GLYCOGEN SYNTHASE KINASE-3 PHOSPHORYLATION, T-CELL FACTOR SIGNALING ACTIVATION AND CELL MORPHOLOGY CHANGE FOLLOWING STIMULATION OF THROMBOXANE RECEPTOR α

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Running title: Activation of GSK-3 and Tcf/Lef by TPα

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ABBREVIATIONS: bFGF, basic fibroblast growth factor; DMEM, Dulbecco modified Eagle’s medium; EGFR, epidermal growth factor receptor; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G-protein coupled receptor; GSK-3α/β, glycogen synthase kinase-3 α or β form; HEK293-TPα, HEK293 cells stably overexpressing TPα receptor; HRP, horseradish peroxidase; I-BOP, [1S-α,2α(Z),3β(1E,3S),4α]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; Lef, lymphoid enhancer factor; MAPK, mitogen-activated protein kinase; PDGF, Platelet Derived Growth Factor; PMSF, phenylmethylsulfonyl fluoride; PVDF, Polyvinylidene fluoride; PTX, pertussis toxin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tcf, T-cell factor, TXA₂, thromboxane A₂; TPα, thromboxane A₂ receptor α form.

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ABSTRACT

Previous reports showed that activation of the thromboxane receptor (TP) induced some types of cells to proliferate. We report here that TPα activates β-catenin/T-cell factor (Tcf)/lymphoid enhancer factor (Lef) pathway through phosphorylation of glycogen synthase kinase (GSK)-3. TP agonist, I-BOP, induced both α and β forms of GSK-3 phosphorylation in HEK293 cells stably over-expressing TPα (HEK293-TPα). H-89, a PKA inhibitor, totally blocked the phosphorylation of GSK-3, while wortmannin, a PI-3-kinase inhibitor, partially attenuated it, suggesting that PKA as well as PI-3 kinase/Akt pathway were involved in TP-induced phosphorylation of GSK-3. Consistently, I-BOP stimulated about 8 fold increase over basal Tcf/Lef reporter gene activity in HEK293-TPα cells. Furthermore, I-BOP-induced Tcf/Lef reporter gene activity was totally inhibited by H-89 and partially inhibited by wortmannin. I-BOP also induced over-expression of Tcf/Lef downstream target gene cyclin D1. Blockade of the β-catenin expression by siRNA approach attenuated I-BOP-induced expression of cyclin D1, indicating that the induction was mediated by β-catenin/Tcf/Lef pathway. Finally, I-BOP resulted in the morphology change, such as cell rounding and aggregation, in HEK293-TPα cells after 1 h incubation. However, HEK293-TPα cells were not able to revert back to normal shape even 24 h after the removal of the agonist suggesting that the prolonged activation of the Tcf/Lef promoter induced downstream gene expression leading to cell permanent morphology change which was related to cell transformation. Taken together, our results showed for the first time TP agonist-induced phosphorylation of GSK-3 and activation of Tcf/Lef signaling leading to cell proliferation and transformation.
INTRODUCTION

Thromboxane receptor (TP) is a member of the G-protein coupled receptor (GPCR) superfamily. Two isoforms of human TP, TP\(\alpha\) and TP\(\beta\), have been recognized. They differ in their carboxy terminal tails and their mechanisms of desensitization and internalization (Kinsella, 2001). TP is known to couple to multiple G proteins including \(G_{q/11}, G_{12/13}, G_{i}, G_{s}, G_{n}\) and mediates many cellular responses (Halushka et al., 1995). One of the notable responses is the stimulation of mitogenic and hypertrophic growth of vascular smooth muscle. For example, U-46619, a TP agonist, stimulates the proliferation of rat coronary or aortic smooth muscle cells and enhances the mitogenic effects of classical growth factors such as platelet derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) (Ali et al. 1993; Zhao and Foegh 1997). I-BOP, another TP agonist, triggers a protein kinase C (PKC), pertussis toxin (PTX)-, Src- and epidermal growth factor receptor (EGFR)-dependent activation of extracellular regulated kinase (ERK) in a human endothelial cell line, ECV304 (Gao et al. 2001). U-46619 was also reported to induce C-Jun N-terminal kinase (JNK) activation and protein kinase A (PKA) dependent cyclooxygenase-2 (COX-2) expression in porcine aortic smooth muscle cells (McGinty et al. 2000). Recently, Gallet et al. (2003) demonstrated that TP agonist-dependent transactivation of EGFR was mediated partially by matrix metalloproteinases and subsequent activation of ERK in human aortic smooth muscle cells. Stimulation of cell proliferation by TP agonists was observed beyond vascular smooth cells. Lin et al. (2005) reported that U-46619 stimulated the proliferation of oligodendrocyte progenitor cells and promoted the survival of mature ologodendrocyte in central nervous system. It appears that TP- mediated cell proliferation and survival is mainly dependent on two
interactive pathways: one is through transactivation of EGFR leading to phosphorylation of ERK; another one is activation of PKA dependent pathway.

