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Heterodimerization of the α and β isoforms of the human Thromboxane receptor enhances isoprostane signalling.

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SUMMARY

Isoprostanes are free radical catalyzed products of arachidonic acid that are elevated in pro-oxidant disease states. Two isoprostanes, 8-isoprostaglandin $F_{2\alpha}$ ($iPF_{2\alpha}III$) and 8-isoprostaglandin E_2 (iPE_2III) act at the receptor for thromboxane A_2 (the TP) to mediate pro-atherogenic effects *in vivo*. We confirmed dimerization of the human TP isoforms, TPα and TPβ, and determined the impact on isoprostane signaling. No overt changes in ligand binding at the TP were observed as a result of TPα/TPβ coexpression. The response to $iPF_{2\alpha}III$ or iPE_2III was enhanced in HEK293 cells stably coexpressing TPα and TPβ, as measured by inositol phosphate generation or intracellular calcium mobilization, relative to cells expressing TPα or TPβ individually. In contrast, the response to traditional thromboxane analogs was unaltered. Augmented isoprostane signaling was similarly observed in HEK 293 cell transiently transfected with TPα and TPβ. These results indicate that TPα/TPβ dimerization enhances isoprostane-mediated signal transduction.

Keywords

Thromboxane; G-protein coupled receptor; Heterodimer; Isoprostanes

INTRODUCTION

Isoprostanes are free radical catalyzed products of arachidonic acid that are elevated in many oxidant-related disease states including atherosclerosis [1]. Isoprostane levels are elevated within developing atherosclerotic lesions in mice and normalization of isoprostane levels correlates with disease regression [2]. At least two isoprostanes, 8-isoprostaglandin $F_{2\alpha}$ ($iPF_{2\alpha}III$) and 8-isoprostaglandin E_2 (iPE_2III), act at the receptor for thromboxane A_2 (the TP) *in vivo* to induce vasoconstriction and platelet aggregation [3]. Significantly, antagonism of TP, but not aspirin-mediated inhibition of TxA_2 synthesis, reduced atherosclerosis in mice, suggesting that mediators other than TxA_2, possibly isoprostanes, act at the TP to propagate the disease [4]. Additionally, increased lesion formation in response to exogenous $iPF_{2\alpha}III$, was observed in two mouse models of atherosclerosis and was prevented by a TP antagonist [5]. Thus, isoprostanes represent both a marker and mediator of vascular disease.

The TP is a G-protein coupled receptor (GPCR) encoded by a single gene [6] that is alternatively spliced at the carboxyl terminus resulting in two isoforms TPα (343) and TPβ (407). The two isoforms are identical for the first 328 amino acids diverging only in their C-terminal regions[7]. Both TPα and TPβ are coupled primarily to phospholipase C, while the former may activate and the latter inhibit, adenylyl cyclase (AC) activity[7]. Additionally,

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dissimilar patterns of constitutive and agonist-dependent internalization of the membrane expressed TPα and TPβ have been observed, reportedly because of their differential interactions with regulatory proteins Rab11 and arrestin[8,9]. However despite advances elucidating their individual functional and regulatory characteristics, the distinction between TPα and TPβ with respect to their ultimate physiological or pathophysiological roles remains unclear.

Appreciation of the complexity inherent in GPCR signaling increased markedly with the recent realization that receptors from closely related and distinct subfamilies can interact physically forming heterodimers [10]. GPCR heterodimerization results frequently in substantial modifications of receptor function[10]. Indeed, we demonstrated previously that heterodimerization of TPα with the human receptor for prostacyclin (the IP) dramatically altered TP signaling [11]. More recently, TPα and TPβ were shown to heterodimerize with consequent alterations in receptor regulation and signaling in response to a TxA_2 analog [12, 13]. The vasopressor response to $iPF_{2\alpha}III$ was augmented in mice transgenic for TPβ expression in the vasculature [3] leading us to question the consequences of TPα/TPβ heterodimerization for isoprostane signaling.

In the present study we examined if TPα/TPβ heterodimerization altered receptor responsiveness to isoprostanes. We confirmed heterodimerization of co-expressed TPα/TPβ and found that coexpression significantly augmented the response to the isoprostanes, $iPF_{2\alpha}III$ and iPE_2III, but not to traditional TxA_2 analogs. These data suggest that the TPα/TPβ heterodimer may constitute a modified TP receptor with increased sensitivity to isoprostanes.

EXPERIMENTAL PROCEDURES

Epitope tagging of hIP and hTP

The 9 amino acid hemagglutinin epitope (HA; YPYDVPDYA), or 10 amino acid Myc epitope (EQKLISEEDL), were inserted between the N-terminal initiator methionine and the second amino acid of the hTPα or hTPβ to generate HAhTPα or MychTPβ. Generation of HAhTPα was as described previously [11]. To generate MychTPβ, 5′-oligonucleotides which contained 3 miscellaneous bases, 6 bases encoding a HindIII site, the 3 bases immediately 5′ of the initiator methionine, 3 bases encoding a methionine, the epitope tag coding sequence and 21 bases encoding amino acids 2–8 were generated. 3′-oligonucleotides were complementary to the receptor coding sequence downstream of a unique restriction site (Nar I site for hTPβ). Using the hTPβ cDNAs as templates, polymerase chain reactions were carried out to generate the 5′-MychTPβ fragments. The resulting products were cloned into PCR 2.1 (Qiagen, CA) and, following verification of the sequence, were excised using HindIII/Nar I. Using the same enzymes the 3′ fragment in pcDNA3.1 (Hygro for MychTPβ) were generated and the two receptor pieces were ligated to each other. The integrity of the splice site was verified by sequencing.

