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The $P2Y_2$ Nucleotide Receptor Mediates Tissue Factor Expression in Human Coronary Artery Endothelial Cells*[S]

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The discovery of the role of $P2Y_{12}$ receptor in platelet aggregation leads to a new anti-thrombotic drug Plavix; however, little is known about non-platelet P2Y receptors in thrombosis. This study tested the hypothesis that endothelial P2Y receptor(s) mediates up-regulation of tissue factor (TF), the initiator of coagulation cascade. Stimulation of human coronary artery endothelial cells (HCAEC) by UTP/ATP increased the mRNA level of TF but not of its counterpart-tissue factor pathway inhibitor, which was accompanied by up-regulation of TF protein and cell surface activity. RT-PCR revealed a selective expression of $P2Y_2$ and $P2Y_{11}$ receptors in HCAEC. Consistent with this, TF up-regulation was inhibited by suramin or by siRNA silencing of $P2Y_2$ receptor, but not by NF-157, a $P2Y_{11}$-selective antagonist, suggesting a role for the $P2Y_2$ receptor. In addition, $P2Y_2$ receptor activated ERK1/2, JNK, and p38 MAPK pathways without affecting the positive NF-κB and negative AKT regulatory pathways of TF expression. Furthermore, TF up-regulation was abolished or partially suppressed by inhibition of p38 or JNK but not ERK1/2. Interestingly, blockade of the PLC/Ca^{2+} pathway did not affect $P2Y_2$ receptor activation of p38, JNK, and TF induction. However, blockade of Src kinase reduced phosphorylation of p38 but not JNK, eliminating TF induction. In contrast, inhibition of Rho kinase reduced phosphorylation of JNK but not p38, decreasing TF expression. These findings demonstrate that $P2Y_2$ receptor mediates TF expression in HCAEC through new mechanisms involving Src/p38 and Rho/JNK pathways, possibly contributing to a prothrombotic status after vascular injury.

Nucleotides are well known for being the universal currency of intracellular energy transaction, but over the past few decades it has been established that nucleotides also serve as extracellular signaling molecules (1, 2), playing important roles in cardiovascular regulation (3–6). Long term trophic and inflammatory signaling upon nucleotide activation of P2Y receptors are implicated in vascular cell proliferation, migration, and platelet aggregation, leading to vascular remodeling, restenosis, atherosclerosis, and thrombosis. Therefore, at least some subtypes of the P2Y receptor family ($P2Y_{1, 2, 4, 6, 11–14}$) are potential therapeutic targets for cardiovascular diseases. Indeed, the discovery of the role of $P2Y_{12}$ receptor in platelet biology (7, 8) led to the design of clopidogrel (Plavix), which has become one of the most used drugs in treatment of atherothrombosis, myocardial infarction, and stroke. The mechanism for the anti-thrombotic effect of Plavix is the blockade of $P2Y_{12}$ receptor on platelets, preventing platelets from being activated by ADP, thus limiting platelet aggregation and clot formation. Despite extensive study of the $P2Y_{12}$ receptor in platelets, very little is known about the contribution of non-platelet P2Y receptors in thrombosis.

Platelet activation and aggregation is the final stage of thrombosis, which is developed from a hypercoagulable status due to tissue factor (TF)[2] exposure to blood. An abundance of active TF in atherosclerotic lesions plays a key role in atherothrombosis by triggering a hypercoagulable status (9, 10). In normal conditions, TF is constitutively expressed at the subendothelial level by smooth muscle cells in the tunica media and by fibroblasts in the adventitia surrounding the vessels (11), whereas it is virtually undetectable in endothelium. Hence, the endothelial layer prevents TF from direct exposure to circulating blood. However, TF expression in endothelial cells can be induced in pathological conditions such as during inflammatory responses. The exposure of cell surface TF to plasma proteins leads to the binding of factor VIIa to TF, causing ultimate activation of the coagulation cascade, thrombin generation, and thrombi formation. Therefore, identification of novel factors in control of inducible TF expression in endothelial and blood cells will provide new insights into preventing the occurrence of pathological thrombosis in the early initiating stage.

Several endogenous factors, such as interleukin-1, tumor necrosis factor a (TNFα), thrombin, and VEGF, induce endothelial TF expression (12). Because many, if not all, pathological conditions that cause TF expression are associated with nucleotide release, we propose that P2Y receptors may also be involved in regulating inducible TF expression. Although the intracellular regulatory signaling mechanisms responsible for TF induction are not completely elucidated, there is evidence showing that most of these known mediators share similar signal transduction pathways such as MAPKs pathways. Activation of P2Y receptors is commonly associated with the stimulation of these pathways, further raising the likelihood of P2Y

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[2] The abbreviations used are: TF, tissue factor; TFPI, TF pathway inhibitor; VCAM, vascular cell adhesion molecule; PLC, phospholipase C; HCAEC, human coronary artery endothelial cells.

receptor involvement in TF induction. Therefore, we hypothesize that activation of P2Y receptor(s) prompts endothelial TF expression, leading to a pro-thrombotic endothelial phenotype.

The principle objective of this study was to determine whether activation of P2Y receptors can induce TF expression in human coronary artery endothelial cells (HCAEC) and, if so, which P2Y receptor(s) is responsible for this function. Second, we sought to determine the intracellular signaling pathways that are involved in TF induction. Our findings demonstrate that in HCAEC, both ATP and UTP induce TF expression, and the P2Y$_2$ receptor is responsible for UTP-induced TF expression. Furthermore, the Src/p38 pathway is required for, and the Rho/JNK pathway contributes to, nucleotide-induced TF expression.

