Thrombospondin Modulates the Expression of a Set of Genes Including Thrombospondin-1 in Human Microvascular Endothelial Cells

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Thrombospondin-1 (THBS1) is a large extracellular matrix glycoprotein that affects vascular systems such as platelet activation, angiogenesis, and wound healing. Increases in THBS1 expression have been linked to disease states including tumor progression, atherosclerosis, and arthritis. The present study focuses on the effects of thrombin activation of the G-protein-coupled, protease-activated receptor-1 (PAR-1) on THBS1 gene expression in the microvascular endothelium. Thrombin-induced changes in gene expression were characterized by microarray analysis of ~11,000 different human genes in human microvascular endothelial cells (HMEC-1). Thrombin induced the expression of a set of at least 65 genes including THBS1. Changes in THBS1 mRNA correlated with an increase in the extracellular THBS1 protein concentration. The PAR-1-specific agonist peptide (TFLLRNK-PDK) mimicked thrombin stimulation of THBS1 expression, suggesting that thrombin signaling is through PAR-1. Further studies showed THBS1 expression was sensitive to pertussis toxin and protein kinase C inhibition indicating Gi/o- and Gq-mediated pathways. THBS1 up-regulation was also confirmed in human umbilical vein endothelial cells stimulated with thrombin. Analysis of the promoter region of THBS1 and other genes of similar expression profile identified from the microarray predicted an EBOX/EGRF transcriptional model. Expression of members of each family, MYC and EGR1, respectively, correlated with THBS1 expression. These results suggest thrombin formed at sites of vascular injury increases THBS1 expression into the extracellular matrix via activation of a PAR-1, Gq, EBOX/EGRF-signaling cascade, elucidating regulatory points that may play a role in increased THBS1 expression in disease states.

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cell types in cellular function and signaling are evident. For example, macro- and microvascular endothelial cells show heterogeneity and differences both in their signaling activities and protein expression (30–33). To study changes in transcription that result from thrombin simulation in microvascular endothelial cells, we have chosen to conduct studies in the human dermal microvascular endothelial cell line (HMEC-1). HMEC-1 maintain a similar phenotype to non-transformed HMECs (34) and expresses a complete set of G-proteins that are known to couple to PAR-1 (25). Our experiments on microvascular endothelial cells address the possible roles of THBS1 in processes involving these cells such as neoangiogenesis in cancer and wound healing or tissue repair after or coincidental with inflammation.

Here we report the effects of thrombin on THBS1 gene expression in HMEC-1 using a microarray-based approach. We found THBS1 to be up-regulated both in mRNA and secreted protein levels in response to thrombin. Additionally, thrombin-induced up-regulation of THBS1 was pertussis toxin-sensitive, indicating a Gi/o signaling component. Inhibition of protein kinase C also inhibited thrombin-induced THBS1 expression, implicating a Gq-mediated signaling component. This pathway demonstrates a potential mechanism by which THBS1 may be up-regulated at sites of vascular injury and in disease states.

**Experimental Procedures**

**Reagents**—All cell culture reagents were purchased from Invitrogen. α-Thrombin, bisindolylmaleimide I hydrochloride (Bio I), pertussis toxin, and Y-27632 were purchased from Calbiochem. The agonist peptide TFLLRNKPDK (TK) was purchased from GL Biochem (Shanghai) Ltd.

**Endothelial Cell Culture**—In the present studies a human dermal microvascular endothelial cell line that was transformed using SV-40 was used (HMEC-1; obtained from Dr. E. Ades (Centers for Disease Control, Atlanta, GA)). The cells were maintained in MCDB 131 medium supplemented with 5% fetal bovine serum, penicillin/streptomycin (5000 units/ml; 5000 μg/ml), hydrocortisone (50 μg/ml), epidermal growth factor (0.01 μg/ml), and t-glutamine (2 mM) in an atmosphere of 95% air, 5% CO2 at 37 °C. The cells were seeded at 1 × 104 cells/ml and subcultured after detachment with 0.05% trypsin, 0.5 mM EDTA. All of the studies utilized cell passages 15–20.

**HUVEC Treatment and RNA Isolation**—Cells were grown in medium 199 supplemented with 15% fetal bovine serum, endothelial mitogen, penicillin, streptomycin, amphotericin B, and heparin. Passage 2-5 HUVECs were plated in 48-well plates and used after confluent.

