Activation of thromboxane A2 receptors induces orphan nuclear receptor Nurr1 expression and stimulates cell proliferation in human lung cancer cells

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Previous studies implicated that activation of thromboxane A2 receptor (TP) induced cell proliferation and transformation in several cell lines. We report here that the activation of TP by its agonist, [1S-[1α, 2α(Z), 3β (1E, 3S*), 4αi]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo [2.2.1] hept-2-yl]-5-hepatic acid (I-BOP), induced Nurr1 expression and stimulated proliferation of human lung cancer cells. Nurr1, an orphan nuclear receptor of the nuclear receptor subfamily 4A subfamily, has been implicated in cell proliferation, differentiation and apoptosis. I-BOP markedly induced Nurr1 messenger RNA and protein levels as compared with other subfamily members, Nur77 and Nor-1. The signaling pathways of I-BOP-induced Nurr1 expression were examined by using various inhibitors of signaling molecules. The induction of Nurr1 expression by I-BOP appeared to be mediated through protein kinase A (PKA)/cAMP response element binding (CREB), protein kinase C and mitogen-activated protein kinase/extracellular signal-regulated kinase pathways and not related to epidermal growth factor receptor and prostaglandin E2 pathways. Transcriptional activation of Nurr1 gene by I-BOP was further investigated at the promoter level in H157 cells. S'-Deletion analysis, site-directed mutagenesis and luciferase reporter assay demonstrated that Nurr1 expression was induced by I-BOP in a PKA/CREB-dependent manner. Further studies have revealed that Nurr1 may mediate cyclin D1 expression and I-BOP-induced cell proliferation in H157 cells since small interfering RNA of Nurr1 blocked I-BOP-induced cyclin D1 expression and cell proliferation and also decreased cell growth rate. These results provide strong evidence that Nurr1 plays a significant role in cell proliferation and may mediate TP agonist-induced proliferation in lung cancer cells.

Introduction

Thromboxane A2 (TXA2), a product of thromboxane synthase, interacts with its G-protein-coupled receptors, thromboxane A2 receptor (TP), to initiate platelet aggregation, vascular smooth muscle contraction and proliferation (1). Two isoforms of TP, TPα and TPβ, have been cloned and characterized (2). TPα and TPβ are encoded by a single receptor gene and are derived by a novel differential messenger RNA splicing resulting in difference in their C-terminal domains. TPα has been cloned and characterized (2). TP is abundantly expressed in different organs such as lung, liver, heart, uterus, brain and vasculature (3). TP can bind to both the a TP receptor (TPα, to initiate platelet aggregation, vascular smooth muscle contraction and proliferation (1). Two isoforms of TP, TPα and TPβ, have been cloned and characterized (2). TPα and TPβ are encoded by a single receptor gene and are derived by a novel differential messenger RNA splicing resulting in difference in their C-terminal domains. TPα has been cloned and characterized (2). TP is abundantly expressed in different organs such as lung, liver, heart, uterus, brain and vasculature (3).

Materials and methods

Chemicals and antibodies

PGE2, [1S-[1α, 2α (Z), 3β (1E, 3S*), 4αi]-7-[3-[3-hydroxy-4-(4-iodophenoxy)-1-butenyl]-7-oxabicyclo [2.2.1] hept-2-yl]-5-hepatic acid (I-BOP) and SQ29548 were from Cayman Chemical (Ann Arbor, MI). Forskolin (FSK), H89, LY294002, GF109203X and c-jun N-terminal kinase (JNK) inhibitor II were from Calbiochem (San Diego, CA). PD98059 and U0126, AG1478, PD15305, PPI and PD2, MG132, SB203580 and SQ22536 were from Alexis Biochemicals (San Diego, CA). Other biochemicals and chemicals were from Sigma–Aldrich (St Louis, MO). Rabbit polyclonal antibody specific to Nurr1 and mouse monoclonal antibody specific to pERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibody specific to phospho-cAMP response element binding (CREB) (Ser133) was from GenScript (Fiscatway, NJ). Rabbit monoclonal antibody specific to cyclin D1 was from Neomarkers (Fremont, CA). Rabbit polyclonal antibodies specific to extracellular signal-regulated kinase (ERK) 1/2 and CREB were obtained from Cell Signaling Technology (Beverly, MA). Antibody specific for glyceraldehyde-3-phosphate dehydrogenase was generated as described previously (21). Horseradish peroxidase-linked goat anti-mouse and rabbit IgG were supplied by BD Transduction Laboratories (Lexington, KY).

