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Differential regulation of RhoA-mediated signaling by the TPα and TPβ isoforms of the human thromboxane A2 receptor: Independent modulation of TPα signaling by prostacyclin and nitric oxide

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ABSTRACT

In humans, thromboxane (TX) A_2 signals through the TPα and TPβ isoforms of the TXA_2 receptor that exhibit common and distinct roles. For example, Gq/phospholipase (PL)Cβ signaling by TPα is directly inhibited by the vasodilators prostacyclin and nitric oxide (NO) whereas that signaling by TPβ is unaffected. Herein, we investigated whether TPα and/or TPβ regulate G_{12}/Rho activation and whether that signaling might be differentially regulated by prostacyclin and/or NO. Both TPα and TPβ independently regulated RhoA activation and signaling in clonal cells over-expressing TPα or TPβ and in primary human aortic smooth muscle cells (1° AoSMCs). While RhoA-signaling by TPα was directly impaired by prostacyclin and NO through protein kinase (PK)A- and PKG-dependent phosphorylation, respectively, signaling by TPβ was not directly affected by either agent. Collectively, while TPα and TPβ contribute to RhoA activation, our findings support the hypothesis that TPα is involved in the dynamic regulation of haemostasis and vascular tone, such as in response to prostacyclin and NO. Conversely, the role of TPβ in such processes remains unsolved. Data herein provide essential new insights into the physiologic roles of TPα and TPβ and, through studies in AoSMCs, reveal an additional mode of regulation of VSM contractile responses by TXA_2.

1. Introduction

The phosphorylation status of myosin light chain (MLC) of the actomyosin complex plays a central role in regulating the various types of cytoskeletal reorganizations that widely occur within the vasculature including in platelet shape change and aggregation, tonic- or agonist-induced contraction and relaxation of smooth muscle cells (SMCs), cell migration, cell proliferation and stress fibre formation [1]. Many of the physiologic regulators of platelets and vascular smooth muscle (VSM) contraction, including thromboxane (TX) A_2, thrombin, ADP, prostaglandin (PG) I_2 or PGD_2, act through specific G protein coupled receptor (GPCR) -effector systems [1]. While agents such as TXA_2 and thrombin that promote platelet activation or SMC contraction induce Gq-dependent phospholipase (PL)Cβ activation to evoke calcium (Ca^{2+}) -dependent activation of myosin light chain kinase (MLCK) and MLC_{20} phosphorylation [1,2], they may also engage the Ca^{2+}-independent pathway involving receptor co-coupling to G_{12} and RhoA signalling [1]. G_{12} members, particularly $G\alpha_{13}$, activate RGS (regulators of G protein signaling)-box containing members of the Rho guanine nucleotide exchange factor (RhoGEF) family, such as p115 RhoGEF, PDZ RhoGEF or LARG, to activate RhoA and its key effector in this system Rho kinase 1 (also known as p164 ROKα/ROCK2) and 2 (p160 ROKβ/ROCK1), herein termed Rho kinase/ROCK [3–5]. Rho kinases, in turn, phosphorylate, and inactivate, myosin phosphatase, MLC itself and the myosin phosphatase inhibitor CPI-17 resulting in the Ca^{2+}-independent increase in overall levels of phosphorylated MLC through a Rho/Rho kinase-mechanism [2,4,5]. Other targets of Rho kinase include its phosphorylation-dependent activation of LIM kinases which, in turn, phosphorylate and inactivate the actin depolymerizing agent cofilin [4]. The central importance of the Ca^{2+}-independent mechanism of contraction within the vasculature has been highlighted through findings that disorders of the Rho/Rho kinase pathway are major underlying causes of hypertension, vascular spasm and atherosclerosis making Rho kinase an important therapeutic target in the treatment of these diseases [1,2,6].

