Physiological Significance of Thromboxane A₂ Receptor Dimerization

Masako Sasaki¹,², Katsutoshi Miyosawa¹, Satoko Ohkubo¹, and Norimichi Nakahata¹,²,*

¹Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan
²Tohoku University 21st Century COE Program “CRESCENDO”, Sendai 980-8578, Japan

Received September 26, 2005; Accepted February 7, 2006

Abstract. The thromboxane A₂ receptor (TP), one of the G protein-coupled receptors (GPCRs), consists of two splicing variants, TPα and TPβ, which differ in their C-terminal regions. In the present study, we investigated whether TPα and TPβ formed homo- or hetero-dimers and whether the dimerization changed the function of TP. The immunofluorescent analysis using human embryonic kidney (HEK) 293 cells expressing either FLAG-tagged TPα or TPβ showed that TPα is mainly distributed on plasma membranes and TPβ existed on plasma membranes and within the cells. Co-immunoprecipitation analysis using HEK293 cells expressing both TPα and TPβ showed that TPα and TPβ formed homo- and hetero-dimers. U46619, a TP agonist, caused phosphoinositide hydrolysis and elevation of [Ca²⁺]i in a concentration-dependent manner in Chinese hamster ovary (CHO) cells expressing TPα or TPβ. The responses were observed to a greater extent in the cells expressing TPα than TPβ. In the cells expressing both TPα and TPβ, U46619-induced responses were observed to a lesser extent than in the cells expressing TPα alone. Furthermore, [³H]SQ29548 binding showed that the level of the cell surface expression of TP was the following order: the cells expressing TPα > TPα and TPβ > TPβ. These results indicate that TPα and TPβ formed homo- and hetero-dimers, and TP-mediated signaling may be regulated by the hetero-dimer.

Keywords: thromboxane A₂ receptor, thromboxane A₂ receptor (TP) α, TPβ, dimerization, phosphoinositide hydrolysis

Introduction

Thromboxane A₂, one of arachidonic acid metabolites, acts as a stimulator of platelets and a constrictor of smooth muscle cells (1). Thromboxane A₂ receptor (TP) is a member of the G protein-coupled receptor (GPCR) family cloned as a prostanoid receptor for the first time (2), and it is a representative Gq-coupled receptor causing activation of phospholipase C (PLC) (3). Recently, it is shown that TP has a role in immune systems, clarified by the analysis using TP-deficient mice (4).

Similar to many GPCRs (5), human TP has splicing variants, which differ in their C-terminal tails. TPα, a first cloned isoform (2) (also called as placental type), consists of 343 amino acids, while TPβ (also named as endothelial type) consists of 407 amino acids (6). Recent investigations demonstrated that many kinds of cells or tissues including endothelial cells expressed both TPα and TPβ (7). There are some reports that mentioned the different characteristics of TPα and TPβ. Hirata et al. (8) showed that although both isoforms are coupled with the Gq/PLC pathway, the stimulation of TPα results in elevation of cyclic AMP, but the stimulation of TPβ causes a decrease in cyclic AMP. Yukawa et al. (9) indicated that the two isoforms of TP were desensitized in different manners. It has been demonstrated that TPβ undergoes tonic (10) and agonist-induced (11) internalization. However, the difference between TPα and TPβ with respect to their physiological roles remains unclear. Moreover, it has not been identified how the function of the splicing variants are regulated.

Recently, it has been shown that various GPCRs form homo- and/or hetero-dimers (12, 13). However, it is not
clear why GPCRs form dimers. One of the roles of dimerization of GPCRs is suggested to be for maturation and intracellular translocation of GPCRs (14–16). Hetero-dimerization between GPCR splicing variants has been reported, such as vasopressin V2 receptor, CCR5 calcitonin receptor, neurotensin NTS2 receptor, D3 receptor, and α1A adrenoceptor (17–21). On the other hand, some natural mutants of GPCRs retained themselves within the cells, most likely in the endoplasmic reticulum (ER) (17, 18, 20, 22). It is assumed that there is a close relationship between dimerization and quality control of GPCRs. The decrease in cell surface receptor is assumed to result in reduction of the receptor-mediated signal transduction. Since thromboxane A2 is unstable with a chemical half-life of 30 s, the expression level of cell surface TP is crucial to demonstrate the amplitude of receptor-mediated signal transduction.

In the present study, we investigated whether TPa and TPβ formed homo- and/or hetero-dimers. In addition, we investigated TP-mediated signal transduction was changed when TPa and TPβ were co-transfected into the cells.

