Inhibition of Endothelial Cell Migration, Intercellular Communication, and Vascular Tube Formation by Thromboxane A₂

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The eicosanoid thromboxane A₂ (TXA₂) is released by activated platelets, monocytes, and the vessel wall and interacts with high affinity receptors expressed in several tissues including endothelium. Whether TXA₂ might alter endothelial migration and tube formation, two determinants of angiogenesis, is unknown. Thus, we investigated the effect of the TXA₂ mimetic [1S-(1endothelial migration and tube formation, two determinants of angiogenesis, suggests that antagonizing TXA₂ signaling might enhance vascularization of ischemic tissue.

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The eicosanoid thromboxane A₂ (TXA₂) is released by activated platelets, monocytes, and the vessel wall and interacts with high affinity receptors expressed in several tissues including endothelium. Whether TXA₂ might alter endothelial migration and tube formation, two determinants of angiogenesis, is unknown. Thus, we investigated the effect of the TXA₂ mimetic [1S-(1

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Thromboxane A₂ (TXA₂) is a biologically active eicosanoid primarily released from activated platelets, monocytes, and damaged vessel wall (1). The biological half-life of TXA₂ is approximately 30 s in vitro models. Thus, TXA₂ acts as a paracrine/autocrine factor with potent effects on the vasculature including the initiation of platelet degranulation and aggregation, vasoconstriction, smooth muscle cell proliferation, and perturbation of endothelial cells (EC) (1, 2). All of these effects of TXA₂ are mediated through interaction with cell surface receptors that are members of the G-protein-linked, seven-transmembrane domain receptor family (1). Thus far two TXA₂ receptor (TP) isoforms have been cloned (TP-α and -β) (3, 4). These isoforms vary in the length of their cytoplasmic tails and arise via alternate splicing of a single gene, with TP-α the result of a retained intron (4). The downstream signaling pathways of the two TP isoforms differ in their modulation of adenylyl cyclase, with TP-α increasing cAMP accumulation and TP-β inhibiting cAMP accumulation (5, 6). Expression and desensitization following prolonged agonist stimulation of the two receptor isoforms are also regulated differently; prolonged stimulation induces the expression of the TP-β, but not the TP-α, isoform, and the signaling pathway responsible for TP-β desensitization is much more sensitive to protein kinase C activation than is that of TP-α (7).

While expression of TP in the vasculature is widespread, little is known of the physiological effects of TXA₂ on endothelial cell growth or migration. Such effects might be expected to be of considerable clinical relevance; conditions in which TXA₂ synthesis is elevated, such as myocardial ischemia, unstable angina pectoris, and diabetes (1, 8, 9), are also those in which angiogenesis or vessel growth and generation of collateral circulation are important.

Thus, we have investigated the effects of TP stimulation on the migration and tube forming capabilities of endothelial cells. We report here that activation of the TXA₂ receptor by a potent and stable ligand (IBOP) inhibits the formation of vascular networks by HEC in vitro in a time- and dose-dependent fashion. A TXA₂ mimetic also inhibits endothelial cell migration in a denudation-injury model. Based on experiments measuring gap junction function and expression, we suggest that at least one mechanism by which both the antimigratory and antiangiogenic effects of TP stimulation occur is via the inhibition of intercellular communication.

EXPERIMENTAL PROCEDURES

Materials—Sterile plasticware was from Costar (Cambridge, MA). Tissue culture materials and reagents, excluding pooled human serum (Gemini Bio-Products Inc., Calabasas, CA), were from Life Technologies, Inc. The thromboxane A₂ mimetic IBOP and the TP blocker SQ29548 were from Cayman Chemical (Ann Arbor, MI). Type 2 collagenase was obtained from Worthington, and dispase was from Becton Dickinson (Bedford, MA). All other chemicals and reagents were obtained from Sigma unless otherwise stated.

Isolation of Endothelial Cells from Human Umbilical Veins—Human
endothelial cells (HEC) were isolated from umbilical veins as previously reported (10). Culture medium consisted of M199, containing 20% (v/v) newborn calf serum and 5% (v/v) pooled human serum with 2 mM L-glutamine. Cells were plated onto plates precoated with 0.02% (w/v) gelatin with a medium change after 24 h and every 2 days thereafter until confluent. Cultures of HEC were maintained in a humidified atmosphere of 5% CO2 at 37 °C. HEC were passaged weekly with 0.05% trypsin, 0.02% EDTA, and the cells were collected and cell number was determined using a dual threshold cell counter (Coulter Electronics, Luton, UK). Cells were plated at the concentrations described.