Cell Culture and Transfection

HEK293 cells (American Type Tissue Culture Collection, Rockville, MD) were maintained, and stably transfected with Myc-tagged TPβ or HA-tagged TPα, as previously described[11]. For transient transfections 50–80% confluent HEK293 were transfected using Fugene 6 (Roche Biochemicals, IN) according to the manufacturers instructions.

Co-immunoprecipitation

Immunoprecipitation was carried out as previously described [11]. Briefly HEK 293 cells stably expressing HAhTPα or both HAhTPα and MychTPβ, were treated with 3 mM DSP

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(Pierce Biotechnology, IL) for 30min and lysed in buffer A (150 mM NaCl; 1 mM EDTA; 20 mM Tris HCL [pH 8.0]; 10% glycerol and a cocktail of protease inhibitors) for 2 h at 4°C. Supernatants were precleared with protein G-Sepharose. Anti-Myc G-Sepharose was prepared by adding 9 μg anti-Myc ascites per lysate to 10% protein G-Sepharose followed by 1 h rotation. MychTPβ was immunoprecipitated from precleared lysates by adding 150 μl of anti-Myc-protein G-Sepharose to each lysate and rotating for 16 h. Protein G was precipitated at 14,000 rpm for 1 min, washed three times with Buffer A and resuspended in 10 μl of sample buffer (Nupage). Immunobloting for HA was carried out using biotinylated anti-HA antibody (1:500) followed by peroxidase labeled streptavidin.

Membrane preparation and radioligand binding

Membrane preparation and radioligand binding were performed as described previously [11]. Displacement experiments were initiated by the addition of $[^3H]$-SQ 29548 (40 nM) allowed to continue for 30 min at 30°C, prior to termination by the addition of 3 ml ice cold-wash buffer (10 mM HEPES, pH 7.4, 0.01% bovine serum albumin), followed by immediate filtration through GF/C filters. Radioactivity associated with the filters was quantified by scintillation counting. Nonspecific binding was measured in the presence of a 500-fold excess of unlabeled SQ 29548.

Inositol Phosphate Production

Inositol phosphate production was assessed as described previously [11]. Briefly, cells were labeled overnight with 2 μCi/ml $[^3H]$-myoinositol, pretreated with 20 mM LiCl for 30min and stimulated appropriately. Total inositol phosphates were extracted with formic acid for 30 min at room temperature, neutralized using 5 M ammonia and recovered by anion exchange using Dowex 1-X8 AG anion exchange resin.

Calcium measurement

Intracellular calcium ($Ca^{2+}{}_i$) was assessed in stably transfected HEK cells, as previously described [14]. Cells were loaded for 1h at 37°C with 5uM Fura-2/AM (Molecular Probes) in phenol red free-RPMI 1640 culture media. Cells were washed, incubated for 5 min in PBS containing 1mM EDTA and 5mM EGTA and harvested. Cells were resuspended at 10^6 cells/ml RPMI1640 and fluorescence detected spectrophotometrically. Approximate values for $Ca^{2+}{}_i$ were calculated using a Kd of 224 nM for Fura-2.

RESULTS

TPα/TPβ dimerization

We confirmed TPα/TPβ heterodimer formation which has been demonstrated previously [12, 13]; following immunoprecipitation of MycTPβ, immunobloting with anti-HA revealed the presence of HAhTPα in lysates derived from cells co-expressing HAhTPα and MychTPβ, but not lysates from cells expressing HAhTPα alone (Fig. 1A). The co-immunoprecipitated partner appeared primarily in the oligomeric form (Fig. 1A). This is not unexpected as nonreducing conditions were used for optimal visualization of oligomeric receptor. Under reducing conditions both monomeric and oligomeric forms of coimmunoprecipitated HAhTPα were observed (data not shown).

TPα/TPβ coexpression facilitates isoprostane-mediated inositol phosphate generation

To determine if TPα/TPβ dimerization specifically alters signaling in response to isoprostanes, we treated HEK 293 cells stably expressing comparable levels of TPα or TPβ individually or in combination (Fig. 1B), with increasing concentrations of the isoprostanes iPE_2III or $iPF_{2\alpha}III$, or with the TP specific agonists, IBOP and U46619. Treatment with the IBOP or

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U46619 induced a robust increase in inositol phosphate generation, which did not differ significantly between cells expressing TPα or TPβ individually, and those coexpressing both receptors (Fig. 2A and B). In contrast, inositol phosphate generation following treatment with the iPE_2III or $iPF_{2\alpha}III$, while less than that elicited by the TP-agonists, was significantly enhanced in cells co-expressing TPα and TPβ (Fig. 2C and D).