**HMEC-1 Treatment and RNA Isolation**—Cells were grown to 95% confluence, switched to serum-free medium containing 0.03% bovine serum albumin, and incubated for 3 h. Cells were then treated with either 86 μM (10 units/ml) human thrombin or vehicle (phosphate-buffered saline, pH 7.4 (PBS)) (control samples) and incubated for additional 90 min or 6 or 12 h. Afterward, cell monolayers were rinsed three times with ice-cold PBS followed by the addition of the lysis buffer (RT buffer from RNAeasy Midi kit, Qiagen, Valencia, CA), cell scraping, disruption, and homogenization. Total RNA enriched in mRNA was isolated using the RNAeasy Midi kit following the manufacturer’s protocol. Only RNA samples with an A260/A280 ratio > 1.8 and no visible degradation by gel electrophoresis and ethidium bromide staining were used for microarray hybridizations and real-time (RT)-PCR.

**Synthesis of Fluorescent cDNA and Hybridization to Microarray Slides**—Microarray analyses were performed by the Vanderbilt Microarray Shared Resource on a fee-for-service basis. Briefly, 30 μg of total RNA from both control and thrombin-treated samples were reverse-transcribed using SuperScript reverse transcriptase (Invitrogen), incorporating Cy5-dUTP into the control sample cDNA and Cy3-dUTP into the thrombin-treated sample cDNA. The labeled cDNA probes were purified using the QIAquick PCR purification kit (Qiagen). The purified cDNA was then mixed with a hybridization buffer containing 10 μg of poly(A) RNA, 25% formamide, 5× SSC (0.75 M NaCl and 0.075 M sodium citrate), and 0.1% SDS, denaturated and hybridized for 16 h at 42 °C on a 11,000 human cDNA microarray. The cDNAs imprinted in these arrays are available from Research Genetcs. For detailed information about the microarray used in these experiments and the genes represented in the array refer to array.mc.vanderbilt.edu. After hybridization, slides were washed sequentially for 5 min each in 2× SSC, 0.1% SDS, 1× SSC, and 0.1× SSC. After drying, slides were immediately scanned for fluorescence emission from each spot on the array at 532 and 635 nm for Cy5 and Cy3, respectively.

**Normalization**—Fluorescent intensities in each channel, Cy5 and Cy3, for each spot were quantified using GenPix Pro 5.0 from Axon Instrument, Inc. (Union City, CA). The resulting data were then normalized, filtered, and analyzed using GeneSpring 6.1, Silicon Genetics (Redwood City, CA). Experiments were normalized to a signal ratio, and two-color normalizations automatically displayed all measurements per spot-intensity-dependent (Lowess) if more than 100 genes per region divided by control channel if fewer than 100 genes per region. The cutoff used was 10 in raw data or 20% of data used for smoothing.

**Filtering**—To determine statistically significant data points from the 11,409 genes tested, the data set was filtered using a t-test p value test performed by GeneSpring (Silicon Genetics).

GeneSpring correlated the t test values with Student’s t distribution, with t = 1 degrees of freedom to yield a significance value (p value). Data were then filtered by the p values to include only data that had a p value ≤ 0.005; this displayed only the normalized mean gene intensities that differed from 1. Any coincidental data that could have passed the first filtration was removed by using a multiple testing correction based on Bonferroni’s inequality. This statistical correction helped to further limit the chance of false positives to be no more than p = 3.00 × 10−3 by multiplying each nominal p value by N (the total number of genes).

**Clustering**—A k-means clustering algorithm provided by the Gene-Spring software was implemented to divide the genes into interest groups based on their expression patterns. The k-means clusters were constructed so that the average behavior in each group was distinct from any of the other groups. Analyzing the time series experiment this way identified unique classes of genes that were up-regulated in a time-dependent manner were identified.

The k-means clustering algorithm utilized by GeneSpring first divided the genes of interest into a user-defined number (k) of equal-sized groups based on the order in the selected gene list. Four clusters were chosen. Next, the program created centroids (in expression space) at the average location of each group of genes. Choosing to proceed through this design, the genes were assigned to the group with the closest centroid. The standard correlation algorithm was chosen for the similarity measure. After all of the genes had been reassigned, the location of the centroids was recalculated, and the process was repeated until the maximum number of iterations had been reached.

