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Tyrosine Phosphorylation of Phosphatidylinositol 3-Kinase and of the Thromboxane A_2 (TXA_2) Receptor by the TXA_2 Mimetic I-BOP in A7r5 Cells

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ABSTRACT. Thromboxane A_2 (TXA_2) interacts with its G-protein coupled receptor, the TP receptor, to produce contraction and proliferation of vascular smooth muscle cells. We have shown previously that proliferation of primary cultures of vascular smooth muscle cells initiated by [1*S*-(1α, 2β(5Z), 3α(1*E*, 3*R*), 4α]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2yl]-5′-heptenoic acid (I-BOP), a stable TXA_2 mimetic, is mediated by activation of mitogen-activated protein (MAP) kinase. In the present study, we examined further the intracellular mediators involved in TXA_2 activation of vascular smooth muscle cells. Transient transfection of the cDNA for the TP receptor into A7r5 vascular smooth muscle cells resulted in expression of TP receptors with a receptor density, B_{max}, of 0.7 ± 0.2 pmol/mg protein and a receptor affinity, K_d, of 0.6 ± 0.1 nM (N = 7). Mock transfected cells lacked significant receptor expression. In TP receptor transfected cells, I-BOP increased the activation of MAP kinase 2-fold, stimulated tyrosine phosphorylation of cellular proteins of relative molecular mass (M_r) of 140, 85, 60, 56, and 45 kDa, and increased the message for c-*jun*, a nuclear transcription factor involved in mitogenesis, 2.6-fold. Immunoblot analysis indicated that the 85-kDa protein represented phosphoinositide 3-kinase (PI3-K), while the 60 kDa protein was the TP receptor. The activity of PI3-K was increased 3.5-fold by the addition of I-BOP (0.1 μM). In summary, the present study demonstrated that stimulation of the TP receptor results in tyrosine phosphorylation of the receptor and of PI3-K. BIOCHEM PHARMACOL 53;12:1823–1832, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. prostaglandins; thromboxane A_2; vascular smooth muscle proliferation; tyrosine kinases

TXA_2,§ a cyclooxygenase metabolite of arachidonic acid, is a potent initiator of platelet aggregation and of vascular smooth muscle contraction and proliferation (1). The ability of TXA_2 to promote proliferation and to act as a cell cycle progression factor in vascular smooth muscle cells has been demonstrated in studies employing mimetics of TXA_2 (2–4). Previously, we have shown that I-BOP, a stable mimetic of TXA_2, stimulates proliferation of rat and guinea pig aorta smooth muscle cells as well as coronary artery smooth muscle cells (5). Proliferation of guinea pig coronary artery cells coincided with activation of MAP kinase. These findings were confirmed subsequently by others using smooth muscle cells derived from rat aorta (6). PI3-K is an enzyme responsible for producing phosphorylation of phosphatidylinositol 4,5-bisphosphate at the D-3 position on the inositol ring, yielding the product PI(3,4,5)P3. PI3-K consists of an 85 kDa adapter subunit and a 110 kDa catalytic subunit. The 85 kDa subunit can be tyrosine phosphorylated and, through its SH2 domains, can interact with specific phosphotyrosine-containing motifs of other proteins, such as those found in the activated PDGF and insulin receptors (7, 8). The activation of the 110 kDa subunit is produced by growth factor receptors, small molecular weight GTP binding proteins such as Rac and Rho (9), and also by G-protein coupled receptors (10) and the βγ subunits of heterotrimeric G-proteins (11–13). The product of PI3-K activity, PI(3,4,5)P3, has been implicated in numerous cellular responses including glucose transport (14), histamine secretion (15), activation of protein kinase B (16) and PKC_ζ (17), and cell proliferation (18).

Similar to growth factor receptors, certain G-protein coupled receptors have been shown recently to be tyrosine phosphorylated, both in a basal state as shown for the bradykinin receptor (19) and also as a result of stimulation, as seen in the endothelin (20), angiotensin II (21) and β_2

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§ *Abbreviations:* TXA_2, thromboxane A_2; TP, thromboxane A_2 receptor; MAP, mitogen-activated protein; SH2, src homology 2; PDGF, platelet-derived growth factor; HEL, human erythroleukemia cells; PVDF, polyvinylidene difluoride; I-BOP, [1S-(1α, 2β(5Z), 3α (1*E*, 3*R*), 4α)]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1] heptan-2yl]-5′-heptenoic acid; PI3-K, phosphoinositide 3-kinase; PI (3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; DMEM, Dulbecco's Modified Essential Medium; FBS, fetal bovine serum; AB, 1% antibiotic/antimycotic/fungizone; ECL, enhanced chemiluminescence; PMSF, phenylmethylsulfonyl fluoride; and SSC, 150 mM NaCl, 15 mM sodium citrate, pH 7.0.

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adrenergic (22) receptors. The mechanism for these tyrosine phosphorylations has not been delineated. However, the role of these phosphorylations, which have been shown to occur at the carboxy-terminal tail of the receptors, is that of a stimulus for receptor sequestration and desensitization (22–24). Recent evidence suggests that this phenomenon may be related to additional signaling pathways. For example, activation of the AT1a receptor was shown to produce association of the receptor with the cytoplasmic tyrosine kinase JAK2, in that the activated receptor co-immunoprecipitated with JAK2 (25). In platelets, thrombin and U46619, a TXA_2 mimetic, produced activation of PI3-K (26). This suggests direct interactions between G-protein coupled receptors and tyrosine kinase pathways, which are activated by growth factor receptors. This concept is supported by recent findings showing a role for the insulin-like growth factor-1 receptor in thrombin-induced mitogenesis (27).