EXPERIMENTAL PROCEDURES

HCAEC Culture and Stimulation—HCAEC were cultured in EBM-2 supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA 1000 (Lonza), and 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2. HCAEC were used between the third and eighth passages, seeded at 10^5 cells/well in a six-well plate and grown for 24 h, reaching ~80–90% confluence. Then the cells were starved overnight and pretreated with inhibitors or antagonists for 1 h before stimulation with different agonists at the indicated times and concentrations.

RT-PCR Analysis—Total RNA and DNA were extracted from HCAEC using the RNeasy and DNeasy kits, respectively (Qiagen). For the synthesis of the first strand of cDNA, 1 μg of total RNA after DNase (Ambion) treatment was reverse-transcribed using a cDNA synthesis kit (Applied Biosystems). The cDNA samples were then amplified by PCR using 2.5 units of Taq DNA polymerase (Roche Applied Science). The sequences of primers for P2Y receptors are listed in online supplemental Table 1. The PCR condition was for 40 cycles of the following: jump start for 2 min at 95 °C, denaturation for 1 min at 95 °C, annealing for 1 min at 56 °C, and extension at 72 °C for 1 min. The resulting PCR products were resolved on a 1.5% agarose ethidium bromide gel, and the bands were visualized with ultraviolet light.

Real Time RT-PCR Analysis—Real time RT-PCR was performed on an iCycler iQ5 detection system (Bio-Rad) with SYBR Green reagents (Applied Biosystems). The PCR mixture (25 μl) contained 0.6 μM concentrations of each primer, 8 μl of water, 12.5 μl of SYBR Green mixture, and 2.0 μl of cDNA. The samples were placed and sealed in 96-well plates with the following reaction condition: reverse transcription step (30 min at 50 °C), initial PCR activation step (15 min at 90 °C), and cycling steps (denaturation for 30 s at 94 °C, annealing for 30 s at 58 °C, extension for 1 min at 72 °C, 40 cycles). An internal control, GAPDH, was amplified in separate tubes. We used the comparative cycle threshold ΔΔCt method for relative quantification of gene expression.

Immunofluorescence—HCAEC were cultured on 8-chamber glass slides (Nunc). After 1 day in culture, cells were starved and treated with UTP, ATP, or TNFα for 4 h, after which cells were fixed for 10 min in cold methanol. The fixed cells were washed with PBS and blocked with 3% horse serum for 1 h at room temperature. Then the cells were incubated with mouse monoclonal anti-human TF antibody (1:100) overnight at 4 °C followed by incubation with FITC-conjugated anti-mouse IgG for 60 min at room temperature. For negative controls, cells were incubated with non-immune IgG in place of the specific primary antibody.

Cell-based ELISA—Cell surface TF was detected by a cell-based ELISA kit (Columbia Bio LLC) following the manufacturer's instructions. In brief, HCAEC were seeded into 96-well plates and stimulated by UTP for 4 h. Then, the cells were fixed by 50 μl of fixatives (without permeabilization) for 20 min and blocked for 20 min after incubation with the mouse anti-human TF antibody (1:1000) for 1 h at room temperature. After 3 washes, 50 μl of tetramethyl benzidine substrate linked with a second antibody was added and maintained until color developed. The optical density absorbance was read on a Microplate Reader ELX800 (Bio-TEK Instruments Inc.) at 450 nm. Relative cell surface TF protein was presented as change of optical density values in UTP-stimulated cells over control cells.

Cell Surface TF Activity—TF pro-coagulant activity in HCAEC surface was analyzed by a two-stage chromogenic assay (ACTICHROME®, American Diagnostica Inc.) following the manufacturer's instructions. In brief, HCAEC were grown in 6-well plates and stimulated with UTP, ATP, or TNFα for 4 h. After 2 washes, cells were incubated with assay buffer (300 μl), 25 μl of factor FVIIa, and 25 μl of factor X at 37 °C for 15 min. Then 25 μl of Spectrozyme factor Xa substrate was added and incubated at 37 °C for 20 min. Finally, 200 μl of the reaction mixture in each well were transferred into 96-well plates and read on a microplate reader as described above at 405 nm. A TF standard curve was constructed by following the kit instructions.

Western Blot Analysis—After stimulation, cells were lysed, and standard Western blotting was performed as previously described (13). The individual primary antibodies used were anti-TF (1:3000), anti-p-ERK, anti-p38, anti-p-JNK, anti-IκBa, anti-p-p65, anti-p-Src, and anti-p-FAK (1:1000). Equal protein loading was verified by stripping off the original antibodies and re-probing the membranes with the primary antibody β-actin, GAPDH, histone H3, total ERK1/2, p38, or JNK (1:1000).

Intracellular $[Ca^{2+}]_i$ Analysis—Measurement of intracellular $[Ca^{2+}]_i$ concentration was performed using the Fluo-Forte™ Calcium Assay kit (Enzo Life Sciences). Briefly, HCAEC were plated in growth medium in 96-well plates at 6×10^4 cells/100 μl/well. After 24 h, cells were pretreated with U73122 for 1 h, then the growth medium was removed, and 100 μl of Dye-loading solution was added in the presence of U73122. The cells were further incubated for 45 min at 37 °C and 15 min at room temperature before stimulation, after which the cells were challenged with UTP, and a time-response curve of intracellular $[Ca^{2+}]_i$ signal was recorded via real-time monitoring of fluorescence intensity at excitation = 490 nm and emission = 525 nm in a Fluorometric Microplate Reader (FLUOstar Omega).