**Promoter Analysis**—The Genomatix software suite (Munich, Germany) was used to predict and analyze promoter regions. Gene2Promoter was used to define the promoter regions upstream of the gene of interest. Common frameworks were then found using the GEMS Launcher task “definition of common framework” using the complete vertebrate matrix library. FrameWoker runs with different quorum constraint parameters were performed; 40 or 30% of input sequences had to contain the framework. ElDorado using “Annotation and Analysis” was subsequently used to analyze the predicted promoter regions of MYC, EGR1 since they did not fall into the common framework.

**Literature Mining**—Genomatix Bibliosphere was used to mine the literature for co-citations between each of the 65 genes from the filtered list.

**Semiquantitative RT-PCR**—Universal RT reagents and SYBR Green PCR Master mix were purchased from ABI (Branchburg, NJ). Total RNA (1.0 μg) was reverse-transcribed using random hexamers with Universal RT. Reagents were incubated for 10 min at room temperature then 30 min at 42 °C followed by 5 min at 99 °C and 5 min at 55 °C. SYBR Green PCR Master mix was used on an i-Cycler instrument (Bio-Rad) to amplify human cDNAs as well as the cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. All primers used for real-time semiquantitative PCR were designed using Beacon Designer II (Premierbiosoft). Semiquantitative RT-PCR reactions were carried out in a total volume of 50.0 μl containing 5 μl of cDNA with the following thermocycling steps: 95 °C for 8.5 min, then 45 cycles of 95 °C for 15 s, 61.5 °C for 1 min, and 95 °C for 2.0 min then 55 °C for 2 min followed by melting curve data collection with 201 cycles of 0.2 °C/s temperature escalation and analysis. Specificity and sensitivity of the assays were confirmed with amplification of cDNAs in serially diluted total RNA samples. Amplion signals for each target cDNA strongly correlated with serial dilutions of each RNA sample. Data analysis and calculations were done following the 2−ΔΔCT method (35). Each sample was assayed in duplicate. Primer concentration and annealing temperature were optimized for highly specific and reproducible detection of SERPINE1, tissue plasminogen activator (PLAT), THBS1, PAR-1, PAR-2, MYC, EGR1 β-actin, and GAPDH RNA by...
semiquantitative RT-PCR.

**Primers**—Primers used for semiquantitative real-time PCR were purchased from Integrated DNA Technologies (Corvalle, IA): SERPINE1 antisense, 5'-AAT GTT GCT GCC GAG ACA AGT-3'; PLAT antisense, 5'-GGT CCT GAG AAG TCT GGA-3'; and SERPINE1 sense, 5'-CAT TAC TAC GAT CTC GGA CTG-3'; PLAT antisense, 5'-GGT GTG GAG AAG TCT GTA GAG-3'; and PLAT sense, 5'-CCT AGA CTG CAT TCG TCA GAA-3'; THBS1 antisense, 5'-CCT ATG GCC TGG GAT GCC AGA-3'; and THBS1 sense, 5'-GCAA CCT ATG ACA GAA ACT-3'.

All 65 genes identified were up-regulated at each point of the time course. There were no statistically meaningful genes whose expression was suppressed.

Each gene was then clustered into four groups according to expression profiles using a k-means clustering algorithm. Fig. 1B is an expression time-course of the average of all members of each cluster, revealing the general trend of the cluster. Cluster-1 consisted of 32 different genes including: PLAT, natural killer cell transcript 4 (NK4) (40), CDC42 effector protein (Rho GTPase binding) 1 (CDC42EP1), and 7-aminobutyric acid (GABA) A receptor δ (GABRD). Transcription factors such as SRY (sex determining region Y)-box 15 (SOX15), zinc finger protein 205 (ZNF205), and myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2) are also included in this cluster.

When promoters from each gene of the cluster were analyzed and compared, common putative transcription factor binding sites were identified. These transcription factors were then analyzed to find that transcription models consisting of multiple transcription factor binding motifs, which have been empirically shown to function synergistically, are in close physical transcription factor binding motifs, which have been empirically shown to function synergistically, are in close physical proximity to each other. There were no statistically meaningful genes whose expression was suppressed.