In light of these findings, further elucidation of the pathway for G-protein coupled receptors to initiate proliferation is required. In particular, clarification of the mitogenic signal transduction pathway in vascular smooth muscle elicited by TXA_2 is necessary. Previous studies examining the signal-transduction pathway of TXA_2 in vascular smooth muscle cells have employed primary cell cultures. The use of primary cultures suffers from cell line variability, finite cell lineage, and relatively weak responsiveness. In the present study, we have used the cDNA for the HEL cell TP receptor (homologous to the human placental TP receptor (28) as a model G-protein coupled receptor. This receptor was transiently expressed in the established clonal cell line A7r5, derived from neonatal rat aorta smooth muscle, and was used to characterize the cellular responses thought to be involved in proliferation. In this study, we have further characterized the TXA_2 proliferative pathway including possible involvement of PI3-K.

MATERIALS AND METHODS

Materials

A7r5 cells were from ATCC (Rockville, MD). All cell culture reagents were from GibcoBRL (Grand Island, NY). The TXA_2 mimetic I-BOP and its radioiodinated form [^{125}I]BOP were synthesized as described previously (29). Radioisotopes were from Dupont New England Nuclear (Boston, MA). Antibodies were from the following sources: polyclonal anti-PI3-K p85, monoclonal anti-phosphotyrosine and anti-phosphotyrosine-agarose, Transduction Laboratories (Lexington, KY); anti-TP receptor antiserum, raised against the peptide sequence TLCHVYHGQC, representing residues 221–230 located in the third cytoplasmic loop of the TP receptor, coupled to keyhole limpet hemocyanin as a carrier protein, was obtained from Dr. P. V. Halushka, Medical University of South Carolina (Charleston, SC). The antiserum was tested for specificity using a dot-blot analysis against the immunizing peptide.* TP receptor cDNA was a gift of Drs. C. J. Allan and P. V. Halushka, Medical University of South Carolina. Myelin basic protein and 1-α-phosphatidylinositol 4-monophosphate were obtained from the Sigma Chemical Co. (St. Louis, MO), while 1-α-phosphatidylinositol was from Avanti (Alabaster, AL). The reagents for the ECL procedure were obtained from Amersham. Photographic film used for the ECL procedure and for the northern blot and PI3-K autoradiograms were from Eastman Kodak (Rochester, NY).

Cell Culture and Transfection

A7r5 cells were maintained in DMEM supplemented with 1% AB and 10% FBS in a humidified atmosphere (95% O_2, 5% CO_2). Passaging of cells for maintenance and for studies was done with trypsin/EDTA (0.05%/0.5 mM). For the purposes of placing the cells into a state of quiescence prior to studies involving I-BOP-stimulated responses, serum concentrations were decreased to 0.5% for 24 h. Cell monolayers were preincubated in serum-free DMEM or Hanks' balanced salt solution with indomethacin (10 μM, 15 min) prior to study.

For transient transfections, confluent monolayers of A7r5 cells in 150-mm petri dishes were exposed to the cDNA for the TP receptor in the pCDNAIII plasmid. The transfection mixture [10 mL/dish; polyornithine (10 μg/mL), TP cDNA (50 μg), FBS (1%) in DMEM] was removed after a 5-h incubation, and the cells were exposed for 1 min to DMEM, 10% FBS, and 20% glycerol. The cell monolayer was washed with PBS and then DMEM, 10% FBS, 1% AB was added. Forty-eight to seventy-two hours later, the time at which TP receptors reach a maximum expression (data not shown), cells were used for assays. For permanent transfections, the cDNA for the TP receptor was introduced into the cells by a standard calcium phosphate precipitation protocol. Selection of clones was performed 2 days post-transfection using a high concentration of G418 (400 μg/mL) which, when individual clones were identified and passaged, was decreased to 100 μg/mL. Screening for clones expressing TP receptor was performed by radioligand binding assays.

Radioligand Binding and MAP Kinase Assays

Equilibrium radioligand binding assays using [^{125}I]BOP were performed on monolayers of transfected cells according to previously published procedures (5). The TP receptor antagonist SQ29548 (10 μM) was used to measure specific binding (86 ± 2%, N = 7). Measurement of I-BOP activation of MAP kinase in the transfected cells was as described previously (5). Briefly, subsequent to stimulation, monolayers of transfected A7r5 cells were washed two times

* Martin M., Davis-Bruno K. and Halushka P. V., personal communication. Cited with permission.

with cold PBS, scraped into PBS, and centrifuged for 30 sec. Ice-cold lysis buffer (20 mM HEPES, pH 7.5; 80 mM β-glycerophosphate; 10 mM EGTA; 2 mM EDTA; 0.3 mg/mL dithiothreitol) was then added to the pellet, tip-sonicated, and centrifuged (100,000 *g*, 20 min, 4°). The supernatant was removed and used for assay of MAP kinase activity. Transfer of ^{32}P from [γ-^{32}P]ATP to the substrate myelin basic protein was used as an indication of MAP kinase activity. Stimulated activity was corrected for protein and compared with that seen in unstimulated control cells.