Cell culture

Human lung carcinoma cell lines H157, A427, H1299, H460 and H358 were supplied by the American Type Culture Collection (Manassas, VA). These cells were maintained in RPMI 1640 medium supplemented with 10%
heat-inactivated fetal bovine serum (FBS), 0.1 mg/ml streptomycin and 100 U/ml of penicillin G (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Western blotting

Cells were plated in 12-well plates to achieve ~80% confluence and then were starved in RPMI 1640 medium without FBS for 24 h before stimulation. For inhibitor study, cells were pretreated with the respective inhibitors at working concentrations or vehicle (0.1% ethanol) for 30 min in serum-free medium prior to stimulation. Cells were washed with phosphate-buffered saline and lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM NaF, 5 mM pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were determined using Bio-Rad reagent (Richmond, CA). Western blotting was carried out as described previously (21). Proteins were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Membranes were blocked with 5% non-fat milk in Tris-buffered saline at room temperature for 1 h and incubated with primary antibodies overnight at 4°C. The membranes were then incubated with horseradish peroxidase-linked goat anti-mouse or rabbit secondary antibodies for 1 h at room temperature and developed using enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Cardiff, UK). All membranes were stripped using stripping buffer before reprobing with anti-glyceraldehyde-3-phosphate dehydrogenase, anti-ERK1/2 or anti-CREB polyclonal antibodies to ensure equal protein loading.

Semiquantitative reverse transcription–polymerase chain reaction analysis

Total RNA was isolated from cells using the TRI Reagent (Sigma–Aldrich) and reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen). The polymerase chain reaction (PCR) primers for Nurr1 gene amplification were 5′-AACCTGCTACTAATGAGTAGT-3′ (sense) and 5′-CAATGCA-GAGAAGCCAGAAAT-3′ (antisense); for Nur77 gene amplification, 5′-TCTC-GTCTACACCTGCTAC-3′ (sense) and 5′-GGGACCAAAATACTCCAC-GCTTG-3′ (antisense); for Nor-1 gene amplification, 5′-TCCGAGACCTCTCCTAG-3′ (sense) and 5′-GGTTGCTGTCGCTGATCT-3′ (antisense); for TPα gene amplification, 5′-GAGATGATGGCTCAGCTCTCT-3′ (sense) and 5′-CCAGCCCTGAACTCTCCA-3′ (antisense); for TPβ gene amplification, 5′-CGAGTTCTCTGCTGTCG-3′ (sense) and 5′-CTACCTCTTCTGCAGAC-3′ (antisense). For GAPDH, the primer pair was 5′-GCTTG-3′ (sense) and 5′-GCTTGTCCACGTAGTGGG-3′ (antisense) for gene amplification, 5′-GTTGGCAGGTCCTCCA-3′ (antisense); and CRE mutant, 5′-TGTTGGCAGGTCCTCCA-3′ (antisense) and 5′-GCCCTTTTGTTGTATGACG-3′ (antisense). PCR conditions were as follows: 95°C for 2 min, followed by 35 cycles (30 s for 5′-beta) of 95°C for 1 min, 55°C for 30 s and 72°C for 1 min.

Transient transfection and small interfering RNA treatment

Cells were transiently transfected with Nurr1 small interfering RNA (siRNA) or vehicle (0.1% ethanol) for additional 16 h. Thereafter, cells were collected and further detected by using a microplate luminometer (MTX Lab Systems, Vienna, VA). Relative luciferase activity was normalized as fold induction.

Proliferation assay

Proliferation of H157 cells was studied using 3H-thymidine incorporation assay. Cells were plated in 24-well plates at 1 × 104 cells per well, incubated overnight in RPMI 1640 with 10% FBS and starved without FBS for 24 h, then treated with I-BOP or vehicle for 48 h. 3H-thymidine was added at 1 μCi/ml for 4 h at the last day of I-BOP or vehicle treatment. 3H incorporation assay was performed on β-scintillation counter. The results were normalized as percent of control. The images of cells were captured using a Kodak digital camera under Olympus Tokyo CK inverted microscope.