**RESULTS**

**Thrombin Consistently Up-regulates the Expression THBS1 and Other Genes in HMEC-1**—To identify candidate genes whose expression is modulated by thrombin stimulation in microvascular endothelial cells, analyses using a microarray were performed. The cDNA library used to make the microarray, developed by Research Genetics, consists of ~11,000 different human genes. HMEC-1 cultures were stimulated with a saturating concentration of thrombin, 86 nM (10 units/ml). Total RNA was then isolated from treated and control cells at 1.5, 5, and 12 h after thrombin stimulation. Control and treated cDNAs were simultaneously hybridized to the microchip. The raw data obtained was then quantified, normalized, and filtered according to criteria outlined under "Experimental Procedures." Of the ~11,000 genes analyzed, only 65, including THBS1, showed consistent and statistically meaningful differences in expression when cells were treated by thrombin. Fig. 1A is a heat-map representation of the expression patterns of the 65 candidate genes (for a complete list of the genes, accession numbers and numeric-fold increases see the supplemental data) All 65 genes identified were up-regulated at each point of the time course. There were no statistically meaningful genes whose expression was suppressed.

Cluster-2 contained 12 genes including endothelin-1 (EDN-1), platelet-derived growth factor β, monocyte chemotactic protein 1 (CCL2), and ras homolog gene family, member B (RHOB). Other regulated proteins of this cluster include transcription factors such as Fos-related antigen-1 (FOSL1), core promoter element binding protein (COPEB), and myelocyte-tamoid virus oncogene homolog (MYC). A similar promoter-cluster analysis was performed for the genes of Cluster-2 identifying five models, ZSPF being the most common transcription factor family.

Fourteen genes clustered into group 3 including some well-characterized proteins of the vascular system; fibronectin-1
(FN1), plasminogen activator inhibitor type 1 (SERPINE1), vasodilator-stimulated phosphoprotein (VASP), plasminogen activator, urokinase receptor (PLAUR), and the RhoGEF triple functional domain protein (TRIO) as well as THBS1. Promoter-cluster analysis was again performed for the genes of Cluster-3 identifying 11 models; EBOX, EGRF, PAX5, and AP2F were the most common transcription factor families.

Cluster 4 contained 10 genes, some of which are marked by their functions in the NFκB-β signaling pathway; nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α (NFKBIA), tumor necrosis factor, α-induced protein 3 (TNFAI3), and syndecan 4 (SDC4). Transcription factors such as serum response factor (SRF) and Fos-related antigen-2 (FOSL2) were also expressed as well as the growth factor connective tissue growth factor (CTGF). Promoter-cluster analysis was performed for the genes of Cluster-4 identifying nine models, NFKB being the most common transcription factor family.

**Thrombin Induction of THBS1 Expression Confirmed by RT-PCR**—To confirm the microarray results obtained for THBS1 and a subset of other thrombin-induced genes, semiquantitative RT-PCR was performed. Samples were harvested 6 h after thrombin treatment, the time of maximal THBS1 induction. Thrombin induced THBS1, SERPINE1, PLAT, and connective tissue growth factor by 3.1, 4.2-, 3.0-, and 1.8-fold, respectively (Fig. 1C). These data correspond well with the microarray data of 2.6-, 3.1-, 3.1-, and 1.9-fold, respectively, confirming those results. In addition, expression of both PAR-1 and PAR-2 was analyzed via RT-PCR and found to be induced by 3.3- and 1.8-fold, respectively. These data confirm previous studies that quantified thrombin-induced PAR-1 expression in HMEC-1 using Northern blot analysis (41) and demonstrate that thrombin can induce PAR-2 expression. These results suggest that, although corresponding cDNAs to the PARs are found on the chip used in the present studies, the results were below the strin-
**Confirmation of PAR-1-induced THBS1 protein expression in HMEC-1 and HUVECs.** A, the concentration of secreted THBS1 into the culture media from HMEC-1 was determined via competition ELISA from thrombin (86 nM) or PAR-1-TK (100 μM)-stimulated cells and control cells at the time points indicated. Statistical analysis was performed using the two-tailed t test; ** indicates a p value <0.01 for both thrombin or PAR-1-TK compared with control; *** indicates p values <0.001 for both thrombin or PAR-1-TK compared with control. B, the effects of thrombin (86 nM) treatment on THBS1 induction in HUVECs were determined via competition ELISA from cultured media harvested 24 h after stimulation using similarly treated HMEC-1 as a control. Data represent at least three separate experiments. Statistical analysis was performed using the two-tailed t test; ** indicated a p value <0.01. All results are shown as the mean ± S.E.