Immunoprecipitation and Immunoblotting

For the immunoprecipitation studies, monolayers of cells, subsequent to stimulation, were solubilized in buffer (50 mM HEPES; 10 mM NaPP; 100 mM NaF; 4 mM EDTA; 2 mM sodium orthovanadate; 10 mM CHAPS; 1 mM PMSF; 60 mU/mL aprotinin). After rotating for 30 min at 4°, the lysates were equalized for protein content (Dc protein assay; Bio-Rad, Hercules, CA), incubated with an anti-phosphotyrosine antibody coupled to agarose, and rotated for 1 hr, 4°. The immunoprecipitates were centrifuged and washed twice with buffer (1% Triton X-100; 150 mM NaCl; 10 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA; 0.5% NP-40; and freshly added 0.2 mM PMSF and 0.2 mM sodium vanadate). The immunoprecipitated pellet was then prepared for SDS–PAGE and immunoblotting by addition of 2× Laemmli sample buffer and boiled for 5 min. Subsequent to separation of immunoprecipitated proteins by SDS–PAGE, samples were transferred to a PVDF membrane according to standard techniques (Hoeffer semi-dry transfer apparatus; Hoeffer, San Francisco, CA). Following transfer, the blot was blocked [5% BSA in Tris buffered saline (T-TBS); 10 mM Tris, pH 7.5; 100 mM NaCl containing 0.05% Tween-20] for 1 hr and then placed directly into the same buffer containing the primary antibody of interest. After an overnight incubation at 4°, the blots were washed three times for 20 min in the antibody free 5% BSA T-TBS and, in the case of the monoclonal horseradish peroxidase coupled anti-phosphotyrosine antibody, immediately detected using the ECL procedure or placed into 5% BSA T-TBS containing the secondary antibody (horseradish peroxidase-coupled goat anti-rabbit IgG, 1:7500; Bio-Rad) and incubated at room temperature for 90 min. After washing the blot three times for 20 min each in T-TBS, the immunodetected proteins were visualized using the ECL technique. Quantitation of the visualized proteins was performed by scanning densitometry (Hoeffer GS300 scanning densitometer).

PI 3-K Assays

Measurement of the activation of PI3-K was based upon published procedures (30). Subsequent to serum deprivation, cells were exposed to a 1-hr incubation with serum-free DMEM containing 2 mM sodium orthovanadate. Cells were then stimulated in Hanks' balanced salt solution (pH 7.4), containing 0.2 mM sodium orthovanadate, for 5 min, at 37°. At the end of the incubation, dishes were placed on ice, the cells were washed twice with ice-cold PBS, followed by the addition of ice-cold lysis buffer (50 mM HEPES, pH 7.5; 150 mM NaCl; 1.5 mM $MgCl_2$, 1 mM EGTA; 10% glycerol; 1% Triton X-100; 10 mM Na_4PP_i; 100 mM $NaFl_4$ with the following inhibitors added prior to lysis: 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM PMSF, 1 mM sodium orthovanadate, 0.5 mM dithiothreitol: 0.25 mL/100-mm dish). Lysis was allowed to proceed for 15 min by gentle shaking on ice. The lysed cells were scraped, and the lysate was centrifuged for 5 min (16,000 *g*, Eppendorf centrifuge, 4°). An equal amount of cell lysate (250–500 μg) was added to 5 μg of anti-p85 PI3-K polyclonal antibody (total volume adjusted to 1 mL with lysis buffer). Immunoprecipitation was carried out for 2 hr, rotating at 4°, followed by addition of 50 μL of 10% activated Pansorbin (Calbiochem, La Jolla, CA). Following an additional 1-hr incubation, the immunoprecipitate was sequentially washed 4 times with lysis buffer and 2 times with 200 mM HEPES, pH 7.2, 5 mM EDTA. To the washed immunoprecipitated pellet was added the following: 5 μL of 200 mM HEPES, pH 7.2, 5 mM EDTA; 5 μL of substrate (phosphatidylinositol/phosphatidylserine, 2 mg/mL each in 50 mM HEPES, pH 7.5, freshly made); and 10 μL H_2O. The samples were vortexed and incubated at room temperature for 10 min, followed by the addition of 5 μL of [γ-^{32}P]-ATP solution (100 μCi/mL, 22 mM $MgCl_2$, 0.22 mM ATP), vortexed, and incubated for an additional 10 min. Samples were then placed on ice, followed by the addition of 3 μL of cold HCl (concentrated). The product, phosphatidylinositol 3-phosphate, was extracted overnight by chloroform:methanol (1:1) followed by TLC under chloroform:methanol:ammonium hydroxide (9:7:3.5). 1-α-Phosphatidylinositol 4-monophosphate was used as the standard for the migration of phosphatidylinositol 3-phosphate and was visualized using iodine. Following development and visualization of the standard, the plate was exposed to film and subsequently developed. The area corresponding to the migration of the standard was scraped and counted by liquid scintillation. The amount of radioactivity found in the stimulated samples was compared with that seen in unstimulated cells.

Northern Blot Analysis and [^{3}H]Thymidine Studies

Analysis of the mRNA for c-*jun* was performed by northern blot analysis using a specific probe for c-*jun* as previously described (31). Briefly, total RNA was isolated from monolayers of transfected cells, subsequent to stimulation, using the guanidine isothiocyanate method. Total RNA (20 μg) was run on a 1.5% agarose gel. The RNA was transferred to a Nytran membrane by positive pressure, fixed by ultraviolet cross-linking, and pre-hybridized for 3 hr. Subsequently, hybridization was performed with the appropriate ^{32}P-labeled cDNA probe for 18–20 hr at 60°. The membranes were washed three times for 15 min each at room