Materials and Methods

Materials

U46619 was purchased from Cayman Chemical (Ann Arbor, MI, USA). The expression vector pEGFP-N1 and monoclonal anti-GFP antibody was from Clontech (Palo Alto, CA, USA), and anti-FLAG antibody was from Sigma (St. Louis, MO, USA). The expression vector pcDNA3.1(+) and Lipofectamine 2000 was from Invitrogen (Merelbeke, Belgium). The HRP conjugated anti-mouse IgG and Enhanced Chemiluminescence reagent were from Amersham Biosciences (Piscataway, NJ, USA). FITC conjugated anti-mouse IgG was from Serotec (Oxford, UK). Alexa Fluor 594 conjugated anti-mouse IgG was from Molecular Probes (Eugene, OR, USA). G418 solution was purchased from Calbiochem (San Diego, CA, USA). Fura-2 AM was from Dojindo (Kumamoto).

Cell culture and transfection

Human embryonic kidney (HEK) 293 cells and Chinese hamster ovary (CHO) cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Nissui, Tokyo) containing 10% FCS, 50 unit/ml penicillin, and 50 μg/ml streptomycin in a humidified incubator with a 5% CO2 atmosphere at 37°C. Transfections were done with Lipofectamine 2000 according to the manufacturer’s recommendations. HEK293 cells were stably transfected with FLAG-TPα or FLAG-TPβ, and cell clones were selected with 400 μg/ml G418.

Construction of plasmids

The cDNAs encoding wild type TPa (HPL/pBlue-script) was generous gift from Dr. S. Narumiya (Kyoto University, Kyoto), and TPa/pcDNA3.1(+) was constructed by inserting their cDNAs digested with BamHI/HincII into pcDNA3.1(+) digested with BamHI/EcoRV. Plasmid encoding wild type TPβ was constructed by inserting two fragments into pcDNA3.1(+).

The first fragment was obtained by digestion of HPL/pBlue-script at the EcoRI/BsiHI site. The second fragment was obtained by RT-PCR using RNA from 1321N1 human astrocytoma cells with a sense primer (5'-AAAGTCGACAAGAGCCGTCTCAGGC GTTCCTAGCC-3') and an antisense primer (5'-TTGCG GCCGCTCAATCCTTTCTGGACAGGCCCTTCCC-3'), and the fragment was digested with BstNI/HindIII. Resulting cDNA fragments were cloned into pcDNA3.1(+) digested with EcoRI/BsoI together. FLAG-tagged and HA-tagged clones of TPa or TPβ were constructed as described before (11). For construction of GFP-tagged TPa, the cDNA encoding TPa was subcloned into pEGFP-N1 at NheI/PsiI site. GFP-tagged TPβ was constructed by RT-PCR with a sense primer (5'-GGAGATGATGGCTCAGCTCCTG GCCAGAGCTTCCC-3') and an antisense primer (5'-AAACTGCAGATCCTTTCTGGACGGCCTTCCC-3') including the BglII/PstI site. All PCR was performed by pfu turbo DNA polymerase (Stratagene, Tokyo) and the DNA sequence was confirmed by ABI310 (Applied Biosystems, Foster City, CA, USA).

Immunofluorescence

HEK293 cells stably expressing FLAG-TPα or TPβ were grown on coverglasses coated with 100 μg/ml poly-L-lysine (Sigma) and fixed with 4% paraformaldehyde/PBS. After washing the cells twice with PBS, the cells were blocked and permeabilized with 0.05% Triton X-100/5% nonfat milk/PBS for 30 min at 37°C. Then, the cells were incubated with anti-FLAG M1 antibody (1:50) for 1 h and incubated with FITC conjugated anti-mouse IgG (1:40) for 1 h. The cells were washed 5 times with PBS and the fluorescence was analyzed by OLYMPUS microscope (Olympus, Tokyo).

Co-immunoprecipitation and Western blot analysis

HEK293 cells were co-transfected with the indicated plasmid mixtures. Empty pcDNA3.1(+) vector or pEGFP-N1 vector was always added in order to transfect a constant amount of plasmid DNA. At 48-h post-transfection, the cells were washed with TBS (20 mM
with four hours after transfection, the cells were labeled and added to be transfected equal amount of DNA. Twenty-Assay of inositol phosphates (Leica, Wetzlar, Germany). 

Confocal microscopy

HEK293 cells transfected with both GFP-TPα and FLAG-TPβ were grown on coverglasses coated with 100 μg/ml poly-l-lysine and fixed with 4% paraformaldehyde/PBS. After washing the cells twice with PBS, the cells were blocked and permeabilized with 0.05% Triton X-100/5% nonfat milk/PBS for 30 min at 37°C. Then, the cells were incubated with anti-FLAG M2 antibody (1:500) for 1 h and incubated with Alexa Fluor 594 conjugated anti-mouse IgG (1:500) for 1 h. The cells were washed 5 times with PBS and the fluorescence was analyzed by Leica confocal microscopy (Leica, Wetzlar, Germany).