Some GPCRs are known to functionally link to T-cell factor (Tcf)/β-catenin signaling pathway. Activation of mouse F9 clones expressing the chimera receptor constructed from the β2 adrenergic receptor and the Frizzled-1 receptor with the β-adrenergic agonist, isoproterenol, stimulated stabilization of β-catenin, activation of a β-catenin-sensitive promoter, and formation of primitive endoderm (Liu et al., 2001). Subsequently, it was shown that activation of FPB, an isoform of prostaglandin F$_{2α}$ receptor, led to a decrease in the phosphorylation of cytoplasmic β-catenin and a stimulation of Tcf/Lef-mediated transcriptional activation (Fujino and Regan, 2001). The same group also demonstrated the activation of the Tcf/Lef signaling pathway through EP receptors, EP$_2$ and EP$_4$, using stably transfected receptor in HEK293 cells (Fujino et al., 2002). Recently, GPCR and Tcf/Lef cross-talk was expanded to colon cancer cells. Yang et al. (2005) showed that activation G protein-coupled lysophosphatidic acid receptors stimulated proliferation of colon cancer cells through the β-catenin pathway and induced cell proliferation.

An important enzyme in the β-catenin/Tcf signaling pathway is glycogen synthase kinase-3 (GSK-3) (Doble and Woodgett, 2003). This enzyme is responsible for the phosphorylation and degradation of β-catenin through ubiquitin proteosome pathway. Two isoforms of GSK-3, GSK-3α and GSK-3β, encoded by two distinct genes have been identified (Hoeflich et al., 2000). GSK-3 is well known to be phosphorylated by Wnt signal mediated by frizzled receptor (Nelson and Nusse, 2004). GSK-3 also can be phosphorylated by Akt/PKB, PKC and PKA (Fang et al. 2002). Direct inhibition of the
GSK-3 either by its inhibitors or by phosphorylation prevents the phosphorylation and degradation of β-catenin. Stabilized β-catenin accumulates in the cytosolic compartment of the cell, translocates to the nucleus and binds to members of Tcf/Lef transcriptional factors and induces expression of several downstream genes that have important roles in the development and progression of carcinoma, including cyclin D1, c-myc, vascular endothelial growth factor (VEGF), COX-2, matrix metalloproteinase 7 (MMP-7), urokinase-type plasminogen activator (uPA) and CD44 (Wong and Pignatelli, 2002).

Since TP can activate Akt/PKB, PKC and PKA (Miggin and Kinsella, 2002), we propose that there is a possible crosstalk between the TP and the Tcf/Lef signaling pathway. Using HEK293 cells stably transfected with TPα gene, we report here that the stimulation of TPα by its agonist I-BOP led to GSK-3 phosphorylation, Tcf/Lef signaling activation and permanent cell morphology change. These effects appear to be related to cell transformation.
MATERIALS AND METHODS

Materials - Culture medium, lipofectamine 2000, heat-inactivated fetal bovine serum (FBS) and restriction enzymes were from Invitrogen or Gibco (Carlsbad, CA). I-BOP, U-46619 and SQ-29548 were from Cayman Chemical (Ann Arbor, MI). H-89, wortmannin and GF109203X were obtained from Calbiochem (San Diego, CA). Other biochemicals, chemicals and rabbit polyclonal anti-β-catenin antibody were obtained from Sigma (St. Louis, MO). Mouse monoclonal antibody specific to pERK, and β-catenin siRNA and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody specific to pGSK-3α/β was from Cell Signaling (Beverly, MA). pcDNA3 encoding TP-α and rabbit polyclonal antibody specific to N-terminal sequence of the thromboxane receptor (TP) were generated as described previously (Zhou et al., 2001). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were prepared as described (Wu et al., 1997). pGSK-3 antibodies recognizing both α and β forms were supplied by the Cell Signaling Technology (Beverly, MA). pERK antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit monoclonal antibody specific to cyclin D1 was provided by Lab Vision (Fremont, CA). Horseradish peroxidase (HRP)-linked goat anti mouse and rabbit IgG were supplied by Transduction Laboratories (Lexington, KY). The HEK293 cell lines were supplied by the American Type Culture Collection (Manassas, VA). ECL Western blotting detection was supplied by the Amersham Pharmacia Biotech (Cardiff, UK). The Tcf/Lef reporter plasmids kit including TOP flash, containing two sets (with the second set in the reverse orientation) of the three copies of the Tcf site upstream of the luciferase open reading frame and FOP
flash, containing mutated Tcf elements served as a background were purchased from Upstate Biotechnology (Lake Placid, NY).