Statistical analysis

Luciferase assay and proliferation assay results were expressed as mean ± SD. Statistical significance was assessed by Student’s t-test. Differences were considered statistically significant when P values were <0.05.

Results

I-BOP induces Nurr1 expression in lung cancer cells

Reverse transcription–PCR was used to determine the relative expression of the three NR4A subfamily members, Nurr1, Nur77 and Nor-1, in response to TP activation in H157 cells. All the three NR4A subfamily members were rapidly and transiently induced at the transcription level (Figure 1A). Nurr1 induction was the most dramatic one suggesting that Nurr1 could be the major member in the NR4A subfamily induced by TP activation. Subsequent studies were focused on this orphan nuclear receptor.

Western blot analysis indicates that I-BOP induced Nurr1 expression in H157 cells in a time- and dose-dependent manner. Nurr1 expression was rapidly induced by I-BOP within 2 h and the expression lasted for 4 h and rapidly declined afterward (Figure 1B).

Nurr1 expression was induced by increasing concentrations of I-BOP reaching maximum at 50 nM (Figure 1B). Nurr1 expression was also induced by I-BOP in other lung cancer cells (A427, H460, H358 and H1299) (Figure 1C). H157 cells showed the most significant I-BOP-induced expression among those lung cancer cells examined and were, therefore, used as a model system for subsequent experiments.

Signal transduction pathways involved in agonist-induced Nurr1 expression

TP is known to initiate several signaling cascades to regulate gene expression upon ligand binding. We further investigated the involvement of signal transduction pathways that mediate the expression of Nurr1 in response to TP activation in H157 cells (Figure 2A). As shown in Figure 2A, protein kinase A (PKA) inhibitor, H89, and protein kinase C (PKC) inhibitor, GF109302X, significantly inhibited I-BOP-induced Nurr1 expression, whereas phosphatidylinositol 3-kinase (PI-3K) inhibitor, LY294002, did not show inhibitory effect providing evidence that PKA and PKC pathways might mediate I-BOP-induced Nurr1 expression. FSK, a cyclic adenosine 3′,5′-monophosphate activator, strongly induced Nurr1 expression consistent with the inhibitor study. Furthermore, mitogen-activated protein kinase (MAPK) pathways, ERK1/2, p38 and JNKs were also examined to evaluate their contributions to I-BOP-induced Nurr1 expression. Mitogen activated protein kinase kinase (MEK) inhibitors, PD98059 and U0126, completely inhibited I-BOP-induced Nurr1 expression. However, p38 inhibitor, SB203580, and JNK inhibitor II showed little effect on the induction (Figure 2B). Proteosome inhibitor, MG132, which inhibits I kB degradation, did not significantly inhibit I-BOP-induced Nurr1 expression indicating that nuclear factor-kappa B (NF-kB) did not contribute much to Nurr1 induction pathway (Figure 2C). Activation of TP is known to transactivate EGFR via activation of Src kinase in several types of cells (22–24). Epidermal growth factor has been shown to activate Nurr1 gene expression directly. We further investigated the relationship between EGFR and I-BOP-induced Nurr1 expression in H157 cells. Figure 2C shows that I-BOP-induced Nurr1 expression was slightly attenuated by EGFR inhibitors, AG1478 and PD153035, but not affected by Src kinase inhibitors, PP1 and PP2. However, epidermal growth factor-induced Nurr1 expression was completely blocked by AG1478 as expected (Figure 2D). These data indicate that
I-BOP-induced Nurr1 expression is mediated primarily by direct activation of TP not by transactivation of EGFR.

Taken together, our results suggest that H157 cells express Nurr1 mainly through PKA-, PKC- and MAPK/ERK-dependent signaling pathways in response to activation of TP by I-BOP.

**Activation of TP-mediated phosphorylation of ERK1/2**

MAPK/ERK-signaling pathway is crucial in cell proliferation and gene transcription regulation (25). TP-mediated phosphorylation of ERK1/2 was achieved through multiple mechanisms such as PKA-, PI-3K-, PKC- and EGFR-involved pathways in several cell lines (22–24,26). We next investigated if there is a cross talk between the first three pathways and the MAPK/ERK pathway. I-BOP induced rapid and strong phosphorylation of ERK1/2 in a time- and dose-dependent manner (Figure 3A). PKA inhibitor, H89, and PI-3K inhibitor, LY294002, did not inhibit significantly I-BOP-induced ERK1/2 activation. PKC inhibitor, GF109203X, significantly decreased phosphorylation of ERK1/2, but it also inhibited basal level of pERK1/2 (Figure 3B). These results indicate that PKC but not PKA or PI-3K was involved in I-BOP-induced activation of MAPK/ERK pathway and suggest that attenuated Nurr1 expression induced by inhibition of PKC may be due to inhibition, at least in part, of the activation of ERK.