Assay of inositol phosphates

CHO cells were transiently transfected with the indicated plasmid, and then empty vector was always added to be transfected equal amount of DNA. Twenty-four hours after transfaction, the cells were labeled with myo-[3H]inositol at 2 μCi/mL for another 24 h. After labeling, cells were washed twice with modified Tyrode’s solution (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, 0.18 mM CaCl₂, and 5.6 mM glucose, pH 7.4) with 20 nM [3H]SQ29548 for 30 min at 37°C. The reaction was stopped by addition of ice-cold washing buffer (10 mM Tris-HCl, 140 mM NaCl, pH 7.4) and filtrated with a GC50 filter paper (Advantec, Tokyo). After an additional two washes with washing buffer, the radioactivity in the filter paper was counted. Specific binding was obtained by subtracting non-specific binding in the presence of 10 μM SQ29548.

Statistical analysis of data

All data were expressed as the mean ± S.E.M. Statistical significance was determined with two-way ANOVA or Tukey’s post-hoc test for multiple comparisons. P<0.05 was considered to be significant.

Results

The cell surface expression of TPα and TPβ

To examine the subcellular localization of TPα and TPβ, we generated HEK293 cells stably expressing either FLAG-TPα or FLAG-TPβ. In these cells, the FLAG-TPα fluorescence was only found at the cell surface, while FLAG-TPβ fluorescence was found at both the cell surface and inside of the cell (Fig. 1).

Dimerization of TPα and TPβ

To determine whether TPα and TPβ dimerize and/or oligomerize, we performed co-immunoprecipitation analysis using HEK293 cells expressing FLAG-tagged TPα or TPβ with HA-tagged TPα or TPβ. The immuno-
Fig. 1. Subcellular localization of TPα and TPβ. HEK293 cells stably expressing either FLAG-TPα or FLAG-TPβ were permeabilized and incubated with anti-FLAG antibody. The cells were further incubated with FITC conjugated anti-mouse IgG. The figure shows HEK293 cells expressing FLAG-TPα (a) and FLAG-TPβ (b).

Fig. 2. Co-immunoprecipitation of TPα and TPβ. A: HEK293 cells were transiently co-transfected with FLAG-TPα/HA-TPα (lane 1), FLAG-TPα/HA-TPβ (lane 2), FLAG-TPβ/HA-TPα (lane 3), FLAG-TPβ/HA-TPβ (lane 4), vector/HA-TPα (lane 5), or vector/HA-TPβ (lane 6). Forty-eight hours after transfection, the cells were solubilized in lysis buffer and FLAG-TPs were immunoprecipitated with anti-FLAG agarose. Immunoprecipitated proteins were subjected to SDS-PAGE and detected by anti-HA antibody. Figure shows 27 – 33-kDa (non-glycosylated) and 50 – 65-kDa (glycosylated) proteins for HA-TPα and 33 – 40-kDa (non-glycosylated) and 50 – 67-kDa (glycosylated) proteins for HA-TPβ. B: HEK293 cells were transiently co-transfected with FLAG-TPα/GFP-TPα (lane 1), FLAG-TPβ/GFP-TPα (lane 2), FLAG-TPα/GFP-TPβ (lane 3), FLAG-TPβ/GFP-TPβ (lane 4), or vector/vector (lane 5). Forty-eight hours after transfection, the cell lysates obtained from these cells were immunoprecipitated with anti-FLAG agarose and subjected to SDS-PAGE. The proteins were detected by anti-GFP antibody. Figure shows 35 – 52-kDa (non-glycosylated) and 70 – 83-kDa (glycosylated) proteins for GFP-TPα and 40 – 58-kDa (non-glycosylated) and 75 – 85-kDa (glycosylated) proteins for GFP-TPβ.