**Cell culture and stable transfectants** - HEK293 cells were cultured as monolayer in DMEM supplemented with 10% heat inactivated FBS, gentamicin and antibiotic-antimycotic at 37°C in a humidified atmosphere of 95% air and 5% CO₂. The cells were subcultured in six well plates or 12 well plates to achieve 80% confluence on the next day, and then the cells were transfected with pcDNA3-TPα using lipofectamine reagent. For stable transfection, the cells were allowed to grow in 10% FBS medium for 48 hr after transfection and then the cells were diluted 10 fold and treated with 1mg/ml G418 until the colonies appeared as described previously (Zhou et al., 1999). The Western blot detection of the TPα and the [3H]SQ-29548 binding assay of the receptor were used to monitor the expression level among the different colonies. After the colony with a high level of expression of TPα was obtained, it was maintained in DMEM media supplemented with 10% heat inactivated FBS and 200 µg/ml G418.

**Whole cell radioligand binding assay** - Cells were cultured in 10 cm plates and were harvested when 90% confluence was achieved. The cells were washed in ice-cold PBS buffer for three times. Then 1x10⁶ cells were resuspended in 95 µl PBS buffer using 1.5 ml plastic tubes and the binding assay was conducted in the same tubes. The total volume of the reaction was 100 µl which contained [3H] SQ29548 with the concentration from 2.6 x 10⁻¹² to 2.6 x 10⁻⁷ M. The non-specific binding was determined under the same conditions except 10 µM SQ29548 was added to the reaction mixture. The binding reaction was performed at room temperature for 1 h and was terminated by placing the tubes on ice for 10 min. The cell suspension was then filtered under vacuum through a GF/C glass filter.
followed by washing three times. The radioactivity retained on the filter was counted in 10 ml scintillation cocktail. The Kd and Bmax were calculated using non-linear regression method by GraphPad Prism 4.01.

**Western blot Analysis** – Cells were cultured in six well plates to achieve about 80% confluence. For ERK and GSK-3 activation, the cells were starved in DMEM media without FBS for 16 h, and then cells were challenged by 50 nM I-BOP for 15 min to achieve significant phosphorylation of ERK, and for 0 to 60 min to monitor the time course of the activation of GSK-3α/β. As to inhibition study, various inhibitors were pre-incubated with cells for 20 min following treatment with I-BOP for 15 min. Cells were harvested and lysed in lysis buffer (1% Nonidet P-40 in 150 mM NaCl, 50 mM HEPES, pH 7.4, 5 mM NaF, 5 mM pyrophosphate, 1 mM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 mM PMSF) for 1 h on ice. Lysate was cleared by centrifugation at maximum speed on a bench-top centrifuge and then subjected to 12% SDS-PAGE. Proteins were then electrophoretically transferred onto PVDF membrane. The membrane was blocked with 5% nonfat milk in 30 mM Tris-HCl, pH 7.4, containing 120 mM NaCl (TBS) at room temperature for 1 h. It was then incubated for 2 h at room temperature with a primary antibody in TBS with 5% non-fat milk and then the membrane was washed three times with TBST (TBS buffer containing 0.05% Tween-20), following incubation with HRP-linked goat anti mouse or rabbit IgG for 1 h at room temperature. Finally, the membrane was washed with TBST for three times. The immunoreactive bands were detected using ECL Western blotting detection system.

**Tcf/Lef reporter gene assay** - HEK293-TPα cells were grown in 6-well plates and transiently transfected with 1 µg/well of either TOP flash or FOP flash plasmids using
Fugene-6 procedure as described in the manufacturer’s protocol. Cells were preincubated with inhibitors at indicated concentrations or vehicle (0.1% DMSO) for 20 min at 37°C followed by challenging with 50 nM I-BOP or vehicle for 1 h at 37°C. Cells were then washed three times each with 1 ml/well of FBS free DMEM media and then incubated with FBS free DMEM media for 16 hr at 37°C. Cell lysate was prepared for luciferase assay. Luciferase activity was measured using a microplate luminometer with 5 µg of protein per sample. The specific reporter gene activity was corrected for background activity by subtraction of FOP flash values from corresponding TOP flash values.

