its release from rafts following their disruption[16] likely leads to loss of function for raft-associated G_s coupled receptors like the IP and IPTPα heterodimer. G_q, in contrast, is reported to function in both raft and non-raft domains[51], consistent with the insensitivity of the TPα-G_q-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 (ER)[34–36]. Indeed, our previous work supports formation of IP and TP containing dimers in the ER[28] and is consisted 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 $BRET_{max}$ for IPIP and IPTPα, but not TPTP, was blunted. This may reflect reduced association of the IP and TP to form the heterodimeric species.

However, this is unlikely given that the $BRET_{50}$, which reflects affinity[53], was not altered. It is instead likely that the process of dimer assembly in the ER proceeds normally in cholesterol loaded cells but that elevated cholesterol in the L_o 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 β_2-AR, was reduced following cholesterol enrichment, consistent with reports that cholesterol loading can modify raft function[37–39].

Plasma cholesterol levels are directly related to cell membrane cholesterol content[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[24, 25, 54], processes that are restrained by the PGI_2-IP- cAMP pathway[5, 55]. We considered that cholesterol loading of cells in culture may mimic increased plasma cholesterol levels associated with a high fat diet and/or 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[46, 47] and increased rigidity of cholesterol rich membranes can modulate raft-associated signaling[48, 56]. It may be that the fluid dynamics of the microdomains

become less favorable to transduction of the signal from receptor to G protein and/or effector. This possibility is currently under investigation.

The IP is well established to reduce disease severity in a range of cardiovascular disease models[5, 55]. Further, IP limits the pro-thrombotic and proliferative actions of the TP *in vivo*[55]. We determined that formation of an IPTP heterodimer contributes this IP-mediated restraint of TP function[10, 28]. The importance of GPCR dimerization for physiological and pathophysiological receptor functions is beginning to emerge[57–59]. We reported a dominant negative influence of a naturally occurring IP mutant (IP^{R212C}) on its wild type counterpart, through dimerization, as a mechanistic basis for the exaggerated loss of platelet responsiveness to PGI_2 analogs in platelets from individuals heterozygous for the mutant[28]. These, and other studies[60–62], indicate that understanding GPCR homo- and heterodimerization is critical to determining the function of these 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, while TPα 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, we identified lipid rafts as a system used by the cell to partition the signaling and function of the IP and TP based on their homo- or hetero-dimerization and determined the importance of membrane cholesterol homeostasis for normal IP and TP function. The internal consistency of our studies across transfected, primary and *ex vivo* models demonstrates that membrane cholesterol homeostasis is critical for normal receptor function and 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 cardiovascular disease as well as the restraint placed by the IP on TP's deleterious cardiovascular actions[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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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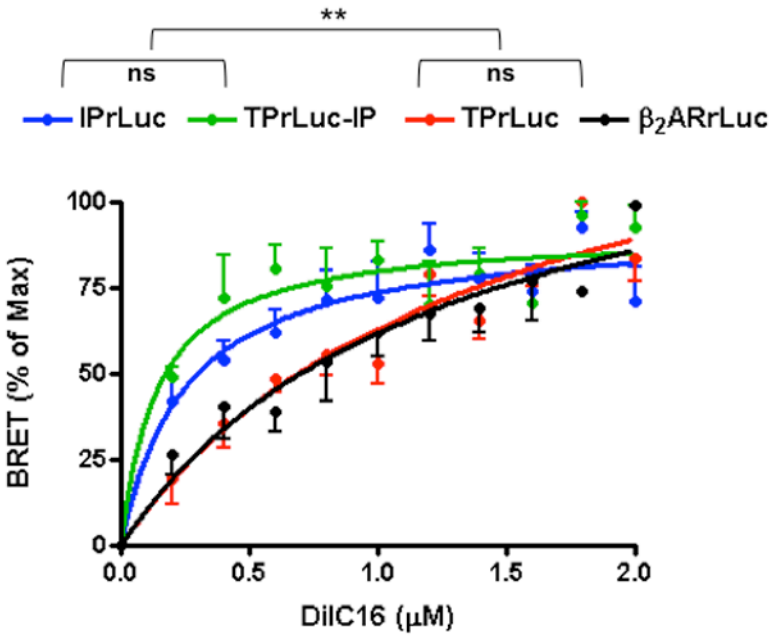