In Vitro Tube Forming Assay—The spontaneous formation of capillary-like structures by HEC on a basement membrane simulating a blood vessel in vivo (Matrigel, Becton Dickinson, Bedford, MA) was used to assess angiogenic potential. Twelve-well plates were coated with matrix gel (10 mg/ml) according to the manufacturer’s instructions. HEC were seeded on coated plates at 1.5 × 10^5 cells/well and incubated at 37 °C for 60 min. The medium was supplemented with the agents outlined, and the cultures were incubated at 37 °C for 24 h. Tube formation was observed and photographed over the 24-h period using a solid state TV camera (COHU Electronics, CA) attached to an inverted phase contrast microscope. Images were captured using a video graphics system (Sony Electronics; monitor model PVM97, printer model UP980MD). The degree of tube formation was assessed by counting the number of tubes contained in two random fields from each well.

Assume migration studies were performed in a monolayer denudation assay as described by Tang et al. (11). Confluent endothelial cells were wounded by scraping with a 2–200 μl pipette tip, denuding a strip of the monolayer 300 μm in diameter. Variation in the wound diameter within experiments was approximately 5%. Cultures were washed twice with PBS and incubated with serum-containing medium supplemented with the agents as indicated. Control cultures were exposed to medium alone. The rate of wound closure was observed and photographed over a 24-h period as for the tube forming assay. The progression of cell migration was quantitated by calculation of the denuded area using the Scion image program (version 1.61, Scion Corp., Frederick, MD).

Determination of Cell Proliferation and Cell Cycle Analysis—For cell proliferation experiments, HEC were plated into gelatin-coated 12-well plates at 1.3 × 10^5 cells/cm² and allowed to attach for 48 h. Cells were washed twice with PBS and incubated in medium with or without 50 nM IBOP. Cells were counted every day for 3 days, and the amount of proliferation was compared. For cell cycle analysis, cells were plated into gelatin-coated 12-well plates at 1.3 × 10^5 cells/cm² and incubated at 37 °C for 48 h. Cells were washed with PBS twice and synchronized by incubation in low serum medium for 24 h. Cells were seeded in fresh medium alone or supplemented with 50 mM IBOP for 24 h. Cell cycle analysis was performed using the method of Giaretti and Nusse (48).

Flow Cytometric Analysis of Integrin Expression—Confluent IBOP-treated and untreated HEC were washed twice with PBS, and the cells were detached using 5 mM EDTA (pH 7.4). Cells on glass matrix were isolated by incubation with dispase. Cells were suspended in 1% bovine serum albumin (BSA) in PBS-B for 60 min. Analysis of integrin surface expression was performed using a flow cytometer using an argon ion laser (exc 510–580nm) imaged with a Nikon RCM8000 real time confocal microscope equipped with UV laser, excitation at 351 nm, small pinhole, and Nikon × 40 UV-corrected water immersion objective (numerical aperture 1.15, working distance 0.2 mm). Ratio images were continuously acquired at 1 Hz after background and shading correction and were accumulated over 500 images. The images were further analyzed during playback using Polygon-Star Software (Nikon) that averages the number of pixels (gray level) within the region of interest (circles spots placed on top of each cell, radii of 4.2 μm containing ~130 pixels) as function of elapsed time. The efficacy of Ca²⁺ wave communication was measured as the number of responding cells per total number of cells in the confocal field. The speed of Ca²⁺ wave propagation between HEC was calculated from the distance between the mechanically stimulated cell and neighboring cells divided by the latencies to half-maximal response (peak fluorescence) determined from sigmoidal curve fitting using Microcal Origin 4.1 software. The wave amplitude was measured as the ratio between the peak maximal increase and basal Indo-1 fluorescence, and the efficacy of Ca²⁺ wave communication was measured as the number of responding cells per total number of cells in the confocal field. All determinations of Ca²⁺ wave propagation were measured from HEC cultures untreated and treated for 2, 8, 16, and 24 h with IBOP (50 mM), 24 h with SQ29548 (5 μM), and both agents together (24 h).