**siRNA treatment**- Cells were transiently transfected with β-catenin siRNA at the concentration of 20 pmol/well in 12 well plates using lipofectamine reagent. The cells were treated with the siRNA for 48 hr to achieve the maximal blockade of the expression of the β-catenin.

**Phase contrast microscope**- For phase contrast microscopic studies, HEK293 or HEK293-TPα cells were grown in 12-well plates to achieve about 40%-50% confluence using DMEM media containing 10% FBS and same antibiotics as mentioned above. Cells were visualized under phase contrast microscope using a Zeiss Axiovert S100 inverted microscope. Images were captured using a Kodak digital camera.
RESULTS

Stable transfection of TP in HEK293 cells and characterization of the functional expression of the receptor - HEK293 cell line was chosen to construct TPα stably transfected cells since it has minimal basal expression of TP. As shown in Fig. 1A, Western blotting using antibody specific to N-terminal peptide of TP demonstrated that HEK293-TPα expressed significantly TPα as compared to HEK293 cells transfected with vector alone. Similar to previous reports, HEK293-TPα had a high affinity to TP antagonist SQ-29548, with a Kd value of 14.6 ± 1.4 nM and Bmax value of 5.3 ± 0.31 pmol/mg protein characterized by [3H]SQ-29548 binding assay (Fig. 1B). The functional assay of the TPα was characterized by agonist stimulated ERK activation. As shown in Fig. 1C, the TP agonist, I-BOP, stimulated significant phosphorylation of ERK in HEK293-TPα cells, while minimal response was observed in HEK293 cells transfected with vector alone. TP agonist-induced activation of ERK has been well documented in several cell lines (Gao et al., 2001; Miggin and Kinsella, 2001). Our results are consistent with previous reports and provide evidence that HEK293-TPα cells expressed functionally active TPα.

TP agonist stimulated phosphorylation of GSK-3α/β in HEK293-TPα cells - Previous reports showed that several prostaglandins such as PGE2 and PGF2α could induce phosphorylation of GSK-3α/β through their specific receptors (Fujino et al., 2002; Shao et al., 2005). We used HEK293-TPα cells to study GSK-3α/β phosphorylation upon TP agonist stimulation. As shown in Fig. 2A, both GSK-3α and GSK-3β were phosphorylated in 2 min following exposure to 50 nM I-BOP in HEK293-TPα cells. Phosphorylation of GSK-3α/β was achieved maximally at 5 min after stimulation by I-
BOP and was gradually decreased during the following 50 min. The time course studies showed that phosphorylation of GSK-3α/β still persisted at high level even after 60 min of incubation with I-BOP indicating that the signal induced by a TP agonist existed for a relatively long time. However, no detectable phosphorylation of GSK-3α/β was found in HEK293 cells (data not shown). As shown in Fig. 2B, I-BOP induced phosphorylation of GSK-3α/β in a concentration-dependent manner. I-BOP at 1 nM clearly led to phosphorylation of GSK-3α/β and phosphorylation reached to a maximum at 50 nM. We then used the specific kinase inhibitors to determine which pathway(s) was involved in the GSK-3α/β phosphorylation. As shown in Fig. 2C, PKA inhibitor H-89 not only totally blocked I-BOP-induced GSK-3α/β phosphorylation, but inhibited basal level of pGSK-3α/β, suggesting that PKA is required to maintain the pGSK-3α/β in unstimulated cells. TP antagonist SQ-29548 can block I-BOP-induced phosphorylation of GSK-3α/β, suggesting that the signal transduction is directly through TP. A concentration dependent inhibition by H-89 is shown in Fig. 2D. However, wortmannin, an inhibitor of PI-3 kinase, partially blocked GSK-3α/β phosphorylation. Surprisingly, the well known PKC activation following TP activation was not involved in this process, since its inhibitor GF 109203X had no effect on I-BOP-induced GSK-3α/β phosphorylation.