Fig. 1. IP and TP membrane distribution in living cells

COS-7 cells were transfected with IPrLuc, TPrLuc, TPrLuc plus untagged IP, or β_2-ARrLuc and the L_o membrane phase labeled with increasing concentrations of DiIC16. Energy transfer (BRET) from rLuc to DiIC16 was quantified as a measure of the receptor's localization to the L_o phase. Data are expressed as % of maximum BRET and are mean ± sem; n=3; **p<0.005 for either IPrLUC or TPrLuc-IP versus either TPrLUC or β_2-ARrLuc; ns = non significant.

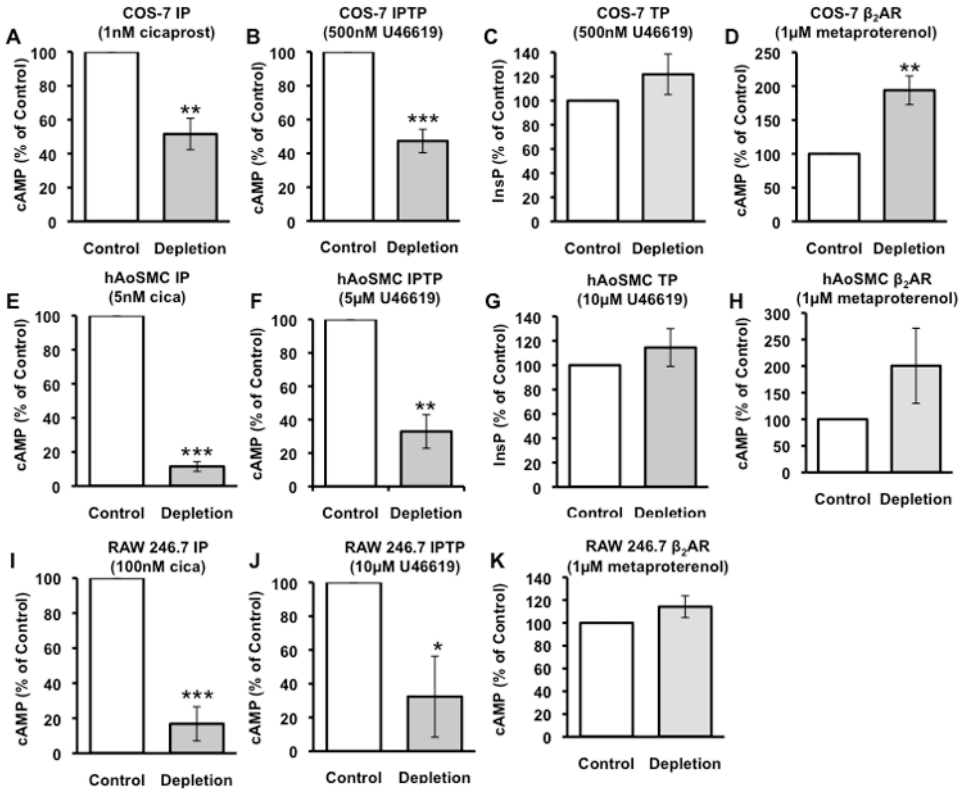

Fig. 2. Effect of cholesterol depletion on receptor signaling

COS-7 cells were transfected with **(A)** IP alone, **(B)** IP plus TP, **(C)** TP alone or (**D**) β_2-AR and, 48hr later, subjected to no treatment (control) or cholesterol depletion (2-hydroxypropyl-β-cyclodextrin 20mM, 1hr). IP and IPTP 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_2-AR signaling as metaproterenol (β_2-AR agonist) stimulated cAMP. In (**A**), (**E**) and (**I**) IP signaling is shown for IP-COS-7 cells, hAoSMC and RAW 246.7, respectively. In (**B**), (**F**) and (**J**) IPTP signaling is shown IPTP-COS-7 cells, hAoSMC and RAW 246.7, respectively. In (**C**) and (**G**) TP signaling is shown in TP-COS-7 cells and hAoSMC, respectively. In (**D**), (**H**) and (**K**) β_2-AR signaling is shown in β_2-AR-COS-7 cells hAoSMC and RAW 246.7 respectively. Results are % of control ± sem from n=3. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$ compared to control.

