Thromboxane A₂ Receptors in Prostate Carcinoma: Expression and Its Role in Regulating Cell Motility via Small GTPase Rho

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Abstract

Thromboxane A₂ (TxA₂) is a prostanoid formed by thromboxane synthase using the cyclooxygenase product prostaglandin H₂ as the substrate. Previously, increased expression of thromboxane synthase was found in prostate tumors, and tumor cell motility was attenuated by inhibitors of thromboxane synthase. This study was undertaken to elucidate how tumor motility is regulated by TxA₂. Here, we report that human prostate cancer cells express functional receptors for thromboxane synthase. This finding is consistent with the hypothesis that TxA₂ is an autocrine factor of prostate cancer cells. We demonstrate that pretreatment with the TP antagonist SQ29548 or pinanethromboxane A₂ (TxA₂), and pretreatment of PC-3 cells with Y27632, a Rho kinase (ROCK) inhibitor, blocked U46619-induced cell contraction. A dominant-negative mutant of RhoA also blocked U46619-induced cell contraction. Taken together, the data suggest that TxA₂ is expressed in prostate cancer and activation of TPs regulates prostate cancer cell motility and cytoskeleton reorganization through activation of Rho. [Cancer Res 2008;68(1):115–21]

Introduction

Cyclooxygenases (COX) convert arachidonic acid to prostaglandin H₂, which can give rise to various bioactive prostanoids via different downstream isomerases. The second isoform of COX (COX-2) is frequently up-regulated in various cancers, including prostate cancer (1). Oncogenes such as ras or Her-2 stimulate COX-2 expression (2), whereas tumor suppressors such as p53 down-regulate COX-2 expression (3). COX-2 has been a promising target for prevention and treatment of cancer (4). However, there are some caveats targeting COX-2 directly for cancer chemoprevention or treatment by using nonsteroidal anti-inflammatory drugs (NSAID), such as aspirin or COX-2-specific inhibitors. Long-term use of NSAIDs, especially aspirin, can have serious side effects, such as gastrointestinal bleeding. In addition, the use of COX-2-specific inhibitors for arthritic pains has been linked to an increased risk of cardiovascular events (5, 6). Therefore, alternative approaches are highly desirable to inhibit COX-mediated cancer initiation or progression.

Among five primary prostanoids from the COX pathway of arachidonic acid metabolism, prostaglandin E₂ is reported to promote tumor angiogenesis (7) and represents a novel angiogenic switch in mammary cancer progression (8). Emerging evidences suggest a possible involvement of another prostanoid, TxA₂, in tumor progression. TxA₂ is a potent vasoconstrictor, mitogen, and platelet activator (9–11). Increased thromboxane synthase expression and/or elevated levels of TxB₂, the stable product of TxA₂, were found in papillary thyroid carcinoma (12), larynx squamous cell carcinoma (13), and renal carcinoma (14). Previously, we reported an increase in thromboxane synthase at mRNA level in renal carcinoma, breast carcinoma, prostate cancer, and uterine cancer when compared with their matched normal tissues in a cancer profiling array (15). In prostate cancer, the expression of thromboxane synthase was increased in tumor specimens of advanced stage and grade and particularly in the areas of perineural invasion (15, 16). Thromboxane synthase expressed in prostate cancer cells was enzymatically active and may play a contributory role in tumor progression, especially tumor cell motility (15, 17, 18).

It is unknown, however, how thromboxane synthase affects tumor migratory phenotype. Herein, we report that functional TP(s), the G protein–coupled receptor (GPCR) for TxA₂, is expressed in prostate tumor cells as well as in tumor specimens. Further, activation of this receptor by TxA₂ mimetics has profound effects on tumor cell cytoskeleton organization and causes contraction through the small GTPase RhoA. Our studies suggest that TxA₂ and its cognate receptor(s) play a signaling role in tumor progression associated with thromboxane synthase expression in prostate cancer.

Materials and Methods

Materials and reagents. SQ29548, pinanethromboxane A₂ (TxA₂), and U46619 were from Cayman Chemical Co. or Biomol. [²H]SQ29548 was purchased from NEN Life Science Products, Inc. Y27632 was from Calbiochem. Rho activation assay kit was obtained from Pierce. Rabbit polyclonal antibodies against TP were kindly provided by Dr. Tai (University of Kentucky, Lexington, KY) or purchased from Cayman Chemical or Santa Cruz Biotechnology.

Cell culture. PC-3 and DU145 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 100 µg/mL each of penicillin/streptomycin at 37°C under an atmosphere of 95% air, 5% CO₂ as previously described (15).