**β-catenin accumulation in cytoplasmic fraction and Tcf/Lef reporter gene activation following stimulation of TPα** Phosphorylation of GSK-3α/β is known to inactivate GSK-3α/β. Consequently, I-BOP-induced phosphorylation of GSK-3α/β may stimulate accumulation of β-catenin in the cytosolic compartment and enhance translocation of β-catenin from the cytosol to the nucleus leading to the Tcf/Lef transcription factor activation. As shown in Fig. 3, I-BOP induced an increase in the amount of β-catenin in
the cytosolic fraction of HEK293-TPα cells. Consistently, I-BOP stimulated an 8-fold increase in the Tcf/Lef reporter gene expression in HEK293-TPα cells as shown in Fig. 4. Since H-89 and wortmannin were found to block I-BOP–induced GSK-3α/β phosphorylation, they were also used to examine the effect on the activation of the Tcf/Lef reporter gene expression. As shown in the same figure, I-BOP-induced activation of the Tcf/Lef reporter gene was totally blocked by H-89 and partially blocked by wortmannin, but was not affected by GF compound. Again, the TP antagonist SQ-29548 can block the TCF/Lef activation. This is consistent with the results of GSK-3α/β phosphorylation induced by I-BOP in HEK293-TPα cells. It is clear that I-BOP activates Tcf/Lef transcription factor through GSK-3α/β phosphorylation, and that PKA and PI-3 kinase/Akt pathways were involved in this process. However, TPα coupled to Gq leading to PKC activation had no direct effect on this process in our system.

**Cyclin D1 expression in HEK293-TPα cells upon TP agonist stimulation**- Previous reports demonstrated that β-catenin regulated cyclin D1 expression (Tetsu et al., 1999). β-catenin/TCF/Lef complex could bind to cyclin D1 promoter to modulate cell cycle progression and proliferation. In HEK293-TPα cells, I-BOP caused a rapid and persisted (up to 8 hr) increase in cyclin D1 expression (Fig. 5A). Inhibition of β-catenin expression by siRNA approach as indicated in Fig. 5B attenuated I-BOP-induced cyclin D1 expression as shown in Fig. 5C. Control siRNA had no effect.

**Cell morphology change in HEK293-TPα cells following stimulation by TP agonist**- Activation of β-catenin/TCF/Lef is often linked to cell phenotypic transformation leading to developmental or malignant transformation. Cell morphology change in HEK-TPα cells began to show after 30 min of incubation with I-BOP and was clearly observed
following 1 h of incubation as shown in Fig. 6. Incubation of I-BOP for 1 h resulted in cell rounding and aggregation in HEK293-TPα cells (Fig. 6A-c & d), whereas I-BOP had no effect on HEK293 cells transfected with pcDNA3 vector alone under the same conditions (Fig 6A-a & b). After 1 h incubation with I-BOP, the HEK293-TPα cells were washed with DMEM medium without FBS to remove I-BOP, but the cells could not revert back to the normal shape even after 24 h (Fig. 6B-a & b), suggesting that a short time incubation with TP agonist was sufficient to induce a permanent change in cell morphology. The permanent morphology change is likely the consequence of the agonist-induced gene expression mentioned above. It may be recalled that in the Tcf/Lef reporter gene assay, H-89 and wortmannin had inhibitory effect on the activation of Tcf/Lef. In accordance with this, pre-incubation of HEK293-TPα cells with H-89 (Fig. 7-E & F) prevented the I-BOP-induced cell morphology change, whereas wortmannin (Fig. 7-G & H) and GF 109203X (Fig. 7-I & J) had little preventive effect. Blockade of the TP signaling by antagonist SQ-29548 could prevent I-BOP-induced permanent cell morphology change, suggesting that the TP was directly involved in the signal transduction leading to cell transformation.
DISCUSSION

We employed HEK293 cells stably transfected with TPα to study the signaling mechanism and its relevance to cell transformation. We demonstrated that the activation of TPα led to GSK-3 phosphorylation, T-cell factor signaling activation and cell morphology change. A detailed scheme depicting the signaling pathway of TP activation was summarized in Fig 8. Previous report indicated that two isoforms of TP may be coupled differentially to Gs and Gi (Hirata et al., 1996). TPα is coupled to Gs leading to an increase in intracellular level of cAMP and activation of PKA, while TPβ is coupled to Gi leading to a decrease in intracellular level of cAMP. It appears that the activation of Tcf/Lef pathway by TPα is mainly mediated by a PKA dependent mechanism. A reasonable prediction is that TPβ may not induce the activation of Tcf/Lef pathway or TPβ activation of Tcf/Lef pathway is mediated by other mechanism(s). It has been shown that the activation of both EP2 and EP3 may induce the stimulation of Tcf/Lef pathway. Activation of Tcf/Lef pathway by EP2 was shown to be mediated by a PKA mechanism, while stimulation of Tcf/Lef pathway by EP4 was demonstrated to be mediated by a PKB/Akt mechanism (Fijino et al., 2002). This is due to the fact that the activation of EP2 induces significantly higher level of intracellular cAMP as compared to that of EP4 (Fujino et al., 2002). Whether or not the activation of TPβ may lead to the stimulation of Tcf/Lef pathway or other pathway(s) is involved in the activation of Tcf/Lef needs to be elucidated in TPβ stably transfected cells.