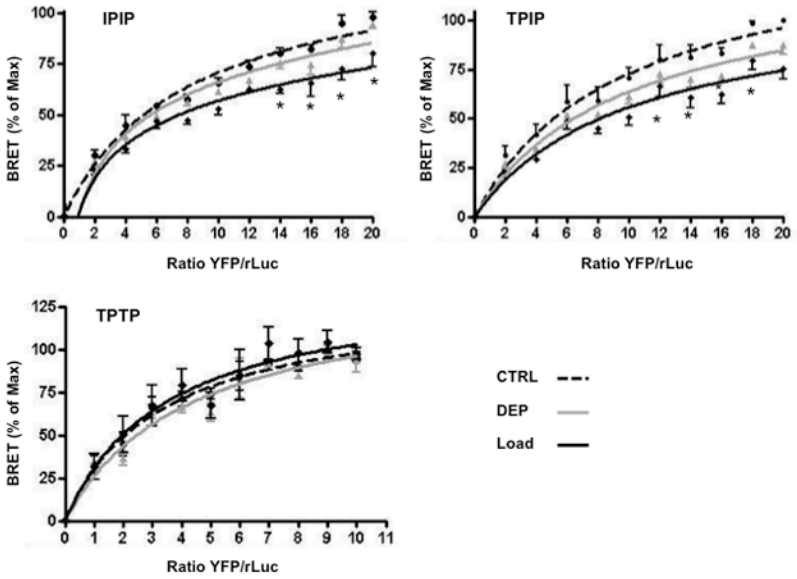

Fig. 3. IP and TP dimerization in living cells

Receptor dimerization was examined by measuring BRET from (**A**) IPrLuc to IPYFP, (**B**) TPrLuc to IPYFP or (**C**) TPrLuc to TP YFP in transfected COS-7 subjected to cholesterol depletion (grey line), loading (solid black line), or no manipulation (dashed line). Data are the % of maximum in control cells vs. the ratio of YFP-receptor/rLuc-receptor and are mean ± sem of n=3.