Silencing of P2Y$_2$ Receptor by siRNA—To knock down the P2Y$_2$ receptor, HCAEC were transfected with the four sequence pool (ON-TARGET plus SMART pool L-003688-00-0005, human P2RY$_2$, NM_002564, Dharmacon) using DharmaFECT 4 Transfection reagent following the manufac-

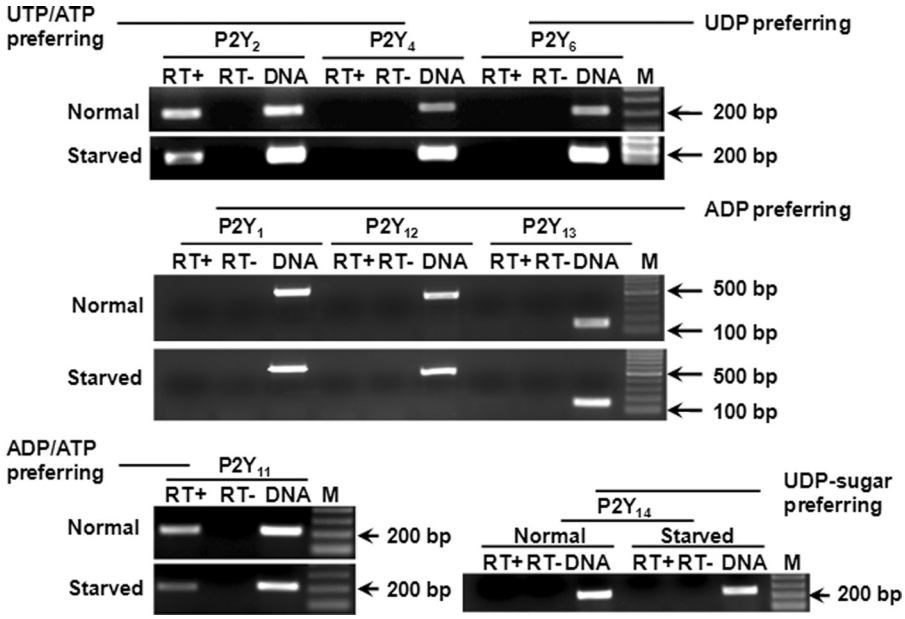

FIGURE 1. **Expression profile of P2Y receptors in HCAEC in normal culture and starvation conditions.** mRNA expression of eight subtypes of P2Y receptors was determined by RT-PCR. *RT−* indicates RT-PCR performed without reverse transcriptase. Genomic DNA was used as the positive controls.

turer's protocol. Briefly, HCAEC were seeded in 6-well plates at 80–90% confluence; the medium was replaced with complete EBM-2 without antibiotics before transfection. DharmaFECT 4 and siRNA products were incubated separately in EBM-2 at room temperature for 5 min. Mixtures were combined, incubated another 20 min, and added to cells at a final concentration of 2 μl/ml DharmaFECT 4 and 25 nM siRNAs. Real-time PCR assay was performed to confirm the decrease of $P2Y_2$ receptor mRNA after 24 h post-transfection. For UTP stimulation, siRNA and transfection reagent were removed 24 h post-transfection, and complete culture medium was added. After overnight starvation, cells were stimulated by UTP as described above.

Materials—HCAEC and endothelial cell basal medium-2 were purchased from Lonza. $P2Y_2$-transfected 1321N1 astrocytoma cells were kindly provided by Dr. Gary A. Weisman (University of Missouri-Columbia). Purified UTP and ATP were obtained from Sigma. Actinomycin D, cycloheximide, U0126, SB203580, SP600125, VX745, TCS-JNK6o, LY294002, L-NIO, U73122, Y-27632, suramin, and NF-157 were purchased from Tocris Bioscience. BAY11-7082, SKI-1, and PP2 were from EMD. Anti-tissue factor mouse mAb (TF9–10H10) was obtained from Calbiochem. Other antibodies were purchased from Cell Signaling.

Data Analysis—Data are expressed as the mean ± S.E. The means of two groups were compared using Student's t test (unpaired, two tailed), and one-way analysis of variance was used for comparison of more than 2 groups with $p < 0.05$ considered to be statistically significant. Unless otherwise indicated, all experiments were repeated at least three times.

RESULTS

ATP and UTP Increase TF Expression and Activity in HCAEC—We first analyzed the expression profile of P2Y receptors in HCAEC, as it has not been determined in human coronary artery endothelium or cultured cells. Our RT-PCR analysis showed that HCAEC expressed $P2Y_2$ and $P2Y_{11}$ receptor mRNAs, with virtually no detectable mRNAs for the other six subtype receptors (Fig. 1). No significant change was observed in receptor expression pattern when the cells were starved overnight in comparison to normal cultures (Fig. 1). This result indicates that HCAEC predominantly express UTP/ATP-sensitive $P2Y_2$ receptor and ATP/ADP-sensitive $P2Y_{11}$ receptor.

To determine whether activation of P2Y receptors can induce TF expression, cells were stimulated with agonists for various time intervals. TF protein expression was increased within 2 h, reached a maximum level at 4 h, and declined to the basal level at 8 h after the serum-starved HCAEC were challenged with 100 μM UTP or ATP (Fig. 2*A*). A dose-dependent increase of TF protein expression was observed after HCAEC were stimulated with different concentrations of UTP or ATP (Fig. 2*B*). Of note, the level of TF induction by UTP/ATP was even comparable with that of TNFα (Fig. 2*A*), a known inducer of TF expression in various cells.