**Thrombin Induction of THBS1 Expression in HUVECs—** Differences in cellular responses and gene expression between endothelial cell types has been well documented (30–33). To determine whether THBS1 expression is up-regulated by thrombin stimulation in another endothelial cell type, similar experiments were performed using HUVECs. HUVEC cultures were stimulated with or without 86 nM (10 units/ml) thrombin, and the fold increase in thrombin-induced THBS1 expression 24 h post-stimulation was determined by competitive ELISA using thrombin stimulated HMEC-1 as a control (Fig. 2B).

**Thrombin-induced THBS1 Expression Is Dose-dependent—** To further characterize the ability of thrombin to regulate THBS1 expression, the thrombin dose-response was investigated. Using competitive ELISA as before, Fig. 3 shows the dose-response curve for thrombin-mediated THBS1 protein expression in HMEC-1. The EC_{50} value was 4.6 (2.6–7.9) nM with an approximate of 1.9 (1.8–2.1)-fold increase at saturation, at the 95% confidence interval.

**Thrombin-induced THBS1 Expression Is PTX- and Protein Kinase C-sensitive—** We have previously demonstrated in HMEC-1 that PAR-1 effectively couples to G_{i/o}, resulting in activation of extracellular signal-regulated kinase (41). To determine whether activation of G_{i/o} is necessary for thrombin-induced THBS1 expression, HMEC-1 were pretreated with PTX, and the effects on THBS1 expression were determined via competitive ELISA as before. Pretreatment with PTX inhibited the response by ~70% (Fig. 4A). The efficiency of PTX treatment was controlled for by a substrate depletion assay. Pretreatment with PTX for 3 h completely inactivates all G_{i/o} subunits (Fig. 4B). These results suggest that activation of G_{i/o} is essential for thrombin-induced THBS1 expression.

Endothelial PAR-1 has been shown to activate ROCK via the G_{12/13}-mediated pathway (50). To determine whether ROCK activity is necessary for thrombin-induced THBS1 expression, HMEC-1 were pretreated with the specific ROCK inhibitor Y-27632. Pretreatment with 25 μM Y-27632 had no statistically significant effect (Fig. 4A). Y-27632 inhibition of ROCK was controlled for using a barrier function assay. PAR-1-induced endothelial barrier dysfunction is dependent upon ROCK activity; thus, HMEC-1 were grown on gold electrodes, and the transendothelial electrical resistance was measured in response to thrombin. Pretreatment for 3 h with 25 μM Y-27632 did not affect the absolute resistance of the monolayer but completely inhibited the transient monolayer barrier dysfunction induced by 10 nM thrombin, Fig. 4C. The data indicate Y-27632 treatment under these conditions completely inhibits...
cells were treated with 0.1 mM Bis I before stimulation with or without 86 nM thrombin. Secreted THBS1 was quantified using the competitive ELISA as before. Inhibition of protein kinase C with Bis I resulted in a 2.7-fold increase in secreted THBS1 levels compared with controls (Fig. 2B). Thrombin-regulation of THBS1 appears also to be a property of the endothelium, as thrombin induced a ~2.7-fold increase in secreted THBS1 levels in culture media harvested 24 h post-stimulation from HUVECs, compared with a ~1.7-fold in HMEC-1 controls (Fig. 2B).

THBS1 protein expression was also shown to be thrombin dose-dependent (Fig. 3). The EC_{50} from the dose-response curve was 4.6 nM. This is similar to the other EC_{50} values reported for other PAR-1-mediated responses such as formation of the second messenger inositol phosphate formation in HUVECs (~3.4 nM) (56) and correlates well with the physiologic range of thrombin (57). Taken together, these results suggest that at physiologic concentrations of thrombin near the EC_{50} value, endothelial cells respond by up-regulating THBS1 expression in culture media harvested 24 h post-stimulation from HUVECs, compared with a ~1.7-fold in HMEC-1 controls (Fig. 2B).