To confirm these findings, HEK 293 cells were transiently transfected with a triple HA tagged (3xHA) hTPα (10 μg; UMR cDNA Resource Center), or cotransfected with 3xHAhTPα and MychTPβ (5μg of each construct), and inositol phosphate generation in response to IBOP or $iPF_{2\alpha}III$ examined. Similar to the results observed in stably transfected cells, the response to the isoprostane iPE_2III (Fig. 3B), but not the TP-agonist IBOP (Fig. 3A), was significantly enhanced by TPα/TPβ coexpression (1.14 ± 0.06 vs 1.48 ± 0.8 fold over basal), demonstrating that this was not an artifact of stable receptor overexpression. Thus, it appears that coexpression of TPα and TPβ promotes specifically the TP-mediated response to isoprostanes.

TPα/TPβ coexpression facilitates isoprostane-mediated Ca^{2+}_i mobilization

We next sought to confirm enhanced isoprostane responsiveness of the TPα/TPβ coexpressing cells, using an alternate index of TP signaling, namely Ca^{2+}_i mobilization. Similar to the inositol phosphate experiments, the increase in Ca^{2+}_i observed in response to U46619 (Fig. 4A) did not differ significantly between TPα-HEK, TPβ-HEK and TPα/β-HEK cells. Once again, the response to $iPF_{2\alpha}III$ (Fig. 4B) or iPE_2III (data not shown) was significantly enhanced in cells coexpressing TPα and TPβ.

Receptor-ligand interactions

One of the characteristics frequently modified as a result of GPCR heterodimerization is the affinity of the individual receptors for their respective ligands [15,16]. We examined if altered ligand binding to the TPα/TPβ heterodimer might underlie the changes we observed in TP-signaling. Membranes from TPα-HEK, TPβ-HEK or TPα/β-HEK cells were labeled with the TP specific antagonist ^{3}H-SQ 29548, which is used commonly to delineate the TP binding site [11,17], and displacement by the traditional TP-ligands SQ 29548 and U46619, or the isoprostanes iPE_2III and $iPF_{2\alpha}III$, assessed.

No difference in the order of displacement was observed between TPα and TPβ: the rank order of potency for the displacing ligands was SQ 29548 > U46619 >> iPE_2III >> $iPF_{2\alpha}III$. Coexpression of TPα and TPβ did not alter affinity for SQ 29548 or U46619 as assessed by displacement (Fig. 5). Significantly, and in agreement with previous reports, iPE_2III bound very weakly, while $iPF_{2\alpha}III$ did not bind at all to TPα or TPβ individually and this was unaltered by receptor coexpression. Thus, although isoprostane signaling was enhanced in TPα/β-HEK cells relative to cells expressing the receptors individually, this did not appear to reflect alterations in the ability of isoprostanes to ligate to the receptor.

DISCUSSION

Studies report ligation of both existing membrane [11,18] and nuclear prostaglandin receptors [19] by isoprostanes. However, the concept that specific isoprostane receptors may exist has been suggested [20]. This is based partially on the apparent inability of isoprostanes to ligate or signal efficiently through either TP isoform *in vitro* [20], despite evidence that their *in vivo* actions are via the TP[3]. However, to date, no molecular evidence for the existence of a distinct receptor for isoprotanes has been found. GPCR heterodimerization frequently alters many of the indices used to characterize receptors, including ligand binding, signaling and regulation [10]. Indeed it has been proposed that the κ2 opioid receptor, described solely on a pharmacological basis, may in fact be the δ-κ opioid receptor heterodimer [21]. Therefore, we

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considered whether the phenomenon of GPCR heterodimerization may provide resolution of the pharmacological discrepancies in isoprostane studies.

Dimerization of the alpha and beta isoforms of the TP receptor was recently shown to mediate alterations in both receptor regulation and signaling [12,13], however, the impact of this interaction on the response to isoprostanes was not examined. Previously, we demonstrated that dimerization of IP and TPα mediated significant alterations in TP signaling and may result in the formation of a novel isoprostane binding site [11]. We confirmed TPα/TPβ heterodimer formation (Fig. 1A) and assessed if the TPα/TPβ heterodimerization might facilitate isoprostane signaling in a similar manner.

In agreement with previous reports, the isoprostanes $iPF_{2\alpha}III$ and iPE_2III stimulated TP-mediated inositol phosphate generation to a lesser extent than the TP-agonists IBOP and U46619 in cells expressing either TPα or TPβ individually (Fig. 2). However, while coexpression of TPα and TPβ did not alter significantly the response to TP activation by IBOP or U46619, inositol phosphate generation (Fig. 2A and B) following treatment with iPE_2III and $iPF_{2\alpha}III$ was significantly enhanced in cells stably expressing both receptors (Fig. 2C and D). We examined $Ca^{2+}{}_i$ mobilization as an additional index of TP signaling and found a similar selective enhancement of isoprostane signaling (Fig. 4).