Analysis of Cx43 Expression (Western Blotting) and Phosphorylation in HEC—Cells were grown to confluence and treated for up to 24 h with 50 mM IBOP. For the last 2 h of incubation, cells were preincubated with protein kinase inhibitors sodium vanadate (1 mM) and okadaic acid (1 μM) were included. For Western blotting, monolayers were washed twice in Hanks’ balanced salt solution, lysed in 200 μl of hot (85 °C) SDS gel-loading buffer (2% SDS, 10% glycerol, 50 mM Tris-HCl pH 6.8), and transferred to microcentrifuge tubes. Samples were boiled for 10 min, sonicated briefly, and centrifuged at 10,000 × g for 10 min. The protein concent
TXA2 Inhibits Angiogenesis and HEC Migration in Vitro

RESULTS

TXA2 Mimetic Prevents HEC Differentiation in Three-dimensional Cultures—HEC on matrigel undergo alignment into cords within 2 h, which establishes the pattern for further tube formation. This is shown by the stability in the number of tubes per field in control cultures (Fig. 1E). By 6 h, tube formation had begun, and by 12 h virtually all cells had fused into continuous tubes, with stabilization and refinement progressing up until 24 h (Fig. 1B). Generation of tubes by HEC was reduced by the TXA2 mimetic IBOP (Fig. 1C). IBOP (50 nM) reduced to 43 ± 14% of controls the alignment of HEC into cordlike structures at 2 h (p < 0.01) (Fig. 1E). By 16 h, IBOP-treated cultures contained only 15 ± 12% of the number of tubes in untreated wells (p ≤ 0.05) (Fig. 1, C and E). HEC incubated with the TP blocker SQ29548 alone formed a number of tubes per field similar to that formed by untreated cells (Fig. 1E), and the simultaneous addition of SQ29548 prevented the effect of the TXA2 mimetic at each time point (p ≤ 0.05 compared with IBOP alone) (Fig. 1, D and E). The inactive hydrolytic product of TXA2, TXB2, did not influence tube formation by HEC, nor did the prostaglandin PGE2 (data not shown).

IBOP was a concentration- and time-dependent inhibitor of tube formation by HEC (Fig. 2, A and B, respectively). Vascular tube formation was significantly decreased by IBOP concentrations higher than 50 nM (p ≤ 0.01), with maximal inhibition (40 ± 20% of control) at IBOP concentrations of 50 nM or more (p ≤ 0.01). Another TXA2 mimetic, U46619, also inhibited tube formation in a concentration-dependent manner. U46619 inhibited HEC tube formation with an IC50 of 100 nM (p ≤ 0.05), with maximal inhibition by U46619 at concentrations above 400 nM, at which tube formation was reduced to 17 ± 5.4% of control (p ≤ 0.01) (Fig. 2A). IBOP also prevented formation of tubes if added to cultures already in the process of tube formation (Fig. 2B). Inclusion of IBOP (50 nM) in the medium late as 18 h after the initiation of tube formation significantly reduced the eventual number of tubes at 24 h (70 ± 21%, p ≤ 0.001). IBOP was most effective at periods greater than 18 h, with the number of tubes suppressed to 40 ± 21% of control (p ≤ 0.0001). Thus, TP activation was found to exert a potent inhibitory effect on in vitro tube formation.

TP Stimulation Also Diminishes the Rate of EC Wound Healing—Tube formation in three-dimensional cultures is dependent on the migration of cells after plating. The inhibition of tube formation by IBOP and U46619 suggested that TXA2 mimetics might also inhibit HEC migration. We used a denudation injury model to assess the impact of IBOP on endothelial cell migration. Confluent, scrape-wounded HEC monolayers were incubated with either IBOP (50 nM) or U46619 (400 nM), and the rate of closure was observed over the following 24 h. Untreated cultures migrated into the denuded area, recovering the exposed surface and reducing the uncovered area to 27 ± 16.5% of the original area (Fig. 3, B and E). IBOP (50 nM) significantly inhibited migration of HEC into the denuded area (Fig. 3C), with inhibition detectable 6 h after initial wounding (p ≤ 0.05). Wound closure was inhibited by 58.4 ± 8.1% at 24 h (p ≤ 0.05). The TP agonist U46619 (400 nM) also inhibited endothelial cell migration with kinetics similar to IBOP with closure of the wound inhibited by 71.7 ± 14.1% after 24 h of treatment (p ≤ 0.005) (data not shown). TP blockade by SQ29548 did not affect HEC migration into the wounded area; however, the migration of IBOP-treated HEC was restored by
inclusion of 5 μM SQ29548 (p ≤ 0.05 versus IBOP alone) for longer than 12 h (Fig. 3, D). The inactive metabolite of TXA2, TXB2, did not affect HEC migration.