Because TF can be stored in some intracellular compartments in addition to cell surface localization, a cell-based ELISA was employed to detect the cell surface TF expression in response to UTP. In line with total TF expression, the cell surface TF expression was significantly up-regulated by UTP in a dose-dependent manner (Fig. 2*C*). Consistent with this, immunofluorescence study showed that TF antigen was barely detectable in quiescent untreated control HCAEC (Fig. 2*D*). However, in cells treated with UTP or ATP, there was diffused TF staining over the entire cell surface and intracellular space as well. A similar result was obtained when cells were stimulated with TNFα (Fig. 2*D*).

To confirm whether enhanced cell surface TF protein translates to increased TF activity, TF pro-coagulant activity

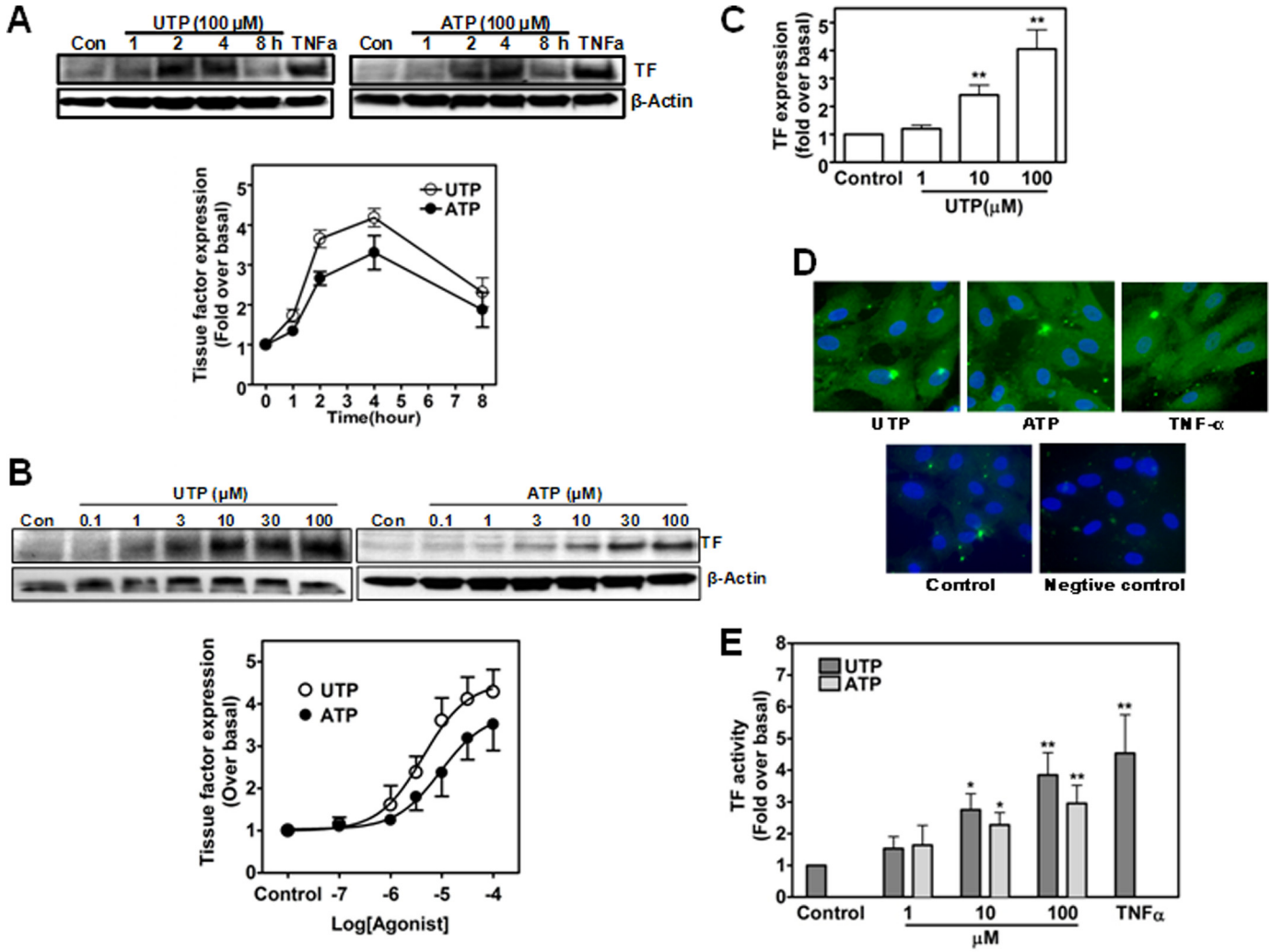

FIGURE 2. **UTP/ATP up-regulation of TF expression and activity.** Total cellular TF protein expression was determined by Western blot assay in HCAEC stimulated by 100 μM UTP or ATP for the indicated times (*A*) or by the indicated UTP/ATP concentrations for 4 h (*B*). Cell surface TF expression was assessed by cell-based ELISA (*C*) or by an immunofluorescence microscopy assay after HCAEC were treated with UTP/ATP (100 μM), TNFα (5 ng/ml), or vehicle (control) for 4 h. *Negative control* stands for mouse IgG control in substituting for anti-human TF first antibody (*D*). Cell surface TF activity was determined by a chromogenic assay after stimulation of the cells with UTP/ATP (100 μM) or TNFα (5 ng/ml) for 4 h (*E*). *, $p < 0.05$; **, $p < 0.01$ relative to respective controls.

was determined by a two-step colorimetric assay based on the ability of TF to promote generation of coagulation factor Xa. Fig. 2*E* shows that UTP or ATP stimulation significantly increased cell surface TF activity over untreated control cells. Together, these results indicate that UTP/ATP stimulation of P2Y receptor(s) can induce TF expression and activity in HCAEC.