The dose response curves for IBOP- and U46619-induced inositol phosphate generation and $Ca^{2+}{}_i$ mobilization were super-imposable, arguing against significant differences in the total receptor expression level across the three stably transfected lines. Moreover no overt differences in TP expression were evident in the binding studies (Fig. 5) or by Western blot analysis (Fig. 1). Therefore, we did not assess directly by saturation ligand binding the total TP expression levels achieved in the three stably transfected cell lines. Instead, we sought to confirm our findings, and establish that variations in total receptor expression levels were not responsible for the enhanced TPα/TPβ isoprostane sensitivity, in an independent set of transiently transfected cells. HEK 293 cells were transiently transfected with 10μg of TPα or cotransfected with 5μg each of TPα and TPβ (10μg total), thereby ensuring equivalent expression levels between the two conditions. The response to TP agonists was lower in the transient model compared to the stably transfected cells, a reflection of the lower overall expression levels that are achieved in transiently compared with stably transfected cells. Importantly, as for the stably transfected cell models, the isoprostane-induced inositol phosphate response was selectively augmented in the TPα/β co-expressing cells (Fig. 3). Taken together these results suggest strongly that interaction between the two TP isoforms specifically promotes isoprostane signaling.

Reports now abound of GPCR heterodimers, formed upon receptor coexpression, mediating alterations in the signaling characteristics of the individual receptors. Dimerization of the β1 and β2 adrenergic receptors enhances cAMP formation in response to isoproternol and has been implicated in regulating cardiac contractility[22]. Indeed, we have previously demonstrated that heterodimerization of TP with the human prostacyclin receptor facilitates cAMP generation in response to TP activation [11]. As such, modulation of isoprostane signaling upon TPα/TPβ dimerization is not unprecedented and likely has physiological or pathophysiological consequences.

Many studies investigating the characteristics of newly discovered GPCR heterodimers describe an altered receptor-ligand-effector profile [10], suggesting that TPα/TPβ heterodimer formation may generate an altered receptor with distinct ligand binding characteristics. However, we observed no distinct differences in the ability of $iPF_{2\alpha}III$ or iPE_2III to displace the TP antagonist SQ 29548 in membranes generated from TPα–, TPβ– or TPα/TPβ–HEK cells. Thus, despite the fact that both isoprostanes signaled more efficiently via the TPα/TPβ

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heterodimer we observed no elevation in their apparent ability to bind to the heterodimer. We previously reported that ^{3}H-SQ 29548 did not fully occupy the binding site for iPE$_2$III in the TPα/IP heterodimer [11]. It is likely that a similar sub-optimal labeling of the TPα/TPβ isoprostane binding site occurred in the present study giving rise to the inconsistency between the signaling and radioligand studies. Thus, while it is apparent that TPα/TPβ heterodimerization does not alter the "traditional" TP binding site, it remains possible that an altered binding site for isoprostanes is created. An alternative explanation may lie in reports that downstream G protein coupling is modified when GPCR heterodimerize. Thus, if the TPα/TPβ heterodimer were more efficiently coupled to Gq in the co-transfected cells elevated inositol phosphate and calcium signals might be expected. However, the absence of a similarly enhanced response to the traditional TP agonists IBOP and U46619 argues against this interpretation and for the generation of an altered binding site that is not adequately labeled by ^{3}H-SQ 29548. Binding studies were attempted with ^{3}H-iPF$_{2\alpha}$III to resolve this issue further. However, because of the extremely low specific activity of the commercially available radioligand useful data was not obtained.

The mechanism through which heterodimerization alters the ability of the signaling of the constituent receptors is currently an area of intense investigation. Several studies have demonstrated that G-proteins exist in a stable complex with their cognate receptor [23,24], suggesting that receptor-effector coupling is mediated through dynamic conformational changes. In accord with this concept, a specific ligand-receptor interaction may mediate agonist activation of one effector pathway, yet act as antagonist for alternate pathway [25], a property likely to be modulated by dimerization. As such the TPα/TPβ heterodimer may stabilize a receptor-G-protein-effector configuration which specifically facilitates the isoprostane-induced conformational changes required for TP activation of PLC.

Evidence from several studies implicates isoprostanes, acting at the TP, in the development of atherosclerosis [4,5,26]. However, the question remains, given their poor efficacy as TP agonists, how is this effected? GPCR dimerization is increasingly being implicated in the regulation of physiological and pathophysiological processes, including atherosclerosis. [27]. Our study demonstrates that TPα/TPβ dimerization enhances isoprostane-mediated signal transduction, and would likely maximize the deleterious effects of isoprostanes in the setting of cardiovascular disease.