The Antimigratory Effects of TXA2 Mimetics Are Not Mediated through Alterations to Endothelial Cell Proliferation, Cytoskeletal Morphology, or Expression of αvβ3 and αvβ5 Integrin Receptors—Among the factors important in the regulation of migration and angiogenesis are the expression of cell surface integrins, cellular proliferation, and architecture of the cytoskeleton (14–17). We examined the effects of IBOP stimulation on endothelial cell proliferation using growth curves and cell cycle kinetics. Cells grown on gelatin-coated 12-well plates in medium alone increased cell number over 3 days with cell number 172 ± 9.5% of the plating density by day 3. IBOP treatment (50 nM) increased cell number to 174 ± 5.7% of the original plating density in the same period, which was not significantly different from control (data not shown). When examining cell cycle kinetics, we found that unstimulated and IBOP-treated HEC progressed through the cell cycle at comparable rates (data not shown). IBOP did not retard cell cycle progression, nor did it inhibit HEC proliferation; thus, the inhibition of HEC tube formation and migration was unlikely to be a result of inhibition of cell proliferation.

We examined the expression of the integrin complexes αvβ3 and αvβ5 on cells from two- and three-dimensional cultures to determine if the effect of IBOP on migration and tube formation was mediated through decreased expression of those integrin receptors (Fig. 4, A and B). Untreated cells from two-dimensional cultures showed a 3- and 5-fold increase in their mean fluorescence intensity for αvβ3 and αvβ5, respectively, over cells stained with preimmune serum. Cells from three-dimensional matrix gel cultures expressed both αvβ3 and αvβ5 integrin complexes similarly to HEC in two-dimensional cultures (data not shown). IBOP (50 nM) did not influence the surface expression of either αvβ3 or αvβ5 in HEC from either culture model. Thus, the effect of IBOP on HEC migration and tube formation was not mediated through decreased integrin expression of those two integrin receptors.

To determine the effects of IBOP on the HEC cytoskeleton, immunofluorescent staining of the cytoskeletal components F-
actin and α-tubulin was performed. Staining for cytoskeletal architecture showed extensive stress fiber formation in HEC that was in a random orientation throughout the monolayer (Fig. 4C). Stress fibers in migrating cells were cytoplasmic and radial (Fig. 4C). The arrangement of F-actin within the monolayer (Fig. 4D) did not decrease stress fiber formation or alter the arrangement of F-actin within the monolayer (Fig. 4D) nor did it prevent the reorganization of the actin cytoskeleton at the wound edge. The pattern of α-tubulin staining in control cells was cytoplasmic and radial (Fig. 4E) with no apparent alterations in the morphology in migrating cells. Cells exposed to IBOP (50 nM) maintained the same morphology of alterations in the architecture of the fibronectin extracellular matrix of the migrating cells. Cells exposed to IBOP (50 nM) were stained with rhodamine-conjugated phalloidin (F-actin) or a monoclonal antibody for α-tubulin, and examined for changes in cytoskeletal morphology. The morphology of F-actin and α-tubulin is shown in untreated (C and E, respectively) and IBOP-treated cells (D and F, respectively). Photomicrographs were taken at ×400 magnification using excitation and emission filters for rhodamine. Results are representative of three individual experiments.

**FIG. 4.** Expression of integrins and cytoskeletal morphology following IBOP. A–B, surface expression of the integrins αβ3 and αβ6 was examined using complex-specific monoclonal antibodies. Untreated and IBOP-treated HEC (24 h) were harvested with EDTA and stained for surface expression of integrins. A FITC-conjugated anti-mouse antibody was used for detection, and expression was analyzed by flow cytometry. Histograms are composites with the expression of integrins on untreated cultures in black, IBOP-treated cultures in white, and negative controls in gray. C–F, confluent HEC were treated for 24 h with 50 nM IBOP, stained with either rhodamine-conjugated phalloidin (F-actin) or a monoclonal antibody for α-tubulin, and examined for changes in cytoskeletal morphology. The morphology of F-actin and α-tubulin is shown in untreated (C and E, respectively) and IBOP-treated cells (D and F, respectively). Photomicrographs were taken at ×400 magnification using excitation and emission filters for rhodamine. Results are representative of three individual experiments.