UTP and ATP Up-regulate TF, but Not Tissue Factor Pathway Inhibitor (TFPI), mRNA Expression—To determine whether the effect of ATP/UTP on TF protein expression is related to TF mRNA expression, real-time PCR assay was performed. A peak elevation of TF mRNA level was detected as early as 1 h, and expression returned to the basal level after 3 h (Fig. 3*A*). Similar to TF protein induction, TF mRNA level was also up-regulated in a dose-dependent manner by ATP or UTP (Fig. 3*B*). This result indicates that activation of UTP/ATP-sensitive P2Y receptor(s) induces TF expression both at the protein and mRNA levels.

To confirm whether increased expression of TF mRNA involves transcriptional mechanism(s) and needs *de novo* synthesis of protein(s), cells were pretreated with the transcription inhibitor actinomycin D or the translation inhibitor cycloheximide for 1 h before UTP stimulation. Fig. 3*C* shows that actinomycin D pretreatment suppressed UTP-induced TF expression both at mRNA and protein levels. Interestingly, cycloheximide pretreatment almost eliminated the induction of TF protein as expected but induced significant TF mRNA up-regulation and further enhanced UTP-induced TF mRNA expression (Fig. 3*C*). These data suggest that the up-regulation of TF mRNA by UTP may involve a transcriptional mechanism(s) but does not need synthesis of new proteins. This result also implies that there is a cycloheximide-regulated mechanism for TF mRNA expression.

To assess the specificity of nucleotide induction of TF expression, we examined the effect of UTP on the mRNA expression of TFPI, a direct physiological inhibitor of the TF-FVIIa complex. Interestingly, stimulation of the cells with UTP did not affect TFPI expression in HCAEC (Fig. 3*D*), suggesting that activation of the P2Y receptor(s) in HCAEC selectively up-regulates TF but not its counterpart TFPI.

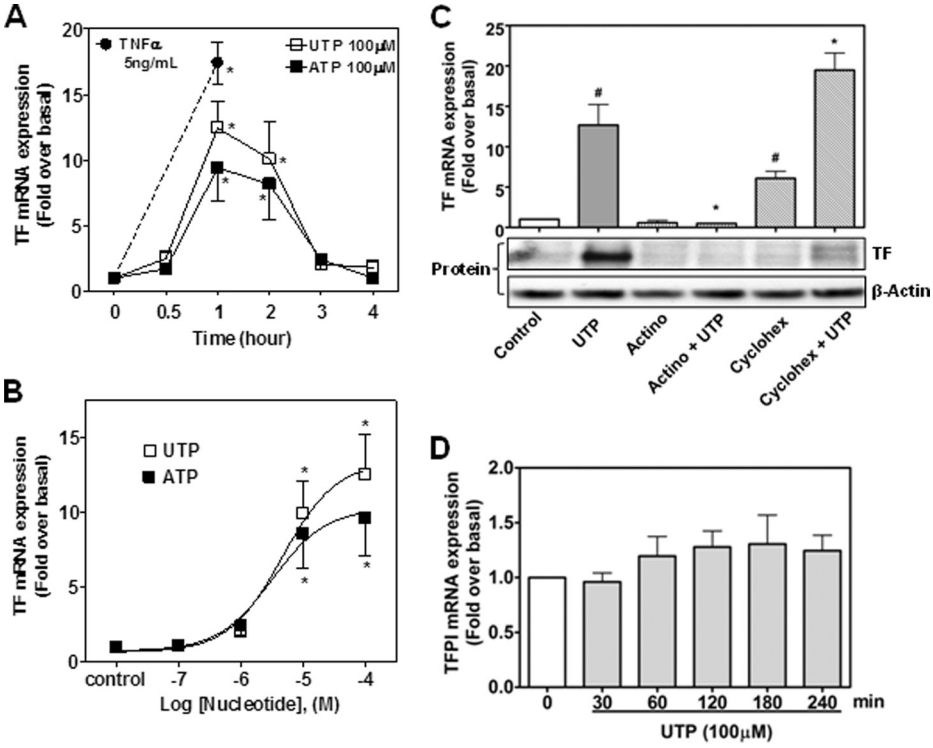

FIGURE 3. **UTP/ATP up-regulates TF but not TFPI mRNA expression.** The time course (*A*) and dose-response curve (*B*) for the change of TF mRNA expression is shown in HCAEC after exposure to UTP/ATP for the indicated time or concentration, as determined by real-time PCR. *, $p < 0.05$ relative to respective controls. *C*, cells were pretreated with actinomycin D (*Actino*, 2 μM) or cycloheximide (*Cyclohex*, 20 μM) for 30 min and then were stimulated by UTP for 1 h (for TF mRNA analysis) or 4 h (for TF protein analysis). #, $p < 0.05$ *versus* control; *, $p < 0.05$ *versus* UTP. *D*, TFPI mRNA expression is shown after stimulation of the cells with UTP (100 μM) for the indicated times.