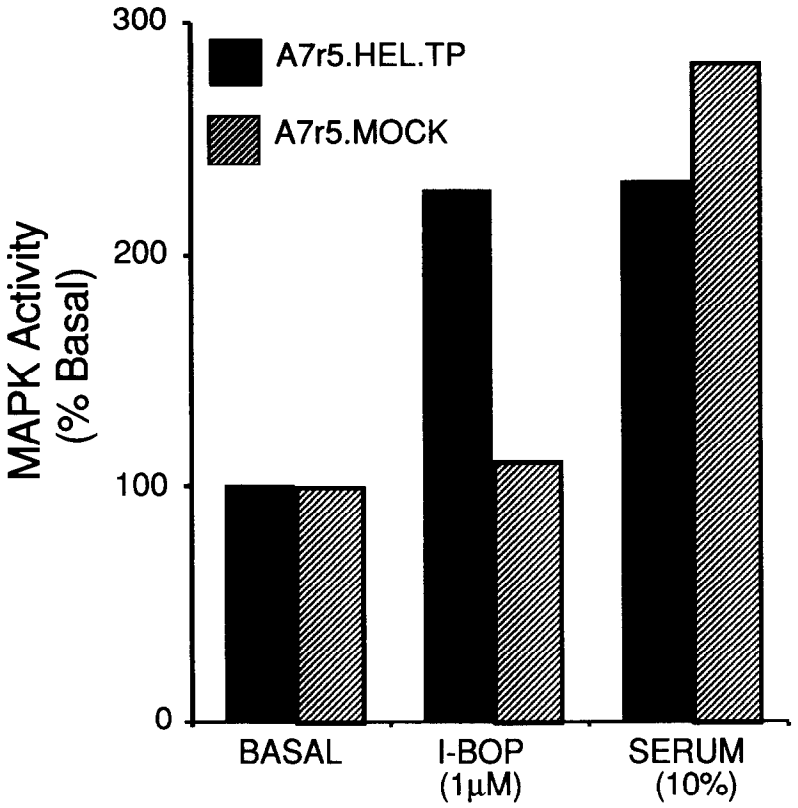


FIG. 1. Activation of MAP kinase. A7r5 cells were transfected with either HEL.TP cDNA in the expression vector pcDNAIII (A7r5.HEL.TP) or with vector alone (MOCK) and exposed to I-BOP (1 μM) or serum (10%) for 10 min, 37°, lysed, and prepared for assay as described. ^{32}P-Phosphorylation of myelin basic protein was used as an index of MAP kinase (MAPK) activity. Activity was corrected for protein content and compared with that seen in the basal state (3 pmol/min/μg protein). Data are the average values for two similar experiments [HEL.TP: I-BOP, 178–276% (range); serum, 221–242%. MOCK: I-BOP, 108–116%; serum, 224–342%).

temperature in 3× SSC and 0.1% SDS and two times at 60° in 0.2× SSC and 0.1% SDS. Autoradiographs of the membranes were obtained and scanned as above. Hybridization with a probe for β-actin was used to correct for unequal loading of RNA.

Measurement of cell proliferation using incorporation of [^{3}H]thymidine into monolayers of stably transfected cells was performed as described previously (5). Monolayers of cells were placed into a state of quiescence by 48-hr exposure to DMEM containing 0.5% serum followed by stimulation with various concentrations of I-BOP for 24 hr. The amount of [^{3}H]thymidine incorporated into the trichloroacetic acid-insoluble extract in response to I-BOP was then compared with that seen under unstimulated conditions.

RESULTS

Radioligand Binding Assays

Monolayers of A7r5 cells were transfected with the cDNA of the HEL.TP receptor. Scatchard analysis of equilibrium radioligand binding assays with the stable TXA_2 mimetic I-BOP revealed the presence of TP receptors with the following characteristics; K_d = 0.61 ± 0.1 nM, B_{max} = 0.7 ± 0.2 pmol/mg total cell protein (N = 7). Nontransfected and mock-transfected (plasmid only) A7r5 cells did not show significant binding or functional responsiveness to I-BOP. These transfected cells, A7r5.HEL.TP, were used to study the proliferative signal-transduction pathway activated by TXA_2 in vascular smooth muscle cells.

Activation of MAP Kinase in A7r5.HEL.TP Cells

Monolayers of A7r5 cells transfected with the vector containing the cDNA for the TP receptor or vector alone (mock) were exposed to I-BOP or to serum. In the mock transfected cells, only serum produced an increase in MAP kinase activity (3-fold greater than unstimulated cells). In the cells transfected with the TP receptor, both serum and I-BOP produced an increase in activity (Fig. 1). In cells expressing TP receptors, I-BOP produced an increase similar to that seen for serum (2.5-fold over basal).

Stimulation of Tyrosine Phosphorylation

Monolayers of A7r5.HEL.TP cells were exposed to serum, I-BOP, or vehicle (control) and examined, by immunoblotting, for resultant tyrosine phosphorylation. In the TP receptor expressing cells, both serum and I-BOP produced similar increases in tyrosine phosphorylation of several cellular proteins (Fig. 2). Proteins of relative molecular mass of 140, 85, 60, 56, and 45 kDa showed an increase in tyrosine phosphorylation in response to I-BOP. A protein of 42 kDa appeared to be dephosphorylated by I-BOP.

Tyrosine Phosphorylation of the 85k Subunit of PI3-K

A7r5.HEL.TP cells were exposed to increasing concentrations of I-BOP, lysed, and immunoprecipitated with an agarose-coupled anti-phosphotyrosine antibody. The immunoprecipitated proteins were separated by SDS–PAGE,