The prostanoid TXA_2 plays an essential role within the vasculature inducing a diversity of cellular responses including platelet shape change, secretion and aggregation, VSMC contraction and migration and is widely implicated in a number of cardiovascular disorders including thrombosis, hypertension, vessel remodelling and atherosclerotic progression [7]. As a predominantly Gq/PLCβ-coupled GPCR, the TXA_2

Abbreviations: GPCR, G protein-coupled receptor; HA, hemagglutinin; HEK, human embryonic kidney; IP, prostacyclin receptor; IP_3, inositol 1, 4, 5-trisphosphate; NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PAGE, polyacrylamide gel electrophoresis; PG, prostaglandin; PK, protein kinase; PL, phospholipase; sGC, soluble guanylyl cyclase; SIN-1, 3-morpholinosydnonimine, HCl; TP, TXA_2 receptor; TX, thromboxane; VSM, vascular smooth muscle.

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receptor or TP can induce both Ca^{2+}-dependent and $G_{12/13}$-mediated RhoA/Ca^{2+} independent responses platelets and VSMCs [1,8]. For example, platelets from $G\alpha_{13}$-deficient mice do not undergo RhoA-dependent shape change in response to low levels of TXA_2 but retain the ability to undergo Gq/Ca^{2+}-dependent shape change and aggregation at higher agonist concentrations [9]. Similarly, both Ca^{2+}-dependent/PLCβ and Ca^{2+}-independent/RhoA mechanisms contribute to TXA_2-induced contraction in isolated bovine aortic (Ao) SMCs and in VSM tissue from various other species [10–12]. Notably however, in humans, but not in non-primates, TXA_2 actually signals through two distinct TXA_2 receptor isoforms termed TPα and TPβ that arise through alternative splicing and differ exclusively in their carboxyl-terminal (C tail) domains [13–15]. Whilst it is currently unknown whether TPα or TPβ independently or indeed differentially modulate RhoA activation and downstream signaling, there is substantial evidence that the TPα and TPβ isoforms can differentially regulate other cellular effectors raising that possibility [16–21].

While both TPα and TPβ are predominantly coupled to Gq/PLCβ activation [22], they can independently regulate other secondary effector systems including opposite regulation of adenylyl cyclase via Gs and Gi, respectively [23]. Additionally, TPα, but not TPβ, couples to PLCδ activation via Gh/tissue transglutaminase [24]. Whereas both TPs are expressed in VSMCs, TPα is the predominant isoform expressed in human platelets [25,26]. Consistent with this, in studies investigating intermolecular cross talk between the pro-aggregatory TXA_2 and the inhibitory prostanoid prostacyclin (PGI_2), it was established that Gq/PLCβ coupling and signaling by TPα, but not TPβ, undergoes prostacyclin- induced desensitization mediated through direct cAMP-protein kinase (PK) A phosphorylation of TPα at Ser^{329} within its unique C-tail domain [21,27]. Moreover, Gq/PLCβ signaling by TPα, but not TPβ, is also desensitized by the platelet antagonist /vasodilator nitric oxide (NO), involving direct NO/cGMP-dependent PKG phosphorylation of TPα also within its unique C-tail [20] The implication from these studies is that TPα plays a critical role in vascular haemostasis acting as the major TP target for regulation/inhibition by prostacyclin and NO, such as within the anucleate platelet that predominantly expresses TPα. However, the impact of such direct inhibitory effects of prostacyclin and NO mediated by PKA and PKG, respectively, on signaling by TPα and TPβ through other effector systems, such as through RhoA, is currently unknown but, clearly, any differential regulatory effects by either prostacyclin or NO on such TXA_2 signaling may have direct clinical implications, for example within human VSMCs that express both TPα and TPβ isoforms. Hence, the aim of the current study was to investigate whether TPα and/or TPβ independently regulate G_{12}/Rho activation and signaling and to establish whether that signaling is differentially regulated by the inhibitory prostacyclin/cAMP/PKA and NO/cGMP/PKG systems. These studies provide essential new insights into the physiologic roles of TPα and TPβ and, through studies in primary human aortic smooth muscle cells (1° AoSMCs), reveal an additional mode of regulation of VSM contractile responses by the potent autocoid TXA_2.