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Immunohistochemistry. For tissue immunostaining for TP expression, paraffin-embedded tissue sections or tissue arrays were deparaffinized, rehydrated, and antigen retrieved by placing in Declere working solution (Cell Marque) in an electric pressure cooker for 15 min. After a hot rinse with boiling Declere, slides were cooled for 5 min. After washing in deionized water, slides were processed for immunohistochemical staining using a Zymed Histostain-SP kit according to the manufacturer’s instructions.

Immunocytochemistry. Cells were grown on glass coverslips in RPMI 1640-10% FBS overnight and changed into serum-free medium. Cells were fixed for 10 min in 3.7% paraformaldehyde solution at room temperature, washed, and then blocked with 3% bovine serum albumin (BSA) in PBS for 30 min at room temperature. For immunostaining for TPs or other proteins, antibodies were diluted in 1% BSA in PBS, added onto cell monolayers, and incubated for 45 min at room temperature. After washing in PBS-1% BSA thrice, cells were incubated with Alexa Fluor 488–conjugated or Alexa Fluor 568–conjugated secondary antibodies (Invitrogen) for 30 min at room temperature. After staining, the cells on coverslips were washed and mounted with Gold antifade mounting medium (Invitrogen).

For live cell staining, cells cultured on cover glass were blocked with 3% BSA for 20 min and then incubated with primary antibody raised against the first 120 amino acids at the NH2 terminus of TP (H-120, 1:25; Santa Cruz Biotechnology) for 45 min at 4°C. After washing with ice-cold PBS, the cells were incubated with Alexa Fluor 488–conjugated secondary antibody. The staining was immediately examined under an epifluorescence microscope.

Cells not incubated with the primary antibody but with the secondary antibody were used as negative controls.

Western blot. PC-3 and DU145 cells were grown to 90% confluence on 10-cm dishes and collected with ice-cold complete radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris (pH 8.0)] containing protease inhibitor cocktail (Sigma). Cell debris was removed by centrifugation. Protein concentration was measured using bicinchoninic acid protein assay kit (Pierce). Proteins samples (30 μg) were mixed with 6× SDS sample buffer and subjected to electrophoresis in 8% SDS-PAGE gels and transferred to 0.45 μm of polyvinylidene difluoride membranes. After transfer, the membrane was blocked with TBS containing 5% low fat milk for 60 min and then incubated with the primary antibody against TP receptor (Cayman Chemical) at the dilution of 1:1,000 in TBS-Tween 20 with 5% low fat milk at 4°C overnight. The membrane was washed (thrice) with TBS-Tween 20 (0.1%) and probed with goat anti-rabbit fluorescently labeled secondary antibody (1:5,000) for 1 h at room temperature and washed (thrice) with TBS-Tween 20 for a total of 15 min. The immunoblots were visualized and the target bands were quantified by IR imaging system (Odyssey).

Determination of the TXA2 receptor binding sites. PC-3 cells were seeded in 24-multiwell plates at a density of 1.5 × 10^4 per well. When the cells reached confluence, the binding test was performed. The binding buffer was prepared by combining 9 volumes of modified HEPES-Tyrode's buffer [10 mmol/L HEPES, 129 mmol/L NaCl, 2.8 mmol/L KCl, 8.9 mmol/L NaHCO3, 1.8 mmol/L KH2PO4, 0.8 mmol/L MgCl2, 5.6 mmol/L dextrose, 0.001% sodium azide, and 0.02% sodium meta bisulfite] with 1% bovine serum albumin. After equilibration for 1 h at 37°C, the cells were incubated with radiolabeled TXA2 receptor agonist (115 nM [3H]-leukotriene B4) and unlabeled TXA2 receptor agonist (1 μM [3H]-leukotriene B4) for 1 h at 37°C. The cells were washed (thrice) with ice-cold saline, harvested, and solubilized in 1% sodium dodecyl sulfate (SDS) and the radioactivity was measured using liquid scintillation spectrophotometer.
Results and Discussion

TP expression in human prostate carcinoma. Previously, we found that thromboxane synthase is expressed in prostate cancer cells and its expression is positively related to tumor progression (15). The arachidonate product of thromboxane synthase, TXA2, can elicit cellular signaling through its cognate GPCRs (TP). To study whether TP is involved in prostate cancer progression, we first evaluated the expression of TP at protein level in normal and cancerous human prostate tissues by immunohistochemistry. Tissue slides were stained with rabbit anti-TP antibody. As shown in Fig. 1A, the benign gland (left) showed weak cytoplasmic staining for TP (the stain on luminal surface is likely artifact), whereas the neoplastic glands from the same patient showed strong cytoplasmic staining for TP (right). The clinical relevance of TP expression is also supported by the observations that increased TP expression was associated with extracapsular extension and invasion of the seminal vesicles by prostate tumors (16).