We have shown that I-BOP leads to a rapid phosphorylation of both GSK-3α and -3β, activation of Tcf/Lef signaling and cell morphology change in TPα stably transfected HEK293 cells. Similar results were obtained with U-46619, another stable agonist of
TPα, in these cells. Furthermore, we also observed I-BOP-induced rapid phosphorylation of GSK-3α and -3β and activation of Tcf/Lef signaling in human lung cancer A549 cells (data not shown). Previously, it was also shown that the activation of EP2 and EP4 led to significant phosphorylation of GSK-3α, but not that of GSK-3β (Fujino et al., 2002). However, in our studies both forms of GSK-3 were rapidly phosphorylated upon stimulation of TPα. The difference in response is not clear and needs to be further explored. In addition to cAMP/PKA pathway, we also found that PI-3-kinase inhibitor, wortmannin, partially inhibited phosphorylation of GSK-3α/β. The activation of Akt/PKB by TP agonists was reported previously by Miggin and Kinsella (2002). Akt/PKB is known to be one of upstream kinases of GSK-3 inducing phosphorylation and inhibition of GSK-3 (Cross et al., 1995). In fact, Akt/PKB dependent activation of the β-catenin/Tcf pathway was reported in ECV304 carcinoma cells (Maupas-Schwalm et al., 2005). One possible explanation is that PKA is an upstream kinase of Akt/PKB pathway and may indirectly phosphorylate GSK-3α/β (Filippa et al., 1999). This may account for why H-89 totally blocked the phosphorylation of GSK-3, while wortmannin partially attenuated it.

The accumulation of β-catenin in the cytosol is a direct result from the phosphorylation and inhibition of GSK-3α/β as shown in this study. Intracellular β-catenin level, however, can be regulated by other pathways as well. It is known that Wnt signaling also regulates intracellular β-catenin level. A large multiprotein complex that includes proteins of the APC and Axin families normally facilitates the phosphorylation of β-catenin by GSK-3 (Nelson and Nusse, 2004). Phosphorylated β-catenin is then rapidly degraded by the ubiquitin mediated proteosome pathway. When Wnt signal is
absent, the signal transduction pathway is off because β-catenin is rapidly destroyed.

When cells are exposed to Wnt signal, it binds to cell surface receptors of the Frizzled family. Receptor activation antagonizes the APC-Axin “destruction complex” by an unknown mechanism that appears to be mediated by the Dishevelled protein. This blocks the phosphorylation of β-catenin and its subsequent degradation. Unphosphorylated β-catenin forms a pool in the cytosol and then translocates to the nucleus where it activates the genes regulated by Tcf/Lef transcription factors. Our findings and reports of studies on other GPCRs such as EP₂ and FP₉ expanded the Wnt pathway to other signaling pathways which could directly phosphorylate and inactivate the GSK-3α/β and regulate β-catenin level.