2. Materials and methods

2.1. Materials

U46619, SQ29,548, BW245C, FK409, FURA2/AM were purchased from Cayman Chemical Company; SIN-1 and Y-27632 from Calbiochem; 3F10 *anti*-HA, 3F10 *anti*-HA-horseradish peroxidase (HRP)-conjugated antibody and chemiluminescence detection kit from Roche; *anti*-RhoA 26C4 (Sc-418), *anti*-phospho-$RhoA^{Ser188}$ (Sc-32954-R), *anti*-$G\alpha_{12/13}$ H-300 (Sc-28588), *anti*-Gαq C15 (SC-392), HRP-conjugated goat *anti*-mouse (Sc-2005), HRP-conjugated mouse *anti*-goat (Sc-2354) and HRP-conjugated goat *anti*-rabbit (Sc-2004) antibodies from Santa Cruz; Glutathione-Sepharose 4B (GE Healthcare) and FITC conjugated goat *anti*-rabbit antibody from Sigma; *anti*-cofilin (# 3312) and *anti*-phospho-cofilin (phospho^{Ser3}, # 3311) were from Cell Signaling; Alexa Fluor® 488 phalloidin (A12379; Excitation / Emission $A_{495/518\ nm}$) from Molecular Probes; *anti*-HDJ2 antibody from Neomarkers; Opti-MEM® and Oligofectamine® were from Invitrogen. All oligonucleotides were synthesised by Genosys Biotechnologies; small interfering (si) RNAs by Qiagen. Cicaprost was a gift from Schering AG (Berlin, Germany). pcDNA3.1(+):hGαq^{Q209l,D277N}, pCis:Gα_{12}^{G228A} and pCis:Gα_{13}^{G225A} were from the UMR cDNA Resource Center (Gαq) or from Dr S. Offermanns, University of Heidelberg, Germany.

2.2. Cell culture and transfections

Human embryonic kidney (HEK) 293 cells were grown in minimal essential medium (MEM), 10% foetal bovine serum (FBS). HEK.TPα, HEK.TPβ, HEK.TPα^{S329A} HEK.TPα^{S331A} and HEK.TPα^{S329,331A} cell lines stably over-expressing hemagglutinin (HA) -tagged forms of TPα, TPβ, TPα^{S329A},TPα^{S331A} and TPα^{S329,331A} respectively, have been previously described [20,21]. For transfections, HEK 293 cell lines were routinely plated 48 h previously at ~2×10^6 cells/10 cm dish in 8 ml media and co-transfected with 10 μg of pADVA and 25 μg of pCMV-based mammalian expression vector using the calcium phosphate/DNA co-precipitation procedure [20].

Primary (1°) human aortic smooth muscle cells (1° h.AoSMCs) were purchased from Cascade Biologics (C-007-5C) and routinely grown in Smooth Muscle Cell Growth Medium 2 (Promocell GMBH, C-22062) supplemented with 0.5 ng/ml epidermal growth factor, 2 ng/ml basic Fibroblast growth factor, 5 μg/ml insulin, 5% FBS.

2.3. Calcium measurements

Measurements of intracellular calcium ($[Ca^{2+}]_i$) mobilization were carried out in FURA2/AM preloaded HEK 293 cell lines transiently co-transfected with pCMV:Gαq and pADVA some 48 h previously, as described [20]. Cells were stimulated with 1 μM U46619, 1 μM Cicaprost, 1 μM BW245C, 5 μM SIN-1 or 10 μM FK409, unless otherwise specified. Data (Supplemental Figs. 1 and 2) are representative of 3–4 independent experiments and calculated as changes in intracellular Ca^{2+} mobilized ($\Delta[Ca^{2+}]_i$ (nM)) as a function of time (seconds, s) following ligand stimulation.