Next, we determined the expression of TPs in human prostate carcinoma cells by immunocytochemistry. As shown in Fig. 1B, TPs were mainly localized in the perinuclear region of the cytosol of PC-3 cells. Western blot analysis confirmed the expression of both TPs and TPβ isoforms in PC-3 cells (Fig. 1C). The level of total TP proteins in DU145 cells (6.3) was approximately a half of those in PC-3 cells (12.07). Further, in PC-3 cells, TPα constituted 28% of
total TP proteins. In contrast, TPα accounted for only 7% of total TP proteins in DU145 cells (Fig. 1D).

**Surface expression and ligand binding activities of TPs expressed in prostate cancer.** The predominant localization of TP in the cytosol of prostate cancer cells led us to examine whether a subset of TPs is expressed at cell surface. A polyclonal antibody raised against the NH₂-terminal 120-amino acid residues of TP was used to stain live, nonpermeabilized cells. As shown in Fig. 2A, PC-3 and, to a less extent, DU145 cells had positivity of staining at cell surface, suggesting that a subset of TPs could be localized at plasma membrane.

To examine the functionality of TPs expressed, ligand binding assays were performed using SQ29548, a high-affinity antagonist of TP (19). Confluent PC-3 or DU145 cells in the 24-well plates were treated with [³H]SQ29548 alone (for total binding) or with [³H]SQ29548 plus excess unlabeled SQ29548 (for nonspecific binding). The specific binding was obtained by subtracting nonspecific binding from total binding. As shown in Fig. 2B, live PC-3 cells can bind SQ29548 in a saturable manner, suggesting the presence of receptors on cell surface. Nonlinear regression analysis of the specific binding data revealed that PC-3 cells bind to SQ29548 with $B_{\text{max}}$ of 120.4 fmol per million cells and $K_d$ of 3.641 nmol/L. The $B_{\text{max}}$ and $K_d$ of DU145 cells toward SQ29548 were 51.47 fmol per million cells and 8.06 nmol/L, respectively. The lower $B_{\text{max}}$ of DU145 cells, when compared with that of PC-3 cells, is consistent with the pattern of TP expression at protein levels (Fig. 1C and D) and TP localization at cell surface (Fig. 2A).

Taken together, the data suggest that at least a subset of TPs is expressed at the cell surface and further prostate cancer cells bind to TP ligand in a specific and saturable manner. Interestingly, the $K_d$ values of PC-3 and DU145 cells are slightly lower than the $K_d$ values of SQ29548 for TPα (12.4 nmol/L) and for TPβ (10 nmol/L; ref. 20). Both isoforms are expressed in PC-3 cells, whereas in DU145 cells TPβ is predominantly expressed. Further studies are needed to determine whether the increased affinity of TP(s) expressed in prostate cancer cells is due to mutations/variations in primary sequences and/or to potential alterations in their binding partners that may lead to alterations in its affinity toward agonists or antagonists, such as SQ29548.

**Induction of cell contraction by TP activation.** When plated in serum-free medium, PC-3 cells presented a spindle-shaped morphology. On treatment with U46619 (300 nmol/L), an agonist of TP (21–23), cells contracted and presented a “round” shape within 5 to 15 min (Fig. 3A). Similar results were also obtained with another TP agonist, IBOP (data not shown; refs. 24, 25). To study whether the induction of cell contraction by U46619 was mediated by TP receptors, we pretreated PC-3 cells with SQ29548 (10 μmol/L) or PTA2 (10 μmol/L), two TxA₂ receptor antagonists that have...
higher affinity to TPs than U46619 does (19, 26). As shown in Fig. 3, SQ29548 and PTA2 blocked the induction of PC-3 cell contraction by U46619. The blockade of U46619-induced cell contraction by TP antagonists suggests that TxA2 mimetic U46619 induces cell contraction through TP receptors and that the induction of cell contraction by U46619 can be blocked by high-affinity antagonists of TPs, such as SQ29548. The data also suggest that TP(s) expressed in prostate cancer cells is able to transduce extracellular signals to the inside of the cells.