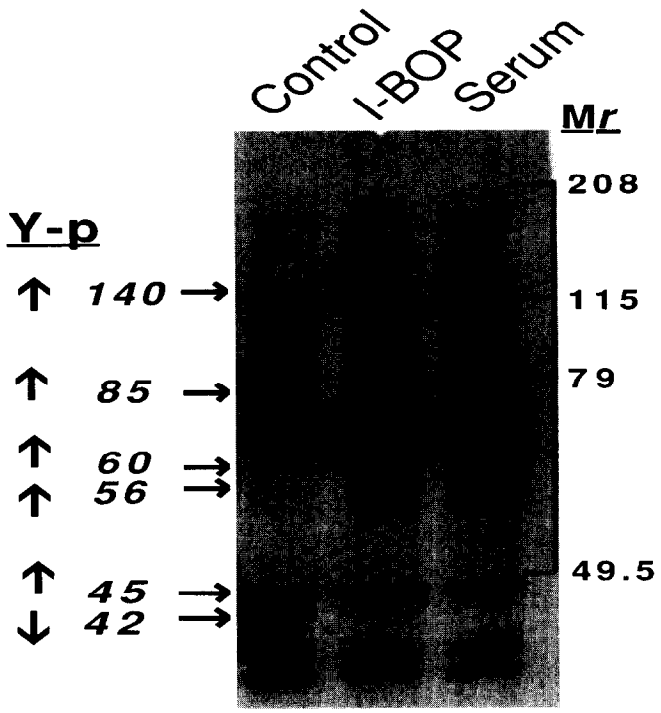


FIG. 2. Immunoblot of tyrosine phosphorylation in A7r5.HEL.TP cells. Monolayers of cells were exposed to vehicle (control), I-BOP (1 μM), or 10% serum for 15 min, 37°. Cell lysates were equalized for protein (50 μg/lane) and separated by SDS–PAGE followed by transfer to PVDF and immunoblotted with an anti-phosphotyrosine antibody and detected by ECL. A representative scan of three similar experiments is shown. M_r standards are indicated. y-P-tyrosine phosphorylation.

transferred to PVDF, and probed with a polyclonal antibody to the 85 kDa subunit of PI3-K. I-BOP produced a concentration-dependent increase in the amount of p85 immunoprecipitated by the anti-phosphotyrosine antibody, reaching a maximum increase of 2.7-fold at 0.1 μM (as measured by scanning densitometry) over unstimulated cells (Fig. 3). In addition, a protein with a relative mass of 110 kDa was also detected, suggesting co-immunoprecipitation of the PI3-K catalytic subunit.

Activation of PI3-K

Tyrosine phosphorylation of the 85 kDa subunit of PI3-K has been associated with activation of the enzyme [7, 8]. Initial experiments were performed to demonstrate activation of PI3-K in A7r5 cells. Addition of 10% serum to monolayers of cells produced an increase in incorporation of radioactivity into a product migrating on TLC comparable to phosphatidylinositol 4-monophosphate in anti-p85 PI3-K immunoprecipitates. This activity produced by 10% serum was inhibited by pretreatment of cells with the PI3-K inhibitor wortmannin (Fig. 4A). In A7r5.HEL.TP cells, both serum and I-BOP produced an increase in PI3-K activity (Fig. 4B). The increase in activity produced by I-BOP was concentration dependent and was inhibited by the TP receptor antagonist SQ29548 (Fig. 4C). These results demonstrate activation of PI3-K by interaction of I-BOP with the TP receptor.

Tyrosine Phosphorylation of the TP Receptor

Addition of I-BOP to monolayers of A7r5.HEL.TP cells produced an increase in the amount of a protein with an apparent molecular mass of 60 kDa immunoprecipitated by an antibody to phosphotyrosine. This protein was recognized by an antibody to the TP receptor (Fig. 5A). I-BOP produced an approximately 3.5-fold increase in the amount of immunoprecipitated protein detected by the TP receptor antibody as compared with unstimulated cells (Fig. 5B). This apparent increase in tyrosine phosphorylation of the TP receptor produced by I-BOP was blocked by the TP receptor antagonist SQ29548 (65% decrease compared with I-BOP alone).

Induction of the Message for c-jun

Initiation of the activation of the early response genes is directly involved in the signal for cell proliferation. Addition of I-BOP to A7r5.HEL.TP cells for 1 hr resulted in induction of the message for the early response gene c-*jun* (Fig. 6). A time of 1 hr was chosen for these studies since, in primary cultures of guinea pig coronary artery smooth muscle cells, induction of the message for c-*jun* peaked at 1 hr (data not shown). In the present study, I-BOP produced a 2.6-fold increase over basal levels.