2.4. Determination of RhoA activation and cofilin phosphorylation

Activated cellular Rho was determined by interaction with a purified glutathione-S-transferase: rhotekin Rho-binding domain (GST-RBD) fusion protein immobilized on Glutathione-Sepharose 4B resin [28]. Preparation of the GST-RBD protein was carried out as previously reported [28]. For the 'Rho-pulldown assay', in brief, HEK.TPα, HEK.TPβ, HEK.TPα^{S329A} HEK.TPα^{S331A}, HEK.TPα^{S329,331A} or 1° h.AoSMCs cells were plated some 48 h previously in complete growth medium onto 10-cm dishes to achieve ~70% confluency; cells were then serum starved for 5 h or 20 h (1° h.AoSMCs cells) in growth media containing 0.1% FBS before stimulation for 0–30 min with 0–10 μM U46619, as indicated in specific figure legends. To assess the effect of prostacyclin, nitric oxide (NO) or PGD_2 on TP-mediated Rho signaling, cells were pre-incubated for 10 min with either 0.01–10 μM Cicaprost; 0.05–50 μM SIN-1; 10 μM FK409 or 1 μM BW245C before stimulation with U46619 (typically 0.1 μM for 10 min). As controls, cells were incubated with an equivalent volume of the drug vehicle, agonist or inhibitor in 0.01% ethanol in HBS (modified Ca^{2+}/Mg^{2+}-free Hank's buffered salt solution) for equivalent incubation times.

Thereafter, cells were lysed in 800 μl Lysis Buffer (125 mM HEPES, pH 7.5, 750 mM NaCl, 5 mM EDTA, 5% NP-40, 10% glycerol, 50 mM $MgCl_2$, and 10 μg/ml each of leupeptin and aprotinin; [29]) and aliquots (600 μl) were subjected to pulldown using Glutathione-Sepharose 4B beads preloaded with 30 μg GST-RBD, essentially as previously described [28]. Following washing, precipitated GTP-bound RhoA was subjected to SDS-PAGE on 12.5% acrylamide gels and immunoblotted with *anti*-RhoA monoclonal antibody (Sc-418), followed by chemiluminescence detection [21]. In parallel, to confirm equivalent RhoA protein expression in the cell lysates and uniform protein loading, aliquots of whole cell lysates (typically 10 μl, corresponding to 1.25% of total cell lysate) were directly immunoblotted with the *anti*-RhoA antibody and/or with the anti-HDJ2 antibody. Similarly, to assess U46619-mediated cofilin phosphorylation and activation, aliquots of whole cells lysates (typically 10 μl, corresponding to 1.25% of total cell lysate) were first immunoblotted with *anti*-phospho^{Ser3} cofilin antibody; thereafter, phospho-cofilin blots were stripped and rescreened versus *anti*-cofilin antibody to normalise for total cofilin protein expression and/or with the anti-HDJ2 antibody to confirm uniform protein loading in each of the assays. All images of RhoA expression/pulldown or cofilin phosphorylation and/or expression were captured using Adobe Photoshop (V6), where band width and intensity was quantified and represented as fold increases relative to basal levels. To account for biological variations in basal activation levels, experiments were normalised to within a comparable range based on measurements from more then 20 individual experiments for each cell type.

2.5. F-actin staining

HEK 293 cell lines or 1° h.AoSMCs, grown on coverslips for 3 days to achieve approximately 50% confluency, were serum-starved for 2 h in growth media containing 0.1% FBS, prior to stimulation with U46619 (0–1 μM; typically 10 nM U46619). To assess the role of prostacyclin or NO, cells were pre-incubated for 10 min with either 0.01–10 μM Cicaprost or 0.05–50 μM SIN-1 before stimulation with U46619 (typically 10 nM for 10 min). F-actin polymerization was stained by the addition of Alexa Fluor® 488 phalloidin essentially as described by the supplier (Molecular Probes) and slides were imaged using an Axioplan 2 Imaging Universal Microscope.