The degree of cell-cell communication was assessed using the fluorescent dye Lucifer yellow. Lucifer yellow was microinjected into the wounded area of confluent HEC, and the extent of dye diffusion was determined. After injection into untreated cells, Lucifer yellow diffused to an average of 5.5 ± 0.7 cells (Fig. 5, A and B). Treatment for 24 h with the TXA2 mimetic IBOP (50 nM) reduced the diffusion of Lucifer yellow to 2.4 ± 0.5 cells (p < 0.05), a reduction in coupling of 64% (Fig. 5, A and C). To examine the electrical properties of IBOP-treated cells directly, pairs of freshly dissociated cells were voltage-clamped, and recordings of junctional conductance were made (Fig. 5D). Treatment of HEC with 50 nM IBOP (24 h) reduced the conductance between cell pairs to 1.9 ± 1 microsiemens, an 85% reduction in conductance in comparison with controls (13 ± 5.8 microsiemens, p < 0.05).

Further characterization of the effects of TXA2 receptor agonists on gap junction-mediated intercellular communication employed real time confocal microscopy to examine the propagation of Ca2+ waves through endothelial cell monolayers. Confluent HEC monolayers were treated with 50 nM IBOP for 0–24 h and loaded with the Ca2+ indicator dye, and the efficacy of Ca2+ wave propagation, defined as the number of cells reached by the Ca2+ signal per total number of cells in the confocal field, was analyzed (Fig. 6). In untreated cells, the intracellular Ca2+ waves spread to 63 ± 4% of the cells in the field (n = 16, Fig. 6). In similarly confluent cultures, exposure to the TXA2 mimetic IBOP (50 nM) for periods longer than 2 h significantly reduced the spread of Ca2+ waves over the same period of study (Fig. 6). After 8- and 16-h exposure, IBOP inhibited the communication of the Ca2+ signal by 35% (p < 0.05) with inhibition maximal after 24 h with Ca2+ signal spreading to only 52% of
The effects of IBOP on HEC migration in vitro

**Materials and Methods**

**Pharmacological Manipulation of Gap Junction Function Mimics the Effects of the TXA2 Receptor on Endothelial Cell IBOP-treated groups (data not shown). Other parameters measured (velocity of wave propagation passing 20-ms, 2-mV pulses in one cell and recording junctional current effects of 18b-glycyrrhetinic acid (18β-GA) on HEC tube formation and migration in vitro. The effects of 18β-GA on HEC migration in the denudation injury assay were assessed (Fig. 7A). Concentrations of 18β-GA as low as 1 μM reduced HEC migration by 64% (p < 0.05) with 57 ± 11.8% of the wound area uncovered after 24 h. 18β-GA inhibited HEC migration in a concentration-dependent fashion with inhibition greatest at 18β-GA concentrations above 5 μM and migration abrogated at 20 μM. Since IBOP inhibited the migration of endothelial cells 50% compared with controls (Fig. 3E), we chose to use 1 μM 18β-GA in further experiments, since it was equivalent in efficacy to IBOP. In the three-dimensional model of vascular tube formation, 18β-GA (1 μM) reduced the formation of tubular networks to 39 ± 20% of control (p < 0.001), thereby implicating gap junction-mediated intercellular communication as a vital part in the angiogenic process (Fig. 7B). The morphology of 18β-GA-treated cultures was identical to that of IBOP-treated cultures (Fig. 1C), which is consistent with the concept that inhibition of intercellular communication through gap junctions by TXA2 is at least one mechanism by which TXA2 can inhibit EC migration and tube formation in vitro.

**Inhibition of Intercellular Communication by IBOP Coincides with Altered Distribution of Cx43 without Changes in Cx43 Abundance—Intercellular communication is regulated by a number of factors, including the phosphorylation state and half-life of the connexin protein, the rate of shuttling of connexin in and out of the membrane, and changes in the rate of transition between the open/closed state (22). Low passage HEC express both Cx40 and Cx43, although Cx43 is the dominant gap junction protein in these cells (23, 24). Thus, we examined the expression levels and intracellular distribution of Cx43 in HEC stimulated with the TXA2 mimetic IBOP. To determine the effect of IBOP on Cx43 expression, lysates of treated cells were subjected to electrophoresis and blotted with an anti-Cx43 antibody. Connexin 43 was found to exist in two different species in HEC, one of which migrated at approximately 44 kDa and another at 45 kDa (Fig. 8A). Exposure to IBOP (50 nM) for up to 24 h did not change the overall expression of Cx43, nor did it alter the ratio of the two species (Fig. 8A). To assess the effect of IBOP treatment on Cx43 phosphorylation, we loaded cells with [32P]H3PO4 and analyzed the phosphorylation of immunoprecipitated Cx43 via autoradiog-
raphy subsequent to SDS-polyacrylamide gel electrophoresis. Analysis of immunoprecipitated Cx43 protein from 32P-loaded cells showed two bands migrating at approximately the same molecular weight as those described for Western blot analysis (Fig. 8B). IBOP was again shown not to influence the amount or ratio of the two 32P-labeled Cx43 species in HEC. Thus, TP stimulation did not control junctional communication through alterations in the amount of Cx43 expressed or the phosphorylation state of Cx43.