The linkage between β-catenin and cyclin D₁ was established from accumulated reports especially in human cancers (Utsunomiya et al., 2001). The cyclin D₁ is associated with cancer growth, invasion and metastasis, through influencing the cell cycle progression. We show here that I-BOP stimulates overexpression of cyclin D₁ and blockade of β-catenin expression by siRNA approach attenuates this effect. Furthermore, our preliminary data showed that TPα agonist stimulated a permanent change in cell morphology, such as cell rounding and aggregation. A same phenomenon was found in the activation of FP receptors stably transfected in HEK293 cells (Fijino and Regan 2001). In this report, FP₉ isoform which activated the Tcf/Lef transcription factors induced a permanent change in cell morphology, while FP₈ which could not activate Tcf/Lef only caused a temporary change in cell morphology. Earlier report showed that overexpression of COX-2 and micorsomal prostaglandin E synthase had the potential to affect cellular proliferation and morphology change (Murakami et al., 2000). Many
studies demonstrated that expression of COX-2 is associated with cell proliferation, anti-apoptosis and differentiation (Mann et al., 2001). As mentioned in previous reports, a permanent change in cell morphology was an indication of developmental or malignant transformation. Thromboxane A$_2$ (TXA$_2$) is one of the downstream metabolites of COX-2, and is involved the proliferation of several cell lines. It also has roles in the tumor growth and metastasis (Daniel et al., 1999; Nie et al., 2000). Therefore, our findings have expanded COX-2/PGE$_2$ pathway to COX-2/TXA$_2$ pathway. In order to verify the possible roles of TP$\alpha$ in cancer, lung cancer A549 cells stably overexpressing TP$\alpha$ receptor and lung cancer H157 cells stably transfected with TP$\alpha$ siRNA are prepared. Preliminary data indicated that I-BOP induced COX-2 expression in A549 cells over-expressing TP$\alpha$ suggesting that a positive feedback might occur in COX-2 and TP$\alpha$ expressing cells. Generation of tumors in the nude mice using these cell lines is currently in progress in our laboratory.
REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Characterization of stable expression of TPα receptor in HEK293 cells. A, Western blotting analysis of TPα stably expressed in HEK293 cells. The whole cell lysates (50 µg) prepared from HEK293 cells stably transfected with TPα (HEK293-TPα) or the pcDNA3 vector alone were respectively subjected to 12% SDS-PAGE and transferred onto PVDF membrane as described under MATERIALS AND METHODS. The immunoreactive bands were detected by antibody against N-terminal sequence of human TPα at a dilution of 1:1000. B, Whole cell binding assay. As described under MATERIALS AND METHODS, HEK293-TPα cells were incubated with various concentrations of [3H]SQ-29,548. The nonspecific binding was determined in the presence of 10 µM unlabeled SQ-29,548. The values of K_d and B_max were determined by non-linear regression model using GraphPad Prism 4.01 software. C, Functional analysis of TPα stably expressed in HEK-293 cells. HEK293-TPα and HEK293 cells transfected with pcDNA3 alone were starved in DMEM medium without FBS for 16 hr and then incubated with 50 nM I-BOP for 10 min. The whole cell lysate was subjected to 12% SDS-PAGE and transferred onto PVDF membrane as described under MATERIALS AND METHODS. The immunoreactive bands were detected by antibody against pERK. Results are a representative of three independent experiments.

Fig. 2. TP agonist I-BOP stimulation of phosphorylation of GSK-3α/β in HEK293-TPα cells. A, Time course of GSK-3α/β phosphorylation induced by I-BOP in HEK293-TPα cells. The HEK293-TPα cells were starved in DMEM medium without FBS for 16 hr and then were incubated with 50 nM I-BOP for indicated time. The cell lysate was
subjected to 12% SDS-PAGE and transferred onto PVDF membrane as described under **MATERIALS AND METHODS**. The immunoreactive bands were detected by antibodies against pGSK-3α/β. B, I-BOP stimulation of phosphorylation of GSK-3 in a concentration-dependent manner. The HEK293-TPα cells were starved in DMEM medium without FBS for 16 hr and then were incubated with I-BOP at indicated concentration for 10 min. The pGSK-3α/β was detected by western blotting as mentioned in A. C, PKA and Akt/PKB but not the PKC are involved in the I-BOP induced phosphorylation of GSK-3α/β in HEK293-TPα cells. The cells were pre-incubated with 10 μM H-89 or 100 nM wortmannin (Wort) or 200 nM GF109203 (GF) or 5 μM SQ-29548 (SQ) for 20 min, and then stimulated by 50 nM I-BOP for 10 min. The pGSK-3α/β was detected by western blotting as mentioned in A. D, H-89 inhibition of phosphorylation of GSK-3α/β induced by I-BOP in a concentration-dependent manner. The cells were pre-incubated with H-89 at indicated concentration for 20 min, and then stimulated by 50 nM I-BOP for 10 min. The pGSK-3α/β was detected by western blotting as mentioned in A. Blots were also probed with polyclonal antibody to GAPDH to ensure equal protein loading. Results are a representative of three independent experiments.

**Fig. 3. Kinetics of β-catenin accumulation in the cytosolic fraction of HEK293-TPα cells after I-BOP stimulation.** After treatment with 50 nM I-BOP for the indicated time at 37°C, cells were lysed and cytosolic and membrane fractions were prepared as described in **MATERIALS AND METHODS**. And then the protein samples were subjected to 12% SDS-PAGE and transferred onto PVDF membrane as described under **MATERIALS AND METHODS**. The immunoreactive bands were detected by
polyclonal antibody against β-catenin. Results are a representative of three independent experiments.