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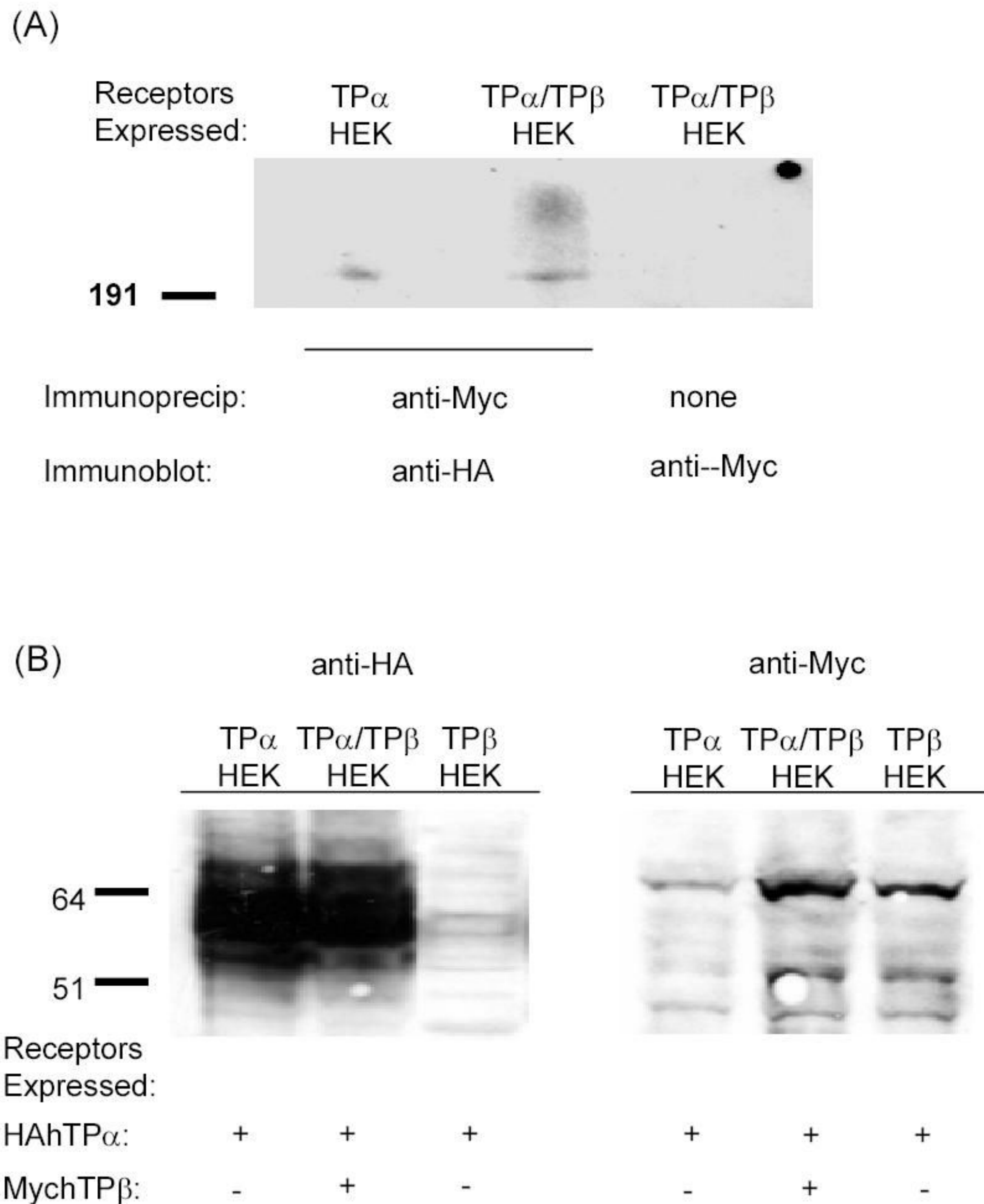

Fig. 1. HAhTPα and MychTPβ expression and dimer formation in stably expressing HEK cells

(A) TPα/TPβ-HEK or TPα-HEK cell lysates were subjected to co-immunoprecipitation using an anti-Myc antibody. Immunoprecipitates were resolved by 10% non-reducing SDS-PAGE and co-immunoprecipitated HAhTPα detected using an anti-HA antibody. Co-immunoprecipitation was only observed when HAhTPα and MychTPβ were coexpressed. (B) Lysates from cells stably expressing HAhTPα, MychTPβ or coexpressing both HAhTPα and MychTPβ, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with an anti-HA or anti-Myc antibody as described under "Experimental Procedures". Molecular masses are in kDa. Western blots are representative of three independent experiments.