To assess the intracellular distribution of Cx43 within HEC, immunofluorescence analysis was performed on cells rendered permeable and then incubated with either medium or IBOP (50 nM) for 24 h. Cells were allowed to form tubes in the absence or presence of 18β-GA (1 μM) for 24 h. The number of tubes in two random fields from each of four wells was quantitated (n = 12). Data are expressed as a percentage of the number of tubes in control wells at 2 h (mean ± S.D.), with # denoting significant difference from control (p < 0.05).

FIG. 8. Expression and subcellular localization of Cx43 in IBOP-treated HEC. A, Western blotting was performed on lysates from cells incubated with the TXA2 mimetic IBOP (50 nM) for 2, 8, 16, and 24 h. Blotting was performed as described under “Experimental Procedures” using a monoclonal Cx43 antibody. B and C, immunofluorescent staining of gap junctions was performed in cells treated with either medium or IBOP (50 nM) for 24 h. The cells were rendered permeable and stained using the same Cx43 antibody used for Western blotting and Cy3-conjugated second antibodies. The morphology of gap junctions in control cells is shown in B, and morphology of those in IBOP-treated cells is shown in C. Membrane (M) and cytoplasmic Cx43 staining are indicated by arrows. Photographs were taken at ×400 magnification and are representative of three experiments.

DISCUSSION

In this study, we found that the TXA2 mimetic IBOP was a potent time- and concentration-dependent inhibitor of in vitro tube formation, with an IC50 of 25 nM. At an IBOP concentration of 50 nM, endothelial cell migration was inhibited by 50%. These effects contrast sharply with those that would be expected from the known effects of TXA2 on signal transduction. TP stimulation causes the activation of phospholipase C, protein kinase C, and extracellular signal-regulated kinase (26–29), which have been associated with enhanced migration and angiogenesis in different systems (30–32). This disparity is unlikely to result from the fact that one of the TP isoforms in
low passage HEC, TP-β, couples to the G-protein Gαi, as well as Gprotein-coupled receptors that activate Gαi, such as PF4, substance P, endothelin, and PGE1, stimulate angiogenesis and enhance migration in similar EC assays (34–36). Thus, despite the similarity that these ligands share concerning other aspects of vascular function (e.g., vasoconstriction), TXA2 receptor stimulation causes effects that are qualitatively different from those of many other G-protein-linked receptor ligands in endothelial cells, in that it inhibits cell migration and tube formation. The fact that TP stimulation inhibited intercellular communication in HEC monolayers and that the pharmacological uncoupling reagent 18β-GA also inhibited HEC migration and tube formation suggests one possible explanation for our results, namely that the inhibition of tube formation and migratory activity of the TXA2 mimetic was due to inhibition of gap junction function. Indeed, endothelial cell migration and angiogenesis are sensitive to inhibition of gap junction-mediated intercellular communication, and antiangiogenic cytokines, such as interleukin-1β, potently inhibit intercellular communication in endothelium (37–39). Inhibition of junctional communication alone is not sufficient to explain the loss of EC function, however, since many proangiogenic G-protein-linked receptor ligands also decrease junctional communication (40, 41). The difference between the effects of TXA2 and other G-protein-linked receptor ligands may be related to the kinetics of the inhibition and the mode of action. Lysophosphatidic acid, thrombin, and endothelin (ET) all inhibit junctional communication in various cells (40, 41). With lysophosphatidic acid, ET, and thrombin, however, maximal inhibition occurs within 5–10 min of ligand binding with the restoration of junctional function at 3 h mediated through receptor desensitization (40, 41). In those studies, gap junction closure by lysophosphatidic acid, thrombin, and ET was not accompanied by changes in the morphology of junctional plaques. Rather, the loss of intercellular communication was associated with changes in the phosphorylation state of Cx43, mediated by either c-Src or MAP kinase activation (40, 41).