**Regulation of tumor cell motility by TxA2-TP signaling.** Cell contraction is an integral part of cell migration. During the migratory process, cells form lamellipodia or filopodia at the leading edge to charter cells forward. At the trailing edge, however, cells must detach and retract to enable cells to move forward (27). To study whether cell contraction, mediated by TxA2-TP, has effects on prostate cancer cell migration, the effects of U46619 and SQ29548 on PC-3 cell migration were examined using the modified Boyden chamber assay. As shown in Fig. 4A, U46619 stimulated the migration of PC-3 cells at very low concentration (30 nmol/L). However, at higher concentration (300 nmol/L or 3 μmol/L), the migration of PC-3 cells was significantly compromised. In DU145 cells, the effects of U46619 were modest; only at high concentration (3 μmol/L), U46619 had significant inhibition on cell migration (Fig. 4A). Interestingly, blockade of TP activation with SQ29548 or PTA2 also significantly reduced PC-3 migration (Fig. 4B), suggesting that TP activation is required for PC-3 cell motility.

Taken together, the data suggest that TP activation has to be controlled in a spatial and temporal manner in its regulation of cell migration. On one hand, blockade of TP activation with SQ29548 or PTA2 was found inhibitory for cell migration, suggesting that TP activation is required for tumor cells (PC-3) to migrate. On the other hand, sustained activation of TP with high levels of U46619 significantly compromised tumor cell motility. Further studies are required to determine when and how TP is activated in a temporal and spatial manner facilitative for cell migration and whether TP activation requires endogenous synthesis of TxA2 or other TP ligands, such as prostaglandin H2.

**Activation of RhoA by TxA2-TP signaling.** As a GPCR, TP activation can elicit a multitude of signaling cascades, including...
the activation of phospholipase C, mobilization of Ca2+, phosphorlyation of p38 and p42/44 mitogen-activated protein kinases (MAPK), formation of cyclic AMP, and activation of protein kinase G, C, and A (28). We examined the involvement of different effectors of TP activation in cell contraction induced by U46619 using various pharmacologic inhibitors. It was found that the activities of p42/44 MAPK, p38 MAPK, protein kinase G (Fig. 5d), phosphatidylinositol 3-kinase, or pertussis toxin-sensitive Gi subunit were not required for U46619 to induce cell contraction (data not shown). The screening revealed that Y27632 (10 μmol/L), a Rho kinase inhibitor (29), was able to block U46619-induced contraction. As shown in Fig. 5c, induced cell contraction, we evaluated the effects of ectopic activation of small GTPase RhoA in cell contraction induced by TP activation.

To further confirm the involvement of RhoA in U46619-induced cell contraction, we evaluated the effects of ectopic expression of RhoA or its mutants on U46619-induced cell contraction as shown in Fig. 5c and d, the constitutively active mutant of RhoA was sufficient to cause cell contraction, whereas the dominant-negative mutant of RhoA blocked cell contraction induced by U46619. The data affirm that activation of RhoA is required for Tax2-TP to induce cell contraction and that RhoA may serve as intermediary for Tax2-TP signaling axis to regulate tumor cell motility.

Rho GTPases, which include RhoA, Cdc42, and Rac, are critical for the dynamic changes in cell shape and adhesions that drive migration (30–33). During the highly coordinated process of migration, cells form lamellipodia or filopodia at the leading edge to chart cells forward (27). At the trailing edge, however, cells must detach and retract to enable cells to move forward (27). Cdc42 is required for cell polarity and filopodial protrusions, whereas Rac1 promotes lamellipodial protrusions. The contraction at the trailing edge is mediated by the small GTPase RhoA (27). Recently, it has been shown that RhoA was activated by GPCR signaling, including those elicited by U46619, through G12 heterotrimeric G proteins during invasion of cancer cells, including PC-3 (34, 35). Our study suggests that TP can regulate cell migration and cytoskeleton reorganization through inducing cell contraction via activating RhoA.

In summary, the present study reports that functional receptors for Tax2 were expressed in prostate cancer cells. Activation of TP by receptor agonists, such as U46619, caused profound reorganization in cytoskeleton and induced cell contraction. Cell motility was significantly inhibited either by sustained activation of TP with high concentrations of U46619 or by blockade of TP activation with SQ29548. Further studies found that RhoA was activated by U46619 through TP activation, which was required for U46619 to induce cell contraction. The present study suggests that Tax2-TP signaling axis may regulate cell migration through elaborating Rho activation and cytoskeleton reorganization. Further studies are required, however, to determine how Tax2-TP signaling axis is activated and regulated in a temporal and spatial manner for cell migration and whether the Tax2-TP signaling axis can be intervened to impede the invasion and metastasis of prostate carcinoma cells.

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