**DISCUSSION**

**Regulation of THBS1 Gene Expression by Thrombin in HMEC-1**—THBS1 is a high molecular weight glycoprotein of the extracellular matrix. Increased THBS1 expression has been linked to pathophysiological states such as atherosclerotic lesions (13), hyperplasia (14), hypertension (15), and cancer (11). The mechanism by which THBS1 is up-regulated in disease states, however, is not well understood.

Although THBS1 can originate from thrombin-induced platelet activation via release from α-granules, THBS1 is also expressed and continuously secreted by many cell types of the vascular system including the endothelium (42, 43). The effects of thrombin on gene expression and specifically that of THBS1 expression in the surrounding endothelium are less well defined. However, THBS1 has been shown to be up-regulated in vasculature cells in response to glucose (55). To characterize on a more complete scale the effects of thrombin on gene regulation and expression in endothelial cells, a microarray analysis approach was used. Of the potential 11,000 targets, surprisingly only 65 different genes including THBS1 were affected over a time course of 1.5, 6, and 12 h after thrombin treatment (Fig. 1A and supplemental data). Four distinct expression patterns were found into which the genes were clustered (Fig. 1B). THBS1 along with a subset other genes identified in the microarray studies were confirmed by semiquantitative RT-PCR (Fig. 1C). Interestingly, of the 65 genes identified, all were up-regulated in response to thrombin stimulation. The microarray did not reveal any statistically valid genes whose expression was suppressed.

Similar microarray studies using HUVECs have been performed by others (26). In common, four transcripts were induced by thrombin; small inducible cytokine A2 or monocyte chemotactic protein-1 (CCL2), immediate early response (IER3), and nuclear factor-κ light polypeptide gene enhancer in B-cells inhibitor-α (NFKBIA). Interestingly, these four hits corresponded in magnitude and temporally. However, the remaining 61 genes including THBS1 identified here differ from those induced in other endothelial cells, demonstrating the need to characterize multiple cell types. Although THBS1 was not identified in previous studies using HUVECs, we show here by ELISA that thrombin induces THBS1 expression in HUVECs.

**Thrombin Regulation of THBS1 Expression in Both HMEC-1 and HUVECs**—Increased levels of secreted THBS1 determined by ELISA correlated with an increase in mRNA both temporally and quantitatively over the 12-h time course (Fig. 2A). Although endothelial cells express PAR-1, -2, and -3, the results of the PAR-1-specific peptide mimicking those of thrombin indicated that PAR-1 activation is sufficient to mediate thrombin regulation of THBS1 gene expression (open squares compared with open circles Fig. 2A). Thrombin-regulation of THBS1 appears also to be a property of the endothelium, as thrombin induced a ~2.7-fold increase in secreted THBS1 levels in culture media harvested 24 h post-stimulation from HUVECs, compared with a ~1.7-fold in HMEC-1 controls (Fig. 2B).

**THBS1 Promoter Analysis**—To identify potential pathways that might lead to PAR-1 induction of THBS1, the promoter region of THBS1 was analyzed. Fig. 5A is a representation of
the predicted promoter site consisting of 600 bp, 500 bp up-
stream to 100 bp downstream of the transcription start site.
The first representation shows all potential binding sites for
the 41 different transcription factor families identified.

An advantage of the high throughput approach of microarray
analysis is the accumulation of large data sets from which
patterns of information can be extracted. To increase the like-
lihood of a potential transcription factor binding site being
functional in the current signaling context, the promoter re-
gions of all genes that clustered by expression profile were also
analyzed. Given that the genes of each cluster display similar
expression profiles within their respective clusters, it is likely
that a population of those genes are transcriptionally under
similar control. The second representation of Fig. 5A
shows only those transcription factor binding sites common to
members of the cluster.

The transcription factors were then further analyzed for
transcription factor models consisting of multiple transcription
factor binding motifs that have been shown empirically to
function synergistically, are in close physical proximity (10–
100 base pairs), and were present in >40% of the promoter
regions of the cluster. Two such models were identified for
THBS1. The final representation of Fig. 5A shows one model
consisting of an EBOX and EGR binding module. The second
predicted model consisted of an EBOX and ZBPF module.