**I-BOP induced Nurr1 expression in CREB-dependent manner**

To further elucidate the transcriptional regulation of Nurr1 gene by I-BOP, we employed luciferase reporter plasmids containing various lengths of 5’-flanking regions of mouse Nurr1 gene promoter and examined transcriptional activity in H157 cells. As reported (27), the sequence of the 5’-flanking region is strongly homologous between human Nurr1 and mouse Nurr1. The regulatory elements NF-κB, CRE and two TGAC sequences that could act as an activator protein 1/c-jun site (TGACTCA) are highly conserved (Figure 4A). The −396/ +112 promoter region with CRE sequence showed more stronger response to I-BOP than the −1329/+132 region with both NF-κB and CRE sequences indicating that CRE sequence was primarily involved in the activation (Figure 4B). This is consistent with our inhibitor study in which NF-κB inhibitor, MG132, only slightly inhibited I-BOP-induced Nurr1 expression. Mutation of CRE sequence in −396/ +132 region greatly reduced the promoter activity induced by I-BOP or FSK further indicating that I-BOP may signal by activation of CREB-dependent pathway. We further examined if I-BOP-induced phosphorylation of CREB at Ser-133 is a pivotal site for the induction of its transcription activity. As shown in Figure 4C, I-BOP stimulated phosphorylation of CREB at Ser-133 through PGE2, PKC and MAPK/ERK-signaling pathways since inhibitors of these kinases blocked I-BOP-induced activation of CREB.

**PGE2 activates Nurr1 expression in lung cancer cells in a cyclic adenosine 3′,5′-monophosphate/PKA-dependent and MAPK/ERK-independent manner**

PGE2 has been shown to induce Nurr1 expression in colorectal cancer cells (18). Whether PGE2 induces Nurr1 expression in lung cancer cells remains to be determined. Compared with TP agonist I-BOP, PGE2 induced Nurr1 expression at significantly higher concentrations in H157 and H460 cells (Figure 5A) but not in A427 and H358 cells (data not shown). Only H1299 cells appeared to be sensitive to low concentration of PGE2. Reverse transcription–PCR analysis showed that both isoforms of TP, TPα and TPβI, and four types of PGE2 receptors, EP1, EP2, EP3 and EP4, are expressed in several of these cell lines (Figure 5B). MEK1 inhibitors, PD98059 and U0126, slightly inhibited PGE2-induced Nurr1 expression in both H157 and H1299 cells. PKA inhibitor, H89, strongly blocked PGE2-induced Nurr1 expression (Figure 5C). Unlike I-BOP that induces ERK phosphorylation in H157 cells, PGE2, on the contrary, strongly inhibited ERK phosphorylation in a dose-dependent manner in H157 and H1299 cells. FSK, an adenylate cyclase activator, also induced strong inhibition of ERK phosphorylation in these cells (Figure 5D). PKA inhibitor, H89, and adenylate cyclase inhibitor, SQ22536, reversed the effect of PGE2-induced inhibition of ERK activation (Figure 5E). Taken together, these results indicated that PGE2-induced Nurr1 expression may involve PKA but not ERK, whereas I-BOP-induced Nurr1 expression may include both PKA and ERK in lung cancer cells.
Nurr1 regulates cyclin D1 expression and mediates I-BOP-stimulated H157 cell proliferation