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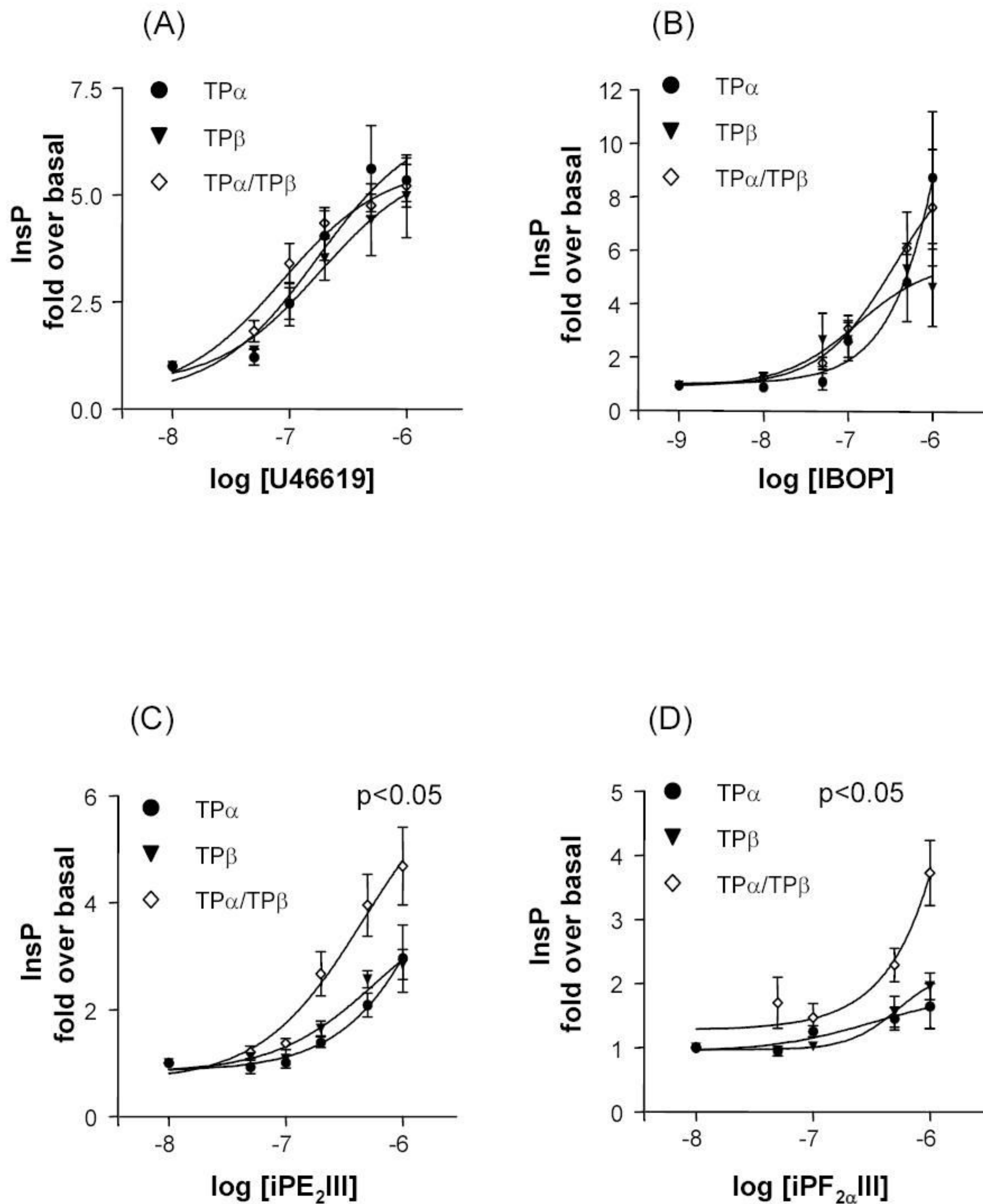

Fig. 2. Isoprostane-mediated inositol phosphate generation in cells stably coexpressing HAhTPα and MychTPβ.

TPα-HEK (closed circles), TPβ-HEK (closed triangles) or TPα/TPβ-HEK (open diamonds) were treated with U46619 (A), IBOP (B), iPE_2III (C) or $iPF_{2\alpha}III$ (D) for 10 min and inositol phosphates quantified as described under "Experimental Procedures". Data are presented as Mean fold over basal ± S.E. from three to five experiments. iPE_2III and $iPF_2\alpha III$, $p<0.05$ TPα or TPβ vs TPα/TPβ as assessed by two way ANOVA.