**Fig. 4. PKA and PI-3 kinase/PKB pathways are involved in I-BOP-stimulated Tcf/Lef-responsive luciferase reporter gene activity in HEK293-TPα cells.** HEK293-TPα cells were transiently transfected with TOP flash or FOP flash reporter vector. The cells were pre-incubated with 10 µM H-89 or 100 nM wortmannin (Wort) or 200 nM GF109203X (GF) or 5 µM SQ-29548 (SQ) for 20 min, and then the cells were treated with 50 nM I-BOP as described under **MATERIALS AND METHODS.** Plotted is the mean ± S.E. of specific luciferase activity values in triplicate as described under **MATERIALS AND METHODS.** Results are a representative of three independent experiments.

**Fig. 5. I-BOP stimulates Tcf/Lef downstream gene cyclin D1 expression in HEK293-TPα cells.** A. Time course of cyclin D1 overexpression induced by 50 nM I-BOP. Cells were cultured in FBS free DMEM medium for 16 hr before treatment of 50 nM I-BOP for indicated time. The cell lysates were subjected to 12% SDS-PAGE and transferred onto PVDF membrane as described under **MATERIALS AND METHODS.** The immunoreactive bands were detected by polyclonal antibody against cyclin D1. B, β-catenin siRNA significantly decreased the expression of β-catenin. As mentioned under **MATERIALS AND METHODS,** HEK293-TPα cells were treated with 20 nM siRNA for 48 hrs, and then the cells were harvested and the cell lysate was subjected to 12% SDS-PAGE and transferred onto PVDF membrane as described under **MATERIALS**
AND METHODS. The immunoreactive bands were detected by polyclonal antibody against β-catenin. C, Effect of β-catenin siRNA on the cyclin D1 overexpression induced by I-BOP in HEK293-TPα cells. The siRNA t before treatment of 50 nM I-BOP for 6 h. The western analysis of cyclin D1 was exactly same as described in A. Blots were also probed with polyclonal antibody to GAPDH to ensure equal protein loading. Results are a representative of three independent experiments.

Fig. 6. The effect of I-BOP on cell morphology in HEK293 cells stably transfected with TPα receptor or pcDNA3 vector alone. A, HEK293 cells transfected with pcDNA3 vector alone (a and b) or HEK293-TPα Cells (c and d) were plated in a 12-well plate at the density of 50,000 cells/well, and allowed to grow for overnight (about 16 hr). The next day the cells were incubated in FBS free DMEM medium and then treated with 50 nM I-BOP (b and d) or vehicle (a and c) for 1 h. B, Removal of I-BOP can not reverse the morphology change in HEK293-TPα cells. As described in A, the cells were treated with 50 nM I-BOP (b) or vehicle (a) for 1h, then washed with FBS free DMEM medium for 3 times and incubated with FBS free DMEM medium for 24 h. Images were obtained as described under MATERIALS AND METHODS. These images were a representative of three experiments that were performed in duplicate.

Fig. 7. PKA inhibitor H-89 prevents the permanent morphology change induced by I-BOP in HEK293-TPα cells. Prior to treatment of 50 nM I-BOP as described in Fig 6, the cells were incubated with vehicle (A and B) or or 5 µM SQ-29548 (C and D) or 10 µM H-89 (E and F) or 100 nM wortmannin (G and H) or 200 nM GF109203X (I and J)
for 30 min. The cells then were washed with FBS free DMEM medium for 3 times and incubated with FBS free DMEM medium for 24 h. Images were obtained as described under MATERIALS AND METHODS. These images were a representative of three experiments that were performed in duplicate.

**Fig. 8. Scheme depicting the activation of TP leading to the phosphorylation of GSK-3α/β and the activation of Tcf/Lef signaling.** TPα activation increases the formation of cAMP catalyzed by adenylate cyclase through Gs leading to the PKA activation. PKA then phosphorylates GSK-3α and β and inactivates them. In the unstimulated cells, β-catenin was phosphorylated by GSK-3α/β and degraded by ubiquitin proteasome pathway. Inactivation of GSK-3α/β by phosphorylation induced by TPα activation results in the accumulation of β-catenin in the cytosol and its translocation into the nucleus where it activates Tcf/Lef signaling leading to the over-expression of the downstream gene, cyclin D1.
Figure 1
Figure 2
Figure 3
Figure 4
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Figure 5
Figure 6
Figure 7
Figure 8