There has been no report showing that TP mediates lung cancer cell proliferation and growth although other studies indicate so in several cell lines (28,29). We examined if I-BOP could stimulate TP-mediated H157 cell proliferation. Figure 6A indicated that I-BOP stimulated H157 cell proliferation in a dose-dependent manner as determined by thymidine uptake. I-BOP at 50 nM increased ~50% cell proliferation within 48 h. I-BOP also induced cell cycle protein cyclin D1 expression within 7 h (data not shown). I-BOP-stimulated cell proliferation was fully blocked by a TP antagonist, SQ29548, indicating that it was a TP-mediated event (Figure 6B). To determine the functional relevance of Nurr1 expression to I-BOP-induced cell proliferation, we employed siRNA approach to knock down Nurr1 expression and examined if Nurr1 was involved in cell proliferation. As shown in Figure 6B, Nurr1 siRNA at 40 pmol/ml strongly knocked down Nurr1 expression and inhibited I-BOP-induced cyclin D1 expression. Nurr1 siRNA also inhibited the basal thymidine uptake significantly more than vehicle.
Fig. 4. Activation of transcription from the Nurr1 promoter in H157 cells. (A) Nucleotide sequences of the human and mouse Nurr1 promoters. The regulatory elements NF-κB, CRE and two TGAC sequences are boxed. Asterisk indicates conserved nucleotides. (B) Effects of I-BOP and FSK on Nurr1 promoter activity. H157 cells were transfected with −1329/+132 or −396/+132 Nurr1 promoter constructs fused to luciferase reporter plasmid and maintained in vehicle (0.1% ethanol) and then treated with 50 nM I-BOP or 25 μM FSK for 24 h. The expression ratio of I-BOP- or FSK-treated cells to control was shown. Values were means ± SDs of three independent experiments. Mutated CRE sequence is shown in upper case. *p values <0.01. (C) MAPK/ERK pathway is involved in activation of CREB by I-BOP. Cells were serum starved for 24 h and then treated with 10 μM H89, 10 μM PD98059 or vehicle (0.1% ethanol) for 30 min before stimulation with 50 nM I-BOP for 15 min. Detection and quantification of phospho-CREB levels were carried out as described under Materials and Methods.

Fig. 5. PGE2 induced Nurr1 expression in lung cancer cells. (A) PGE2-induced Nurr1 expression in lung cancer cells. H157, H460 and H1299 cells were serum starved for 24 h and then treated with 0.1 or 1 μM of PGE2 or 50 nM I-BOP or vehicle (0.1% ethanol) alone for 3 h. Nurr1 levels were determined by western blotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used to ensure equal protein loading. Densitometric analysis of each band was made. The ratio of Nurr1 to glyceraldehyde-3-phosphate dehydrogenase densities with vehicle alone was normalized to 1.0. Results are representative of three independent experiments. (B) Reverse transcription–PCR analysis of TP receptors TPα and TPβ and EP receptors EP1–EP4 in different lung cancer cells. Total RNA was isolated from H157, H460, A427, H359 and H1299 cells and reverse transcription–PCR analysis was carried out as described under Materials and Methods. (C) Effects of various inhibitors on PGE2-induced Nurr1 expression in H157 and H1299 cells. The cells were serum starved for 24 h and then treated with 10 μM H89, 10 μM PD98059, 5 μM U0126 or vehicle (0.1% ethanol) alone for 30 min before stimulation with 1 μM PGE2 for 3 h. Nurr1 levels were determined by western blotting. Detection and quantification of Nurr1 were carried out as described under Materials and Methods. Results are representative of three independent experiments. (D) PGE2 inhibits phosphorylation of ERK1/2 in H157 and H1299 cells. Cells were serum starved for 24 h and then treated with different concentrations of PGE2 (0.01–1 μM) or 25 μM FSK or vehicle (0.1% ethanol) for 10 min. Detection and quantification of phospho-ERK1/2 were carried out as described under Materials and Methods. Results are representative of three independent experiments. (E) ERK1/2 in H157 and H1299 cells. Cells were serum starved for 24 h and then treated with 10 μM H89, 100 μM SQ22536 or vehicle (0.1% ethanol) for 30 min before stimulation with 1 μM PGE2 for 10 min. Detection and quantification of phospho-ERK1/2 were carried out as described under Materials and Methods. Results are representative of three independent experiments.
and Methods for 24 h before stimulation with 50 nM I-BOP for 48 h. 3H-thymidine uptake assay was carried out as described under Materials and Methods. Values were mean ± SD (n = 4). *P values >0.01. (B) Effect of Nurr1 siRNA on I-BOP-stimulated thymidine uptake and Nurr1 and cyclin D1 expressions. Cells were transiently transfected with either Nurr1 siRNA or control siRNA as described under Materials and Methods for 24 h before stimulation with 50 nM I-BOP for 48 h. 3H-thymidine uptake assay was carried out as described under Materials and Methods. Values were mean ± SD (n = 4). *P values >0.01. (C) Cell images were taken before performing proliferation assay as indicated in (B).