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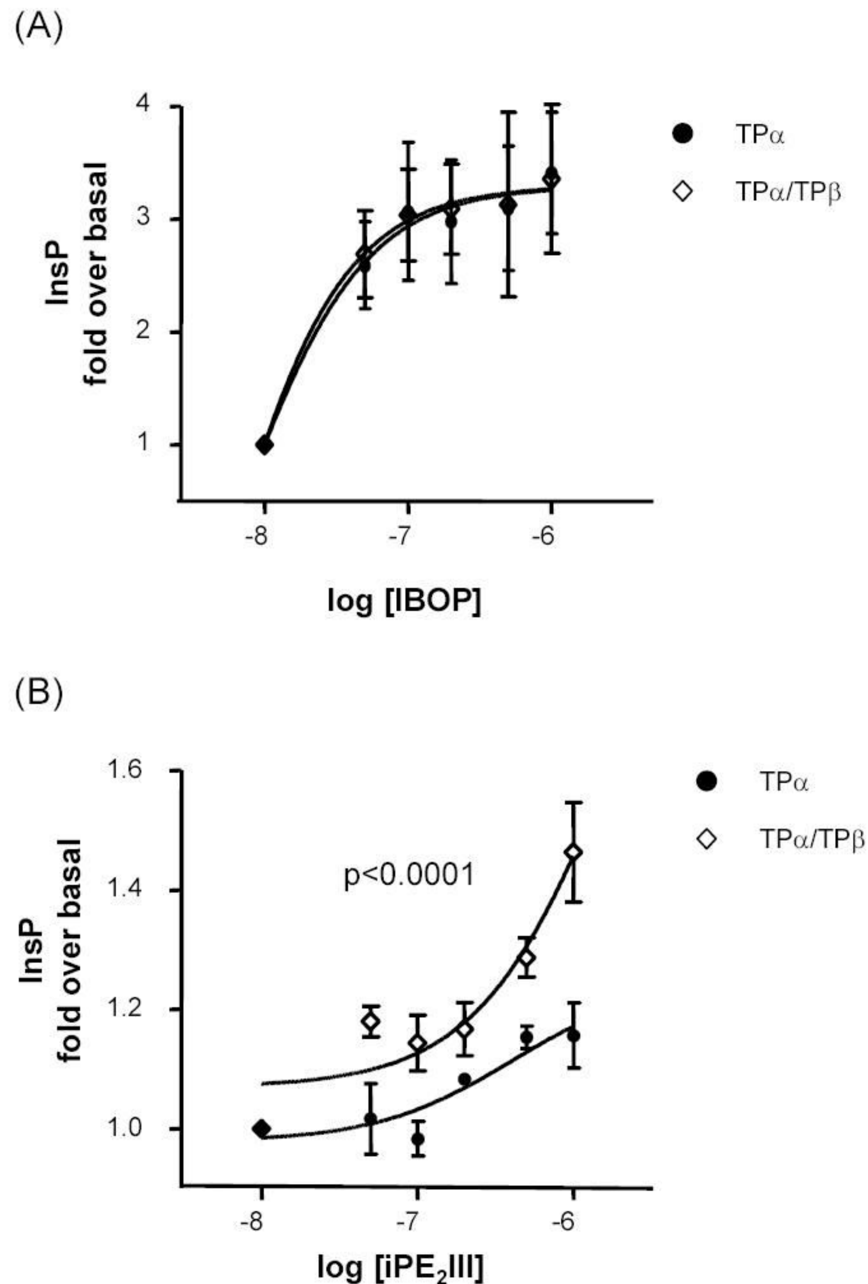

Fig. 3. Isoprostane-mediated inositol phosphate generation in cells transiently transfected with HAhTPα and MychTPβ

HEK 293 cells transiently transfected with 10 μg of 3xHAhTPα (closed circles) or cotransfected with 5 μg each of 3xHAhTPα and MychTPβ (10μg total; open diamonds) were treated with U46619 (A) or iPE_2III (B) for 1h min and inositol phosphates quantified as described under "Experimental Procedures". Data are presented as Mean fold over basal ± S.E. from three to five experiments. iPE_2III, $p<0.0001$ TPα vs TPα/TPβ as assessed by two way ANOVA.

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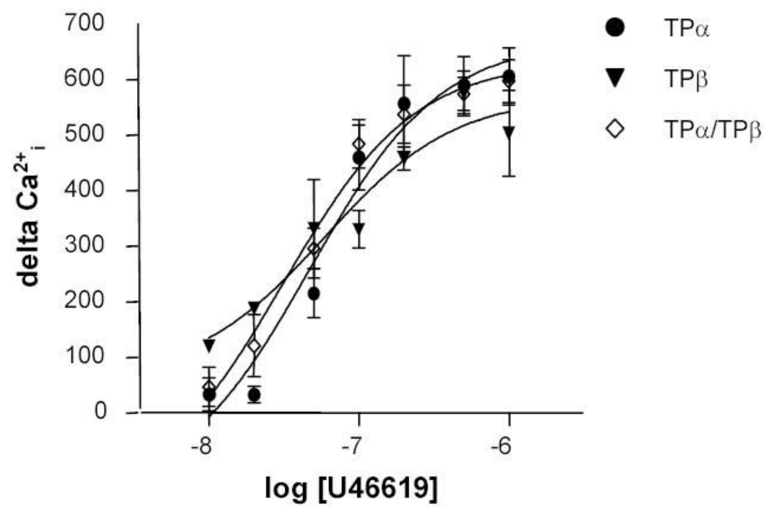