UTP/ATP-induced TF Expression Is Mediated by the P2Y$_2$ Receptor—To determine whether the nucleotide action is through P2Y receptor(s), suramin, a broad-spectrum antagonist of P2Y receptors was employed. Fig. 4*A* shows that suramin dose-dependently reduced UTP-induced TF mRNA and protein expression, suggesting an involvement of the P2Y receptor(s). To further clarify which P2Y receptor subtype(s) is responsible for TF induction, the P2Y$_{11}$ receptor-selective antagonist NF-157 was used because no P2Y$_2$ receptor-selective antagonist is currently available. Fig. 4*B* shows that UTP-stimulated TF induction was not affected by NF-157, suggesting that P2Y$_{11}$ receptor does not contribute to UTP-induced TF expression. Furthermore, UTP was able to induce TF expression in 1321N1 cells stably transfected with P2Y$_2$ receptor cDNA but not in the wild type 1321N1 cells that lack any endogenous P2Y receptors (Fig. 4*C*), further supporting the involvement of the P2Y$_2$ receptor.

To further confirm a role for P2Y$_2$ receptor, we knocked down the P2Y$_2$ receptor by a siRNA approach. P2Y$_2$ receptor mRNA decreased 24 h after transfection of a pool of P2Y$_2$ siRNAs as determined by real-time PCR (Fig. 4*D*). Silencing P2Y$_2$ receptor abrogated UTP- and ATP-induced TF expression, whereas the scramble siRNA did not affect UTP- or ATP-induced TF expression (Fig. 4, *E* and *F*). In addition, ADP, agonist of P2Y$_{11}$ receptor, did not up-regulate TF expression (Fig. 4*F*). Collectively, these data indicate that the P2Y$_2$ receptor mediates UTP- and ATP-induced TF expression in HCAEC.

The Effect of NF-κB and AKT Pathways on P2Y$_2$ Receptor-mediated TF Induction—It is well established that NF-κB is the major positive pathway in control of TF induction. To investigate whether this pathway is involved in P2Y$_2$ receptor induction of TF expression, the IκBa and p-p65 levels were analyzed when the cells were treated with different concentrations of UTP. Fig. 5*A* shows that UTP did not induce IκBa degradation or increase of p65 phosphorylation; however, TNFα as a positive control greatly reduced total IκBa and increased p65 phosphorylation. In addition, BAY11-7082, a well known selective inhibitor of NF-κB pathway, abolished TNFα-induced, but not UTP-induced, TF expression (Fig. 5*B*). These data suggest that the NF-κB pathway is not involved in P2Y$_2$ receptor-mediated TF induction in HCAEC.

The PI3K/AKT pathway negatively regulates TF induction (14). To determine whether this pathway has such a role, we analyzed the effect of UTP on AKT phosphorylation. A basal level of p-AKT was detected that was not affected by UTP (Fig. 5*C*). In addition, both the PI3K inhibitor LY294002 and the endothelial nitric-oxide synthase inhibitor L-NIO did not have significant effect on UTP-induced TF expression (Fig. 5*D*). These data indicate that P2Y$_2$ receptor-mediated TF up-regulation in HCAEC does not involve an AKT-related mechanism.

Differential Roles of the MAPK Pathways in P2Y$_2$ Receptor-mediated TF Induction—To determine the roles of MAPK kinases, we first assessed the effect of P2Y$_2$ receptor activation on MAPK pathways. Fig. 6 shows that UTP caused rapid