To determine which of the two predicted transcription mod-
els was more likely to describe thrombin-induced THBS1 ex-
pression, the expression patterns of all the members of each
transcription factor family predicted by both models were ini-
tially screened using the unfiltered results from the microarray
analysis. Only transcription factors for the EBOX and EGR
families appeared to be thrombin-induced.

Quantitative RT-PCR confirmed prominent transcription factors from each family MYC and EGR1, respectively, are each
up-regulated at the point of maximal THBS1 expression (6 h)
(Fig. 5B). These results suggest that thrombin-induced THBS1
expression may be regulated first by the expression of these
transcription factors. This would be consistent with the obser-
vation that transcription factors of the EBOX and EGR families
have relatively short half-lives in the cell (58, 59).

In support of this model THBS1 expression has been linked
previously to MYC (60) and EGR1 (61–65). In addition, throm-
bin has been shown to regulate the expression of both MYC (66,
67) and EGR1 (54).

Similar promoter analyses of the MYC and EGR1 genes have
revealed possible models of their transcriptional regulation.
This method predicted a well documented NFκB pathway of
MYC expression (68–74). Analysis also predicted an experi-
mentally determined model involving ETS and SRF transcrip-
tion factor family regulation of EGR1 via Gq/o-mediated activa-
tion of extracellular signal-regulated kinase (ERK) via Gq and/or protein kinase C-
deependent (PKC) pathways (53, 54, 75–79).

We have previously shown that thrombin can induce extra-
cellular signal-regulated kinase activity via a PTX-sensitive
pathway in HMEC-1 (41). These data are consistent with our
present findings that pretreatment of cells with PTX inhibited
thrombin-induced THBS1 expression by ~70% (Fig. 4, A and
Thrombin Induces THBS1 Expression

B). Fig. 5C represents a composite pathway for thrombin-induced THBS1 expression.

This pathway model predicts that signaling by both G_{i0} and G_{q} are necessary to increase THBS1 expression. Consistent with that model, we show inhibition of G_{i0} by PTX treatment inhibited the response. Likewise, inhibition of protein kinase C, whose activity depends on G_{q}-mediated calcium mobilization, by treatment with Bis I also inhibited the response, Fig. 4A.

We also observed that treatment with the ROCK inhibitor Y-27632 had no effect (Fig. 4, A and C). It has been reported by others that phosphorylated MYC negatively regulates THBS1 expression in epithelial cells and that the phosphorylation state is ROCK-dependent (80). Our results are consistent, temporally, with the expression pattern of THBS1. They suggest that G_{i1213} activation of ROCK peaks and returns to baseline well before MYC expression is achieved, thereby not affecting the phosphorylation state of MYC. This is consistent with the temporal kinetics of Rho activity after thrombin stimulation of endothelial cells.

In conclusion, thrombin has been shown to activate the transcription factor, Y-box DNA-binding protein B (dbpB) in endothelial cells to induce platelet-derived growth factor β expression (81, 82). Although platelet-derived growth factor β was found to be thrombin-induced in the present microarray studies (clustering to group 2), the promoter analysis of this group found no increase in THBS1 in endothelial cells. In addition, thrombin has been shown to activate the transcription factor, Y-box DNA-binding protein B (dbpB) in endothelial cells. This pathway model predicts that signaling by both Gi/o and Gq is upstream of THBS1 production. Maximal THBS1 transcription is achieved by activation of THBS1 is up-regulated by exposure to thrombin. This induction of an angiogenic factor such as THBS1 at the site of vascular injury at first might seem paradoxical. However, one must consider the time delay involved in THBS1 induction. In addition, inhibition of protein kinase C, Y-27632 had no effect (Fig. 4, A and C). It has been reported by others that phosphorylated MYC negatively regulates THBS1 expression in epithelial cells and that the phosphorylation state is ROCK-dependent (80). Our results are consistent, temporally, with the expression pattern of THBS1. They suggest that G_{i1213} activation of ROCK peaks and returns to baseline well before MYC expression is achieved, thereby not affecting the phosphorylation state of MYC. This is consistent with the temporal kinetics of Rho activity after thrombin stimulation of endothelial cells.

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