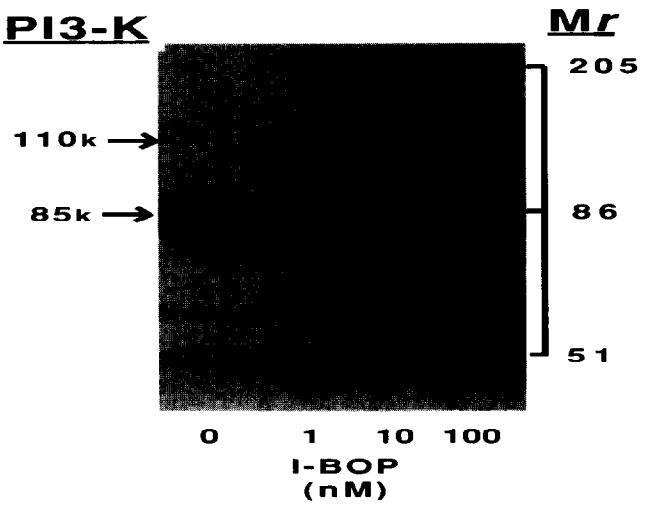
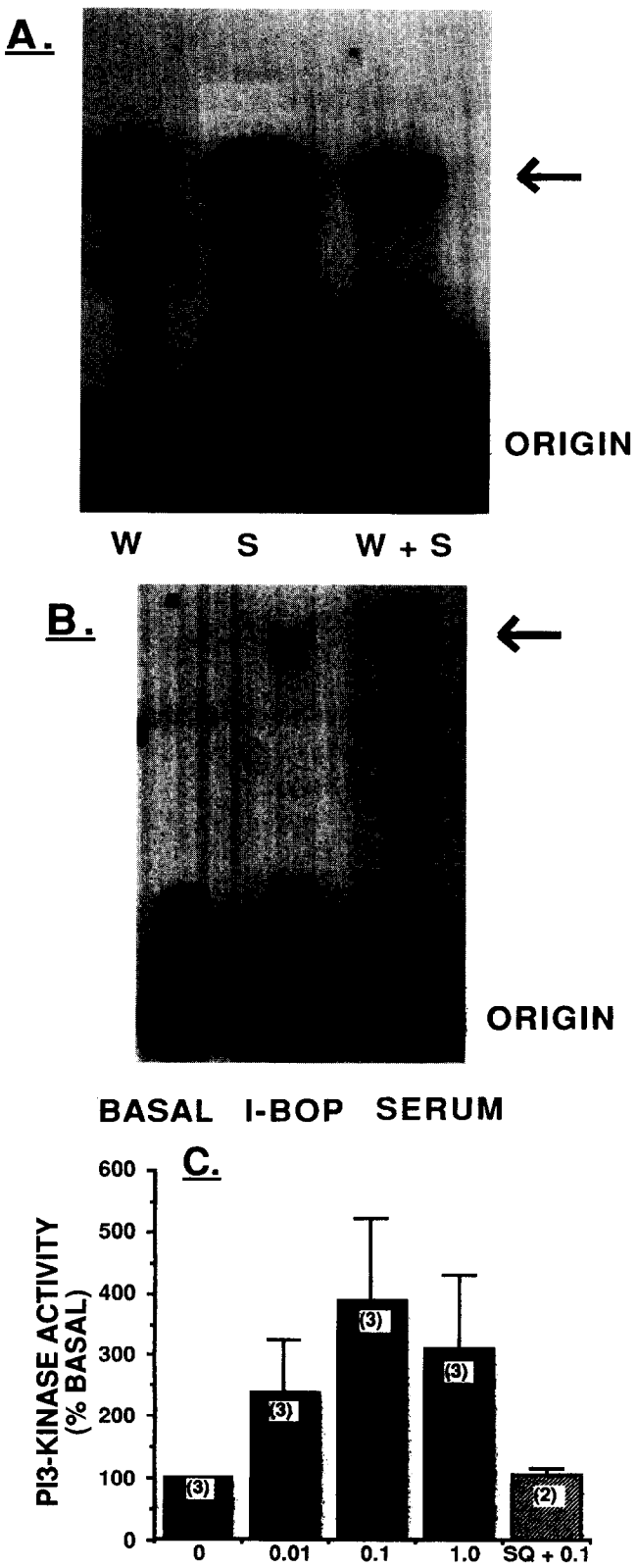


FIG. 3. I-BOP-stimulated tyrosine phosphorylation of p85. A7r5 HEL.TP cells were stimulated with increasing concentrations of I-BOP, lysed, immunoprecipitated with anti-phosphotyrosine-coupled agarose, separated by SDS–PAGE, and immunoblotted with an antibody to the 85kDa subunit of PI3-K. Detection was by ECL. One hundred nanomolar I-BOP produced a 2.7-fold increase in tyrosine phosphorylation of the 85 kDa subunit of PI3-K (N = 2). M_r standards are indicated.



Proliferation of A7r5.HEL.TP Cells

Induction of the message for the early response gene c-*jun* is suggestive of the ability of I-BOP to stimulate proliferation in this cell model. To directly assess the ability of I-BOP to stimulate cell replication, [^{3}H]thymidine incorporation studies were performed in A7r5 cells stably expressing the TP receptor. In these cells, I-BOP produced a significant, concentration-dependent increase in [^{3}H]thymidine incorporation (Table 1).

DISCUSSION

In the present study, we have characterized transiently expressed TP receptors in the clonal vascular smooth muscle cell line A7r5. The TP receptor affinity found in the present study was similar to that seen in previous studies in rat platelets and vascular smooth muscle cells. However, compared with the affinity of this receptor seen in its native state, i.e. HEL cells, that found in the present study was higher (2 vs 0.6 nM, respectively). This difference in affinity may reflect the G-protein coupling efficiency of this receptor as has been described previously (28). The receptor density, however, was from 8- to 14-fold greater, respectively, than that seen previously (32, 33). We have used this cell line as a model system to further elucidate the signal-transduction pathway for cell proliferation produced by TXA_2.

Activation of MAP kinase is known to be one of the key intracellular signals for cellular proliferation. Mimetics of TXA_2 have been shown previously to activate MAP kinase in primary cultures of vascular smooth muscle cells (5, 6). I-BOP, a TXA_2 mimetic, produced activation of MAP kinase in the A7r5.HEL.TP cells and produced increases in [^{3}H]thymidine incorporation into A7r5 cells permanently expressing the TP receptor. These findings indicate that the expressed TP receptors, in a manner similar to TP receptors of primary vascular smooth muscle cells, are coupled to intracellular signals involved in cell proliferation.

Activation of MAP kinase is produced through phosphorylation of tyrosine/threonine residues on the kinase by additional upstream kinases, i.e. MEK kinases. In addition to being involved in MAP kinase activation, the phenom-

FIG. 4. Activation of PI3-K. Measurements of the activation of PI3-K were performed as described in Materials and Methods. Scan of autoradiograms from TLC plates of anti-p85 PI3-K immunoprecipitates from (A) A7r5 cells incubated with wortmannin (100 nM, W), serum (10%, S) or both (W + S) or (B) A7r5.HEL.TP cells incubated with I-BOP (0.1 μM), 10% serum, or vehicle (basal) and assayed for PI3-K activity. (C) Summary of I-BOP activation of PI3-K. Areas on TLC plates corresponding to the migration of standard (indicated by arrows in panels A and B) were scraped and counted for radioactivity. The amount of radioactivity found in samples from stimulated cells was compared to that seen under unstimulated, basal conditions (240 ± 100 cpm). Concentrations of I-BOP were as indicated; SQ29548 (10 μM) is a TP receptor antagonist. Values shown are the means ± SEM where N = 3, and mean ± range where N = 2.