Fig. 3. Co-localization of TPα and TPβ. HEK293 cells expressed with both GFP-TPα and FLAG-TPβ were permeabilized and incubated with anti-FLAG antibody. The cells were further incubated with Alexa Fluor 594 conjugated anti-mouse IgG. The figure shows the fluorescence of GFP-TPα (a) and FLAG-TPβ (b) and fluorescences merged (c).
precipitated materials with anti-FLAG antibody were subjected to SDS-PAGE and blotted with anti-HA antibody (Fig. 2A). The immunoprecipitated proteins obtained from the cells expressing FLAG-TPα and HA-TPβ showed HA-TPα of 27–33 and 50–65 kDa, demonstrating that TPα forms a homo-dimer (lane 1). Lane 2 showed 33–40 and 50–67 kDa bands of HA-TPβ, demonstrating that TPα and TPβ form a hetero-dimer. Lane 3 showed bands of HA-TPα correspondingly to the result of lane 2. The immunoprecipitated proteins obtained from the cells expressing FLAG-TPβ and HA-TPβ showed a band of HA-TPβ, showing that TPβ forms homo-dimers (lane 4). In the cases using GFP-tagged TPs instead of HA-tagged TPs (Fig. 2B), we found GFP-TPα (lane 1, 2: 35–52 kDa, 70–83 kDa) or GFP-TPβ (lane 3, 4: 40–58 kDa, 75–85 kDa) in the immune complexes, respectively. These results suggest that TPα and TPβ dimerize or oligomerize in a homo- and hetero-fashion.

**Co-localization of TPα and TPβ**

We examined intracellular localization of TPα and TPβ immunohistochemistry using confocal microscopy (Fig. 3). After GFP-TPα and FLAG-TPβ were co-expressed in HEK293 cells, the cells were immuno-labeled with anti-FLAG M2 antibody and Alexa Fluor 594-conjugated anti-mouse IgG to visualize TPβ. GFP-TPα labeled with anti-FLAG M2 antibody and Alexa Fluor 700–83 kDa) or GFP-TPβ (open circle) or co-transfected with GFP-TPα and FLAG-TPβ (triangle). Forty-eight hours after transfection, the cells were stimulated by U46619 for 20 min, and accumulated inositol phosphates were measured as described under Materials and Methods. The levels of inositol phosphates are shown as the fold increase above basal, and data are expressed as the mean ± S.E.M. (n = 3). *Significantly different from GFP-TPα (P < 0.05, two-way ANOVA). The figure shows representative result from three independent experiments. B: Expressed level of GFP-TPα in the cells transfected with GFP-TPα (lane 1) or co-transfected with both GFP-TPα and FLAG-TPβ (lane 2). C: CHO cells were transiently transfected with TPα/vector, TPβ/vector, or TPα/TPβ. Cells were stimulated with 1 µM U46619 and the [Ca²⁺]i elevation was measured. Each column represents the mean ± S.E.M. (n = 3). *P < 0.05 (Tukey’s test).

**Effect of co-expression of TPα and TPβ on U46619-induced phosphoinositide hydrolysis and [Ca²⁺]i elevation**

We examined whether the hetero-dimerization of TPα and TPβ changed the function of TP. U46619, a TP agonist, caused the accumulation of inositol phosphates (IPs) in a concentration-dependent manner in both CHO cells expressing GFP-TPα and FLAG-TPβ (Fig. 4A). However, the accumulated IPs were greater in the cells expressing TPα than TPβ. In the cells expressing both GFP-TPα and FLAG-TPβ, U46619 caused IP accumulation to a smaller extent than in the cells expressing GFP-TPα alone (Fig. 4A). The expressed level of GFP-TPα was almost the same between the cells expressing GFP-TPα alone and GFP-TPα with FLAG-TPβ (Fig. 4B). The decrease of U46619-induced phosphoinositide hydrolysis in the cells co-expressing TPα and TPβ was also observed as well as both GFP-TPα and FLAG-TPβ in the cells expressing both wild-type TPα and TPβ (data not shown).

Next, [Ca²⁺]i was measured to confirm the results of phosphoinositide hydrolysis. U46619-induced increase in [Ca²⁺]i was greater in the cells expressing TPα than TPβ, consistent with the result of phosphoinositide hydrolysis. In the cells co-expressing both TPα and TPβ, U46619 increased [Ca²⁺]i to a smaller extent than in the cells expressing TPα alone (Fig. 4C). These findings indicate that the hetero-dimerization of TPα and TPβ reduced TP-mediated signal transduction, and a possible
Incubated with 20 nM \(^3\text{H}\)SQ29548 using this question, we performed a receptor binding assay. The surface expression of TP might be reduced under the condition of co-expression of TP and TP\(_\beta\). It is uncertain where GPCRs form dimers. The dimerization of GPCRs and their quality control. For example, it has been shown that \(\alpha1\text{D}\) and \(\alpha1\text{B}\) adrenceptors form hetero-dimers and that the \(\alpha1\text{B}\) adrenceptor is essential for the cell surface expression of \(\alpha1\text{D}\) adrenceptor (15). Metabotropic GABA\(_{\beta1}\) receptor forms hetero-dimers with GABA\(_{\beta2}\) receptor and the hetero-dimerization masks an ER retention signal of the C-terminus of GABA\(_{\beta1}\) receptor. Consequently, the GABA\(_{\beta1}\) receptor could translocate to the plasma membrane (25).