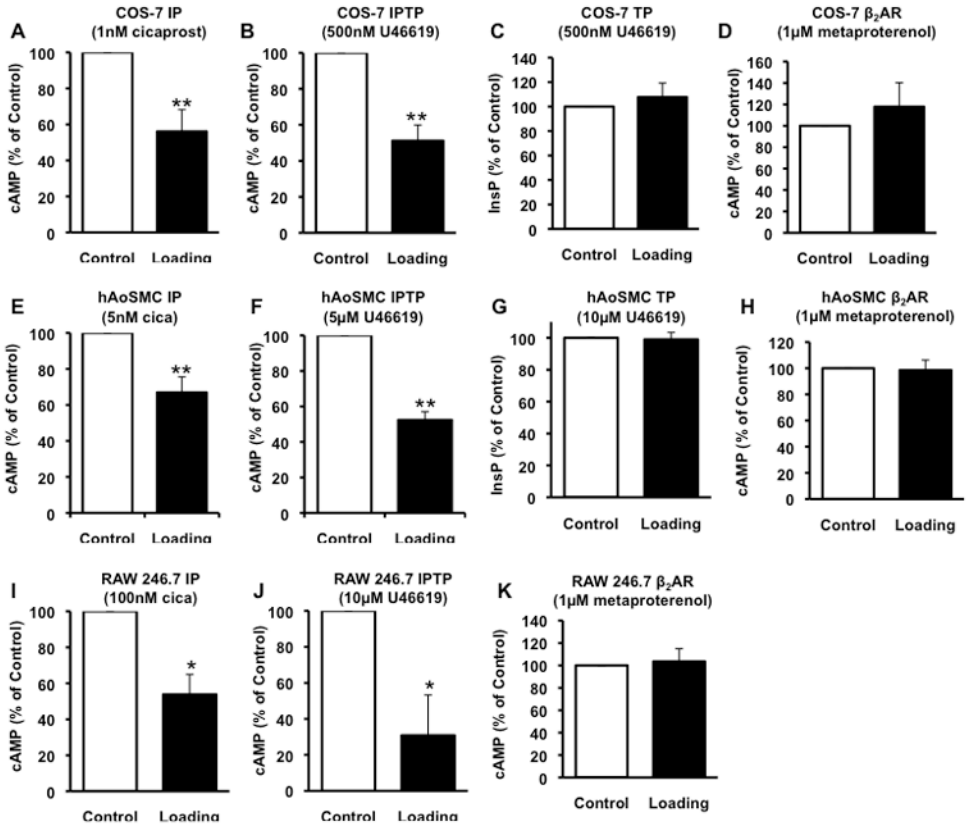

Fig. 4. Effect of cholesterol enrichment on receptor signaling

COS-7 cells were transfected with **(A)** IP alone, **(B)** IP plus TP, **(C)** TP alone or (**D**) β_2-AR and, 48hr later, subjected to no treatment (control) or cholesterol loading (cholesterol-methyl-β-cyclodextrin complex 80μg/ml, 1hr). IP and IPTP 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_2-AR signaling as metaproterenol (β_2-AR agonist) stimulated cAMP. In (**A**), (**E**) and (**I**) IP signaling is shown for IP-COS-7 cells, hAoSMC and RAW 246.7, respectively. In (**B**), (**F**) and (**J**) IPTP signaling is shown IPTP-COS-7 cells, hAoSMC and RAW 246.7, respectively. In (**C**) and (**G**) TP signaling is shown in TP-COS-7 cells and hAoSMC, respectively. In (**D**), (**H**) and (**K**) β_2-AR signaling is shown in β_2-AR-COS-7 cells hAoSMC and RAW 246.7 respectively. Results are % of control ± sem from n=3. * $p<0.05$, ** $p<0.005$ compared to control.

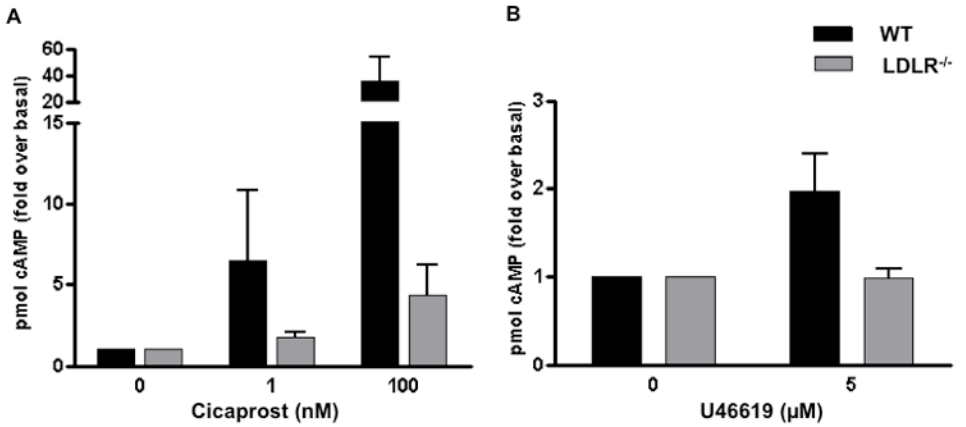

Fig. 5. IP- and IPTP-mediated signaling in mouse peritoneal macrophages

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=6–7; * $p<0.05$ compared to WT.