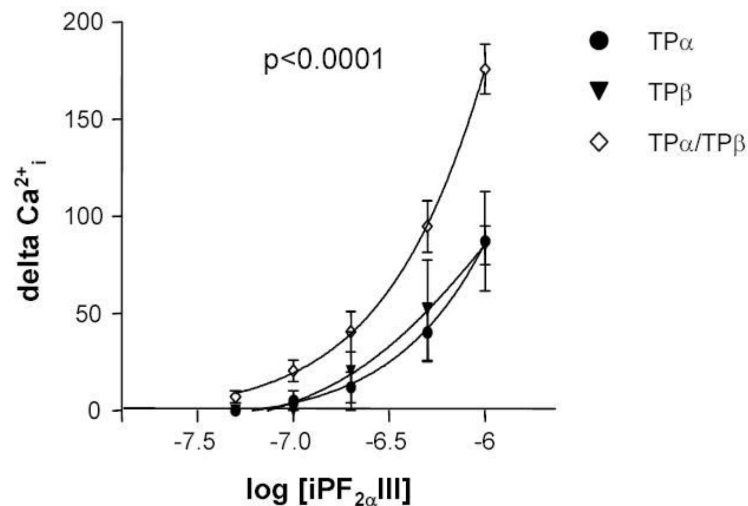

Fig. 4. Isoprostane-mediated $Ca^{2+}{}_i$ in cells stably coexpressing HAhTPα and MychTPβ

TPα-HEK (closed circles), TPβ-HEK (closed triangles) or TPα/TPβ-HEK (open diamonds) were treated with U46619 (A) or $iPF_2\alpha III$ (B) for 10 min and intracellular $Ca^{2+}{}_i$ quantified as described under "Experimental Procedures". Data are presented as Mean fold over basal ± S.E. from four to six experiments. $iPF_2\alpha III$, $p<0.0001$ TPα or TPβ vs TPα/TPβ as assessed by two way ANOVA.

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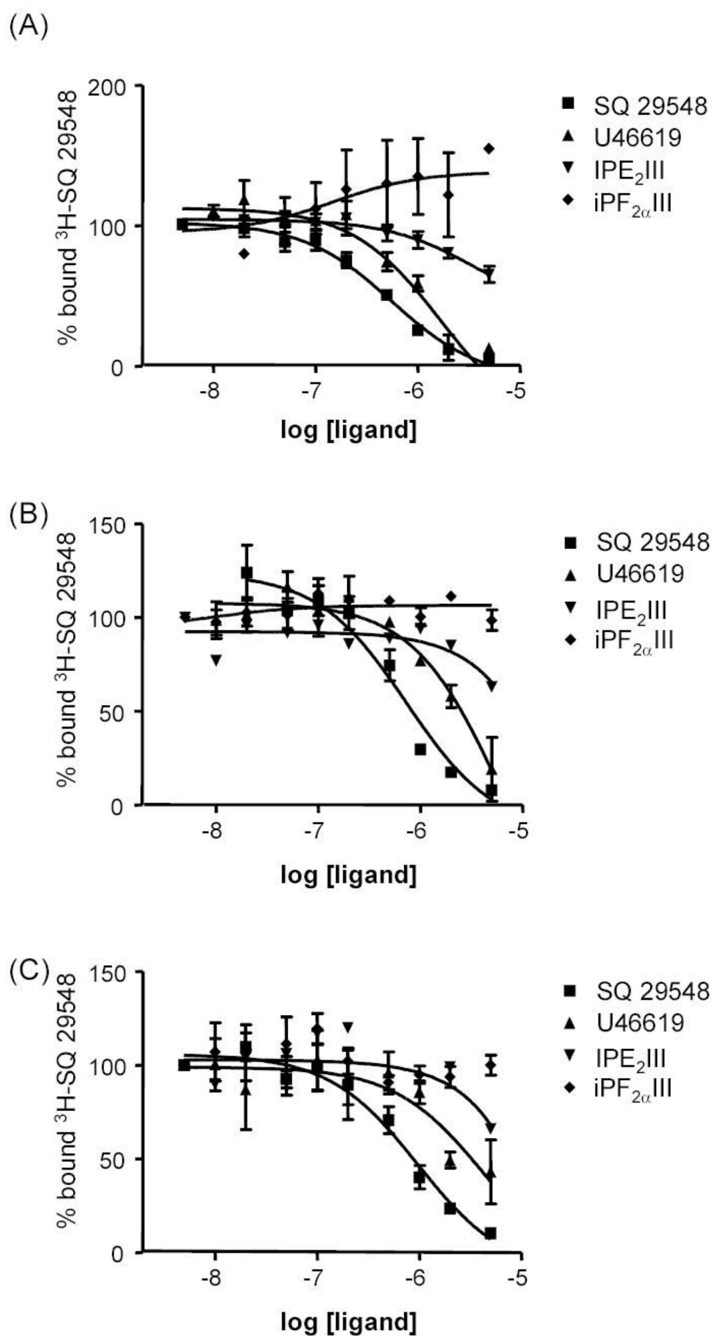

Fig. 5. Effect of TPα/TPβ coexpression on TP ligand binding

Radioligand binding studies were carried out using membrane extracts from (A) TPα-HEK, (B) TPβ-HEK or (C) TPα/TPβ-HEK cells, labeled with [^{3}H]-SQ 29548 as described under "Experimental Procedures". Displacement by increasing concentrations of the TP ligands SQ 29548, U46619 and the isoprostanes iPE$_2$III and iPF$_2$αIII to displace [^{3}H]-SQ 29548 from TP was examined. Data are mean ± S.E. percent of ^{3}H-SQ 29548 displacement from 3 experiments.