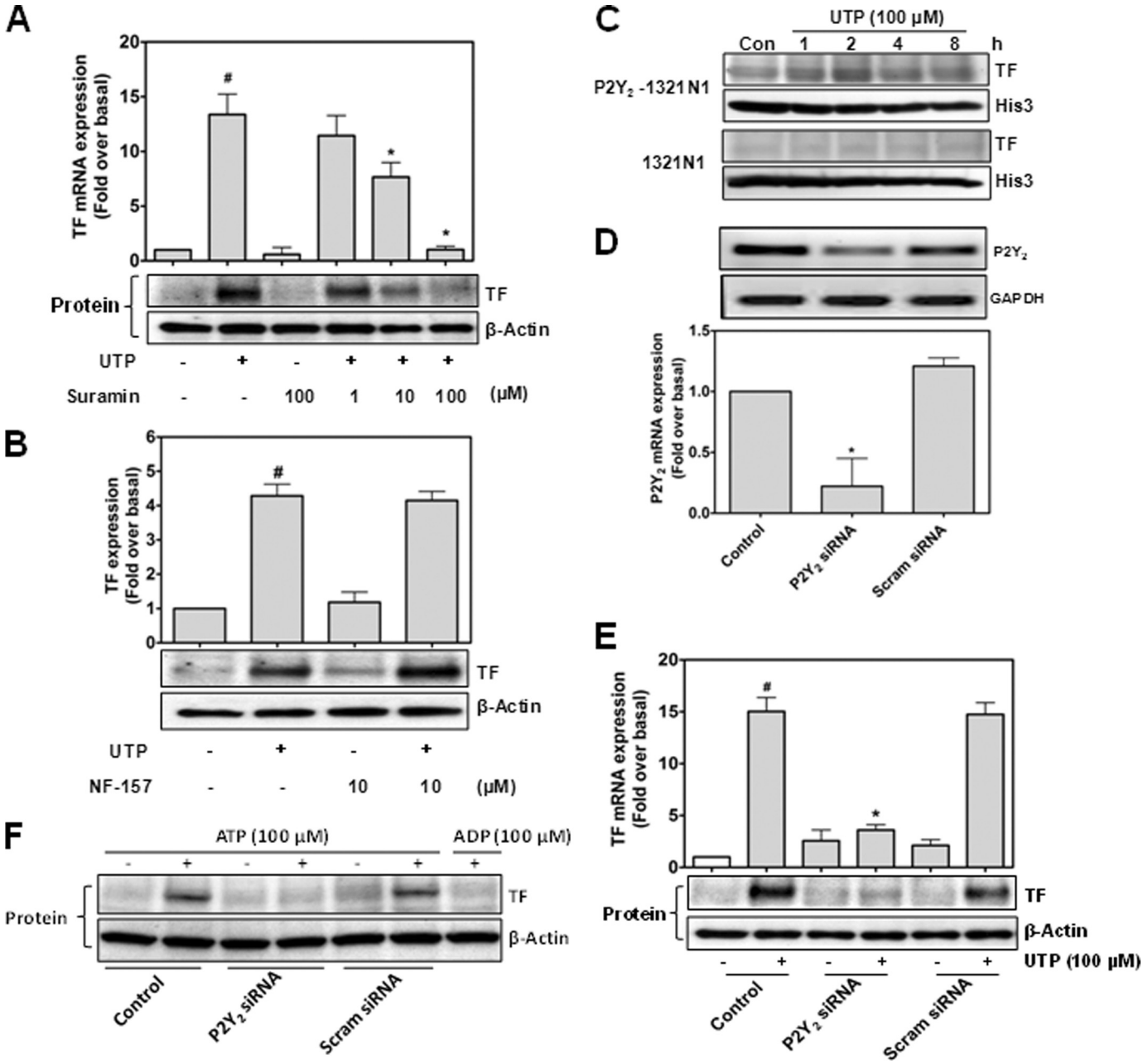

FIGURE 4. **P2Y$_2$ receptor mediates UTP/ATP-induced TF expression.** UTP-induced TF mRNA (1 h) and protein (4 h) expressions were analyzed after the cells were pretreated with suramin, a broad spectrum antagonist of P2Y receptors. #, $p < 0.05$ *versus* control; *, $p < 0.05$ *versus* UTP (*A*). UTP-induced TF protein expression was also determined after the cells were pretreated with NF-157 (P2Y$_{11}$-selective antagonist) for 1 h (*B*). Shown is TF protein expression in P2Y$_2$-transfected 1321N1 cells and wild type control cells after stimulation with UTP for indicated times (*C*). P2Y$_2$ receptor mRNA expressions after HCAEC were transfected with P2Y$_2$-siRNA or scramble siRNA for 24 h. *, $p < 0.05$ *versus* control (*D*). UTP-induced TF protein expression was evaluated after P2Y$_2$ receptor was knocked down by siRNA in HCAEC. #, $p < 0.05$ *versus* non-stimulated control cells; *, $p < 0.05$ *versus* UTP-stimulated control cells (*E*). ATP-induced TF protein expression was evaluated after P2Y$_2$ receptor was knocked down by siRNA in HCAEC. ADP served as a control in comparison to ATP (*F*).

phosphorylation of ERK1/2, p38, and JNK in a dose-dependent manner. However, UTP-induced TF expression was not affected despite the complete inhibition of ERK phosphorylation by the MEK1/2 inhibitor U0126 (Fig. 6*C*). In contrast, SB203580, a selective inhibitor of p38 kinase, dose-dependently inhibited UTP-induced p38 phosphorylation and TF expression (Fig. 6*D*). Phosphorylation of JNK was greatly reduced by the JNK inhibitor SP600125 at 10 μM, whereas UTP-induced TF expression was not affected, which may be due to slight activation of p38 by this inhibitor at this particular dose (Fig. 6*E*). To support this notion, we increased SP600125 concentration to 30 μM, which further decreased JNK phosphorylation and caused a significant reduction of TF expression as well without affecting p38 activation (Fig. 6*E*). We further confirmed this result using structurally different chemical inhibitors targeting on either p38 (VX745, 10 μM) or JNK (*TCS-JNK6o*, 10 μM, Fig. 6*H*). Fig. 6, *F* and *G*, further shows that UTP-induced p38 activation was suppressed by suramin, but not by NF-157, which effectively blocked ADP-induced p38 activation, suggesting a role of