Discussion

The inducible cyclooxygenase (COX-2), a rate-limiting cyclooxygenase isozyme in generating prostanooids, has been found to overexpress in a variety of tumors (30). Among the COX-2-derived prostaglandins, PGE2 is the most studied in its role in inflammation and in tumor progression. Recently, several studies have suggested that another COX-2-derived product, TXA2, may also play important roles in tumor progression (6–9). We previously reported that I-BOP, a TXA2 mimic, could induce COX-2 expression in lung cancer A549-TPα cells resulting in the synthesis of comparable amount of PGE2 and TXA2 (measured as a stable metabolite thromboxane B2) (21). Apparently, both PGE2 and TXA2 are important in lung carcinogenesis. We sought to identify downstream effector genes that are regulated by TXA2 in lung cancer cells. Our results indicate that the nuclear receptor Nurr1 is regulated by TXA2 and PGE2 in lung cancer cells. This is consistent with a previous report that Nurr1 is regulated by PGE2 in colon cancer cells (18). However, these two eicosanoids appear to exhibit different mechanisms in their induction of Nurr1 expression in lung cancer cells. This is evident from following observations. Firstly, I-BOP induced Nurr1 expression at nanomolar concentrations in H157 and H460 cells, whereas PGE2 stimulated Nurr1 expression at micromolar levels in the same cell lines. PGE2 induced Nurr1 expression at nanomolar levels only in H1299 cells in which I-BOP showed minimal response. It appears that the induction of Nurr1 expression by I-BOP and PGE2 at more physiologically relevant concentrations can be cell specific. Whether this is related to the levels of expression of receptor subtypes remains to be determined. Current studies (Figure 5B) as well as others (31) indicate that both TPα and TPβ isoforms and four subtypes of EP receptors were found to express in different degrees in these cell lines. Secondly, inhibitor studies indicated that I-BOP-induced Nurr1 expression was found to be mediated by PKA, PKC and MAPK/ERK pathways but not by PI-3K, src kinase, p38 MAPK, JNK/stress-activated protein kinase and EGFR transactivation pathways in H157 cells (Figures 2 and 3). Interestingly, PGE2-induced Nurr1 expression was found to be mediated by PKA but not by MAPK/ERK in H157 and H1299 cells (Figure 5C). In fact, PGE2 inhibited ERK activation in a PKA-dependent manner in both the cell lines since the inhibition could be blocked by inhibitors of PKA and adenyulate cyclase (Figure 5E). Previously, Krysan et al. (31) reported that PGE2 at high concentration (28 μM) was able to induce ERK activation in H157 and H460 cells. This is in contrast to our current finding of PGE2 at much lower concentrations (<1 μM).

We were also able to confirm that PGE2 at 28 μM activated ERK in the same cell lines and induced Nurr1 expression although the level of induction was not as high as that achieved by low concentrations (data not shown). Divergent effects of PGE2 at low and high concentrations could be due to activation of different EP receptors. Both EP2 and EP4 receptors are known to couple to adenylate cyclase resulting in the activation of PKA and subsequent phosphorylation of Raf-1 (32). However, phosphorylation of Raf-1 at Ser-338 and Ser-259 appears to lead to activation and inhibition of Raf-1/MEK/ERK cascade, respectively (33). A recent report showed that PGE2 at nanomolar level was able to phosphorylate Raf-1 at Ser-259 and inhibit Raf-1/MEK/ERK cascade through activation of EP4 in HCS-2/8 chondrocytes (34). It is tempting to speculate that PGE2 inhibited ERK phosphorylation through EP4 at nanomolar levels but activated ERK phosphorylation through EP2 at micromolar concentrations in lung cancer cells.