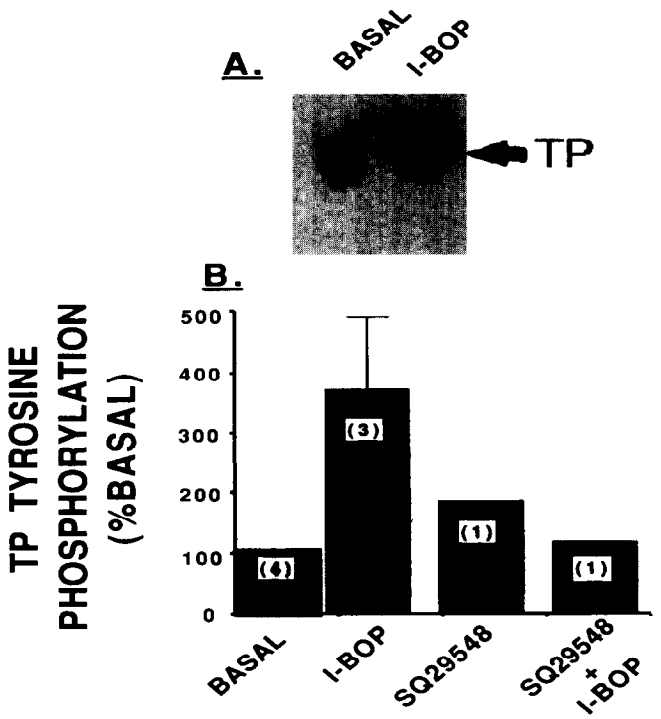


FIG. 5. I-BOP-stimulated tyrosine phosphorylation of the TP receptor. (A) Scan of anti-TP receptor immunoblot of A7r5.HEL.TP cells exposed to I-BOP (1 μM) or vehicle (basal). Cell lysates were exposed to anti-phosphotyrosine-agarose. Immunoprecipitated proteins were separated by SDS–PAGE, transferred to PVDF, and immunoblotted with anti-TP antiserum. Detection was by ECL. (B) Summary of densitometric scanning of immunoblots showing inhibition by the TP receptor antagonist SQ29548 (10 μM).

enon of tyrosine phosphorylation has been associated with growth factor stimulated cell proliferation. In the present study, we have shown that I-BOP stimulated tyrosine phosphorylation of proteins of apparent molecular mass of 140, 85, 60, 56, and 45 kDa. In a study examining the signal-transduction pathway for TXA_2 in primary cultures of rat aorta vascular smooth muscle cells, the TXA_2 mimetic U46619 was shown to produce tyrosine phosphorylation of proteins of comparable molecular mass (34). The apparent decrease in tyrosine phosphorylation of the 42 kDa protein, shown in the present study, may suggest activation of a tyrosine phosphatase.

Comparison of the relative masses of these proteins with proteins known to be tyrosine phosphorylated and involved in cell proliferation suggested that the 85 kDa protein may represent the adapter subunit of PI3-K. I-BOP produced a concentration-dependent increase in the tyrosine phosphorylation of a protein identified by an antibody to this subunit of PI3-K. In addition, the ability of I-BOP to promote tyrosine phosphorylation of this subunit corresponded with its ability to activate the enzyme, with 100 nM I-BOP producing a 2.7-fold increase in tyrosine phosphorylation and a 3-fold increase in activity. In primary cultures of rat aorta smooth muscle cells, Ali *et al.*, (34) also showed tyrosine phosphorylation of a 86 kDa protein produced by a mimetic of TXA_2. However, in a subsequent study, this group suggested that tyrosine phosphorylation of the 85 kDa subunit of PI3-K may not be related to TXA_2 signal-transduction pathways in vascular smooth muscle (35). Comparisons between these previous studies and the present study suggest that the ability of mimetics of TXA_2 to stimulate tyrosine phosphorylation of the 85 kDa subunit of PI3-K may have been masked in the studies of Ali *et al.*, in light of the fact that the smooth muscle cells in those studies were exposed to insulin, a known activator of PI3-K, prior to the TXA_2 mimetic U46619. Additionally, in the present study, unlike those of Ali *et al.*, cells were pretreated with indomethacin to prevent generation of endogenous prostanoids inhibiting I-BOP-stimulated responses in the transfected cells.

Signal-transduction pathways initiated by growth factor receptor tyrosine kinases have been shown to involve tyrosine phosphorylation and activation of PI3-K (see Ref. 36 for review). This would suggest potential involvement of this unique kinase in the signal-transduction pathway for TXA_2 in vascular smooth muscle. G-protein coupled recep-

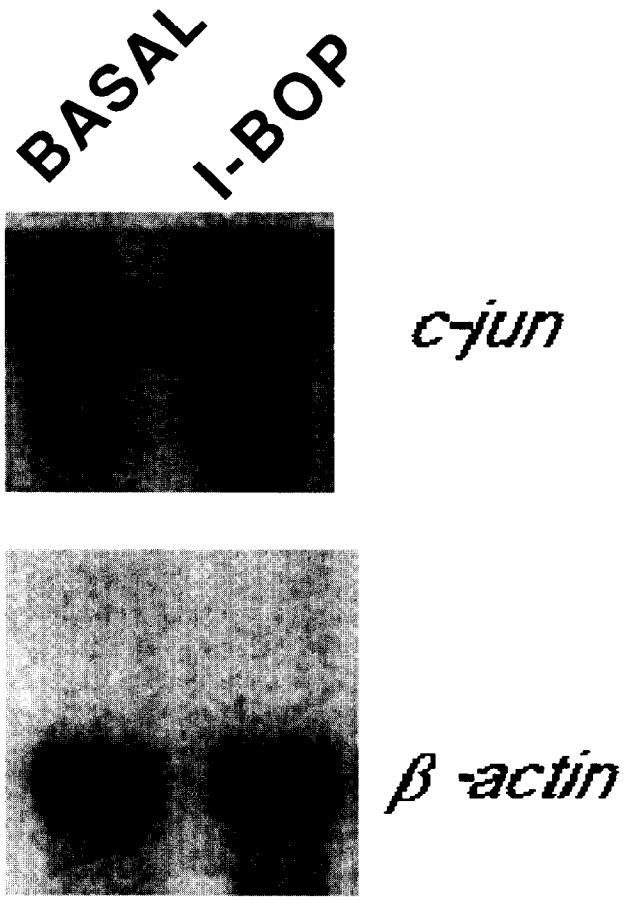


FIG. 6. I-BOP-stimulated induction of the mRNA for *c-jun*. A7r5.HEL.TP cells were treated with vehicle (basal) or I-BOP (0.1 μM). Total RNA was isolated as described and electrophoresed (30 μg), blotted, and hybridized with probes for *c-jun* and *β-actin*. I-BOP produced a 2.6-fold increase in the *c-jun* message, compared to basal. A scan of the autoradiogram is shown; representative of two similar experiments.

TABLE 1. I-BOP-stimulated [^{3}H]thymidine incorporation into monolayers of A7r5 cells stably transfected with the TP receptor

I-BOP (nM)	[^{3}H]Thymidine incorporation (% of basal)
0.1	119 ± 2.4 (9)
1.0	153 ± 7.2* (9)
10	127 ± 4.9* (6)

Values are means ± SEM. Mock transfected cells did not show significant responses. TP receptor characteristics for the stably transfected A7r5 cells: K_d = 0.9 ± 0.2 nM; B_{max} = 1.3 ± 0.7 pmol/mg, N = 4. Basal [^{3}H]thymidine incorporation was 39,110 ± 16,310 cpm/well (mean ± SEM, N = 9).

* $P < 0.05$ vs basal (100%).

tors have been shown to activate PI3-K (10, 37, 38). The mechanism for this latter response has not been defined clearly but has been suggested to involve activation of the 110 kDa subunit of PI3-K by dissociated G-protein βγ subunits (11–13). The involvement of tyrosine phosphorylation of the 85 kDa subunit of PI3-K in its activation produced by G-protein coupled receptors remains to be defined.

Detection of tyrosine phosphorylation of cellular proteins by immunoblot analysis indicated that I-BOP produced tyrosine phosphorylation of a protein with a relative mass of 60 kDa, a mass comparable to that seen previously for the TP receptor. Published data have shown ligand-stimulated phosphorylation of the platelet TP receptor (39). In the present study, I-BOP promoted tyrosine phosphorylation of a 60 kDa protein, which was immunoreactive to an antibody to the TP receptor. These results indicate that the TP receptor transiently transfected into this cell line is tyrosine phosphorylated in its basal state and that this phosphorylated state can be increased in response to receptor–ligand interaction. G-Protein coupled receptors have been shown previously to be tyrosine phosphorylated, both in the basal state as seen for the bradykinin receptor (19) and subsequent to receptor activation (20–23). The role of this phosphorylation in the mitogenic signal-transduction pathway has not been defined.

Receptor-activated pathways involved in cell proliferation converge at the nucleus to promote activation of several early response genes. The activation of these genes results in the synthesis of nuclear proteins such as Jun and Fos, which have been shown to be necessary for DNA replication (40). In primary cultures of vascular smooth muscle cells, mimetics of TXA_2 have been shown to induce the activation of the early response genes for c-*myc* (41) and for c-*fos* and c-*erg* (6). The present study shows the ability of I-BOP to activate this pathway for c-*jun* as well. In light of the role of the activation of these genes in the proliferative response, the present findings once again support a role for TXA_2 as a proliferative agent. We have also shown that other G-protein coupled receptors, such as that for bradykinin, produce induction of the message for c-*jun* in vascular smooth muscle.**

** Jaffa *et al.*, manuscript submitted for publication.

The present study confirms our previous findings in primary cultures of vascular smooth muscle cells showing activation of the proliferative pathway by I-BOP and extends it to include activation of tyrosine kinases, including PI3-K, and nuclear proteins involved in transcription. With these findings, one can propose a potential pathway for proliferation produced by TXA_2. Agonist interaction with the TP receptor activates a cytoplasmic tyrosine kinase, such as $p72^{syk}$, shown to be activated by TXA_2 mimetics in platelets (42). Activation of such a kinase would phosphorylate and activate the ras_{GTP}-MAPkinase pathway, in addition to phosphorylating PI3-K and the TP receptor. The ability of a cytoplasmic tyrosine kinase to phosphorylate a G-protein coupled receptor has been demonstrated previously for the AT1a receptor (21). Subsequent production of PI(3,4,5)-P3 by PI3-K may aid in the translocation of MAP kinase to the nucleus and allow for activation of nuclear transcription events. Such involvement of the products of PI3-K activity in the movement of MAP kinase has been demonstrated previously in serum-stimulated fibroblasts (43). Alternatively, activation of the MAP kinase pathway and of PI3-K by the TP receptor may be mediated by subunits of G-proteins, as has been described for other G-protein coupled receptors (11–13, 44).

In conclusion, this cell model will allow us to further examine the role of tyrosine phosphorylation of the TP receptor and of PI3-K in the signal-transduction pathway of cell proliferation produced by TXA_2 in vascular smooth muscle.

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