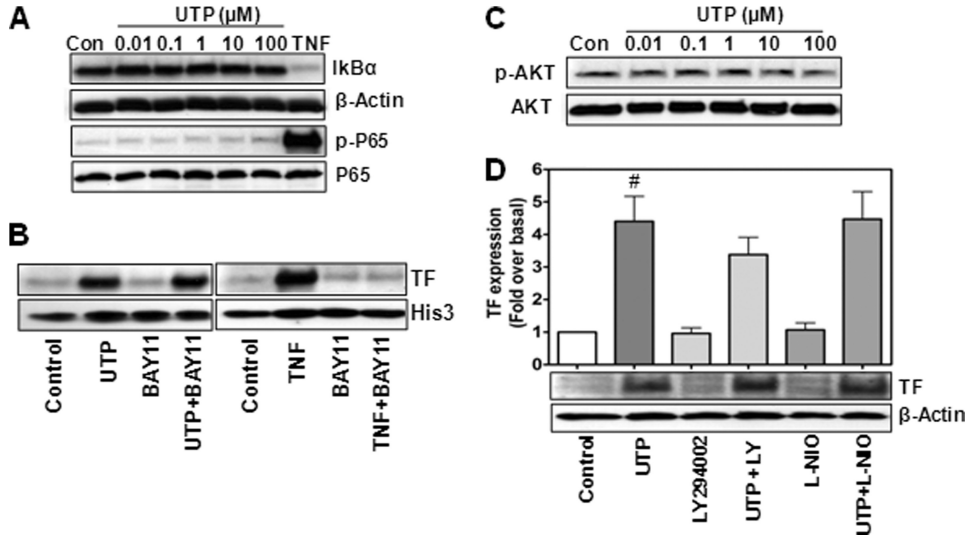

FIGURE 5. **Effect of NF-κB and PI3K/AKT pathways on UTP-induced TF expression.** IκBa degradation and p65 phosphorylation were analyzed after HCAEC were stimulated with indicated concentrations of UTP or TNFα (5 ng/ml) for 15 min (*A*). UTP- and TNFα-induced TF protein expression was assessed after the cells were pretreated with BAY11 (20 μM) for 1 h before agonist stimulation (*B*). Phosphorylation of AKT was determined by Western blotting after UTP stimulation of the cells for 15 min (*C*). UTP-induced TF expression was determined after the cells were pretreated with PI3K inhibitor LY294002 (10 μM) or endothelial nitric-oxide synthase inhibitor L-NIO (10 μM) for 1 h (*D*). #, $p < 0.05$ *versus* control.

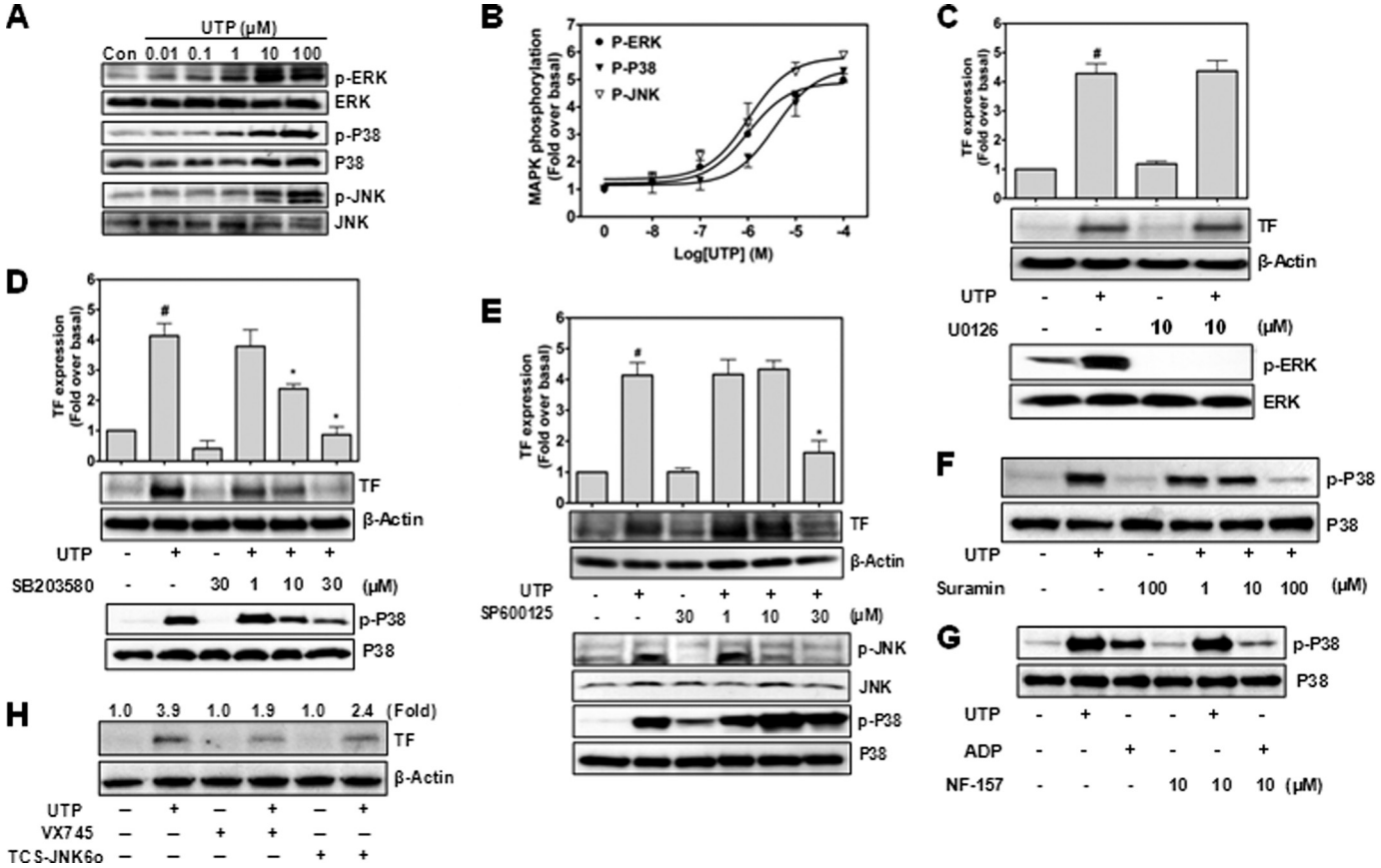

FIGURE 6. **Differential roles of MAPK pathways in P2Y$_2$ receptor-mediated TF expression.** Phosphorylation levels of ERK1/2, p38, and JNK after HCAEC were stimulated by