The positive role of ERK in cell growth and proliferation has been extensively studied. TP-mediated ERK activation has also been reported in several cell lines (23,24). PKA and PKC have been shown to be involved in ERK activation in these cell lines. However, our current study indicated that PKA was not probably an upstream kinase of I-BOP-induced ERK activation since H89 could not block the ERK activation (Figure 3B). This suggests that there is another target mediated by PKA in I-BOP-induced Nurr1 expression in H157 cells. CREB protein was known to be a principal mediator of positive changes in gene expression in response to cyclic adenosine 3',5'-monophosphate following phosphorylation by PKA. PKA-CREB pathway was reported to be involved in the induction of N4R4A subfamily members by cytokine and growth factor (11,12,18). Our results of Nurr1 promoter study demonstrated that CREB did play an important role in TP-mediated Nurr1 expression in H157 cells. PKA
inhibitor, H89, totally blocked I-BOP-induced Nurr1 expression and CREB phosphorylation, further supporting that PKA–CREB pathway is involved in I-BOP-induced Nurr1 expression in H157 cells. Furthermore, ERK pathway inhibitor, PD98059, partially inhibited CREB activation providing the evidence that ERK may function as an upstream kinase of CREB. PKC inhibitor, GF109203X, partially inhibited I-BOP-induced ERK and CREB activations suggesting that PKC mediated I-BOP-induced Nurr1 expression through both of the two downstream signaling molecules.

Nurr1 promoter has CRE and NF-kB motifs that are known to involve in the agonist-induced transcription of Nurr1 gene. NF-kB pathway was shown to be active in interleukin-1β- and tumor necrosis factor-α-stimulated Nurr1 expression (35), whereas CRE-binding site is essential in PGE2-stimulated Nurr1 expression (18). We investigated the role of CRE and NF-kB in the regulation of I-BOP-induced Nurr1 gene transcription using luciferase reporter assay. Our results demonstrated that CRE but not NF-kB motif plays an important role in the regulation of TP-mediated Nurr1 gene transcription. CREB also has been shown to be required for the transcription of Nur77 and Nor-1 gene (36). Furthermore, the promoter activity induced by I-BOP is stronger than that by FSK suggesting that there may be other target signaling molecules for ERK beside CREB to induce Nurr1 expression. Torii et al. (27) reported that more conserved regulatory elements such as Sp1, CuxG-like and two TGAC sequences exist in both human and mouse Nurr1 promoter which might be responsible for the regulation of the transcription of Nurr1 gene.

NR4A receptors are known to function as transcription factors to activate target genes. A number of NR4A target genes have been elucidated in central nervous system (37,38). Cyclin D1 and cyclin D2 were found to be regulated by Nor-1 in vascular smooth muscle cells (17). Our previous study showed that I-BOP caused a rapid and persistent increase in cyclin D1 expression in HEK-293 cells transfected with TPα (39). Our present studies using siRNA to knock down Nurr1 expression indicated that Nurr1 is not only required for I-BOP-induced H157 cell proliferation but also is essential for cell survival. Knock down of Nurr1 expression resulted in the inhibition of cyclin D1 induction by I-BOP indicating that cyclin D1 may be also the target gene of Nurr1 in H157 cells to regulate cell growth.

Nurr1 was found to elevate most dramatically among NR4A subfamily members when H157 and other lung cancer cells were stimulated by I-BOP. Nurr1 appears to play a significant role in lung cancer cell proliferation. These lung cancer cells we used were found to express both TPα and β isoforms (Figure 5B). Which of the two isoforms or both are involved in inducing Nurr1 expression remains to be determined. TPα is known to couple to Gs and Gq, whereas TPβ is shown to couple to Gi and Gq (32). Gs and Gq have been shown to lead to activation of PKA and PKC, respectively. Judging from the inhibitor studies that the induction was both PKA and PKC dependent, we speculate that both isoforms of TP are probably to be involved in I-BOP-induced Nurr1 expression.

In conclusion, our study demonstrated that Nurr1 is the downstream target gene of TP signaling and is essential in I-BOP-stimulated lung cancer cell proliferation by regulating TP-mediated cyclin D1 expression. Signaling study indicated that the event of I-BOP-induced Nurr1 expression is mainly through PKA/CREB, PKC and MAPK/ERK pathways and not related to EGFR and PGE2 pathways in H157 cells. Our results indicate that TXA2 may play its own specific role independent of PGE2 in stimulating cell proliferation. Our studies highlight the significant role of TXA2–TP signaling in lung carcinogenesis. A summary of signaling pathways leading to TP-mediated expression of Nurr1 is shown in Figure 7.

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References

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