In contrast to the cases of \(\alpha1\) adrenceptor and GABA\(_{\beta}\) receptor, some natural splicing variants of GPCRs cause receptor retention within the cells, most likely in the ER, such as CCR5 receptor, vasopressin V\(_2\) receptor, and D\(_1\) dopamine receptor (17, 18, 20). In these cases, the receptor-mediated signals were weakened in the presence of the corresponding splicing variant. For example, cell surface expression of calcitonin receptor was abrogated by its alternative splicing variant, followed by decrease in calcitonin-induced cyclic AMP elevation and phosphorylation of ERK (22). Such a functional regulation was also shown in other GPCRs such as vasopressin V\(_2\) receptor (17). One possible role of the splicing variant of GPCRs is, therefore, thought to decrease its cell surface expression and to decline its signal as an apparent dominant-negative mutant.

It is uncertain where GPCRs form dimers. The dimer could be formed on the plasma membrane or inside of the cells, for example, ER, an organelle that has a crucial role in the quality control of protein synthesis (26). TP has been reported to have N-glycosylation sites at its N-terminus (27), and the glycosylation was believed to be necessary for its cell surface expression. The glycosylated TPs showed a broad protein band of 55 – 70 kDa in SDS-PAGE, and the deglycosylated forms of TP and TP\(_\beta\) were 28 kDa and 32.5 kDa, respectively (28). We have shown in Fig 2 that immunoprecipitated proteins contain both non-glycosylated and glycosylated TPs, and the major TP is a non-glycosylated form. Figure 3 shows that TPs co-localize on plasma membranes and intracellular regions. Considering these results, the dimerization of TPs may have occurred principally before the glycosylation, most likely in the ER; and TP\(_\beta\), which tends to be retained in an intracellular compartment, holds TP\(_\alpha\) within the cell according to their heterodimerization.

It is important whether dimers were formed constitutively or in an agonist-dependent manner. Several reports showed that the dimerization occurred in an agonist-dependent manner (29, 30), while some reports

![Fig. 5. Effect of co-expression of TPs and TP\(_\beta\) on cell surface expression of TPs. CHO cells were transiently transfected with TP\(_\alpha\)/vector, TP\(_\beta\)/vector, or TP\(_\alpha\)/TP\(_\beta\). Forty-eight hours after transfection, the cells were lysed and 200 \(\mu\)g of the cell membranes per tube were used for the determination of TPs on the membrane. The expressing TPs were measured by receptor binding assay using \(^3\text{H}\)SQ29548. Each column represents the mean \(\pm\) S.E.M. (n = 3). *P<0.05 (Tukey’s test).](image-url)
showed that it was constitutive (22, 31). In our preliminary experiments of co-immunoprecipitation, 1 μM U46619 did not affect the formation of the heterodimer of TPα and TPβ until 20 min (M. Sasaki and N. Nakahata, unpublished observation). Thus, it is suggested that TPα and TPβ form a hetero-dimer in the ER rather than in the plasma membrane, and TPβ may inhibit the cell surface expression of TPα, probably through their hetero-dimerization. Physiological role of hetero-dimerization between TPα and TPβ probably involves the quality control of newly synthesized TPs and the translocating of TPs toward the plasma membrane. Because the immunoprecipitated complex may contain other proteins in addition to TPα and TPβ, we cannot exclude a possibility that TPα and TPβ are associated indirectly. Thus the further study was necessary to clarify the direct association of TPα and TPβ on the molecular level.

In the present study, we demonstrated that TPα and TPβ formed hetero-dimers, and the formation of hetero-dimers resulted in the reduction of TP-mediated signaling through the decrease in the cell surface expression of TP. In other words, the proportion of expressed TPα and TPβ is important for eliciting the function of TP. Thus a further study would be necessary to determine the mechanism regulating the proportion of splicing variants to clarify the molecular regulatory mechanism of TP-mediated signal transduction.

Acknowledgments

We are grateful to Dr. Shuh Narumiya (Kyoto University, Kyoto) for providing HPL/pBluescript plasmid. This work was partly supported by Grants-in-Aid for Scientific Research from Japan Society for the Promotion of Science (No. 14370737 to N.N.).

References

27 Kelley LP, Kinsella BT. The role of N-linked glycosylation in determining the surface expression, G protein interaction and effector coupling of the alpha ($\alpha$) isoform of the human thromboxane A$_2$ receptor. Biochim Biophys Acta. 2003;1621:192–203.