Indeed, Cx43 phosphorylation has been shown to be altered by changes in Ca2+/inositol 1,4,5-trisphosphate generation; protein kinase C, protein kinase A, and mitogen-activated protein kinase activation; cAMP accumulation; and c-Src activity (42). Binding of TXA2 to the receptor has been shown to activate all of these signal transduction pathways (29, 43). Yet, in low passage HEC, stimulation of TP with ligand produces no discernable change in the expression level or phosphorylation state of Cx43, as detected by immunoblotting. Along with the alterations in the morphology of junctional plaques found with IBOP, these observations may indicate alternate pathways used by TXA2 to uncouple the cells.

The parameters examined that correlated with decreased functional coupling in IBOP-treated HEC were a change in the size of the junctional plaques, with IBOP causing clustering of Cx43 into larger gap junction plaques and redistribution of Cx43 aggregates from membrane to cytoplasm. Agents that increase cytoplasmic levels of cAMP have previously been shown to induce aggregation of Cx43 into larger sized plaques (44, 45); such CAMP-induced enlargement of gap junctions has been associated with decreased intercellular communication in myocytes (46). The mechanism by which cAMP causes these effects is not certain. Cyclic AMP has effects on coupling that vary among multiple cell types. In many cases, but not all, cAMP induces alteration in junctional communication with simultaneous alteration in phosphorylation of the Cx43 protein (42, 43, 45). Thus, one mechanism by which TXA2 receptor stimulation could control gap junction-mediated intercellular communication in HEC is by elevation of cAMP. Evidence in favor of additional mechanisms includes the fact that TXA2 mimetics decrease coupling, independently of changes in Cx43 phosphorylation.

A recent report by Jordan and colleagues (25) demonstrated internalization of Cx43-GFP chimeras after insertion in the membrane. Cx43 proteins inserted in the membrane coalesced into larger aggregates and were then internalized and assumed a perinuclear location. Confocal micrographs of control and IBOP-treated HEC revealed that IBOP treatment increased the number of perinuclear Cx43-positive particles from 3.8 ± 1.8 to 7.5 ± 2.2 particles/cell. We believe that the increased particles are internalized gap junction aggregates, which represent a redistribution of Cx43 from membrane to cytoplasm. The coalescence of Cx43 into larger aggregates preceded the internalization of Cx43 (25). The induction of larger aggregates by IBOP may be a preliminary step in the internalization process. Thus, the redistribution of Cx43 by IBOP may be the primary cause for the decrease in intercellular communication present in TXA2-treated HEC.

Migration can be inhibited through mechanisms that change neither integrins nor cytoskeleton; in particular, inhibition of migration caused by alteration of gap junction function is independent of changes in the cytoskeleton (19). We found that stimulation of HEC with the TXA2 mimetic IBOP did not induce changes to the endothelial cell cytoskeleton after 24 h of treatment, in contrast to the observation that TXA2 decreased stress fibers in endothelial cells within 15 min, which is associated with an increase in vascular permeability (47). The effects of TXA2 on vascular permeability are more acute than those on intercellular communication, however, and the inhibition of TXA2 on stress fiber formation are reversed at the time when TXA2 most profoundly inhibits migration. The lack of effect on cytoskeletal morphology may also exclude the activation of Rho family members as the mechanism of the anti-migratory effects of TP stimulation. TXA2 mimetics also did not alter the surface expression of integrin complexes αβ3 or αβ5. However, we cannot exclude the possibility that TP stimulation may cause changes in the expression of other integrins important for angiogenesis or the signaling by these integrins.

Thus, we have found that TXA2 receptor stimulation suppresses HEC migration and tube formation in association with inhibition of gap junction-mediated intercellular communication. Intercellular communication is important in these events and thus potentially for angiogenesis and vessel wall function in general. TXA2 has effects on endothelial cell biology that are unusual among G-protein-linked receptors and inhibits cell-cell coupling through an as yet undetermined mechanism. Further understanding of the biochemical basis of these events may lead to strategies to antagonize better the inhibitory effects of TXA2 on endothelial cell function and thus potentially enhance re-endothelialization and vascularization in ischemic or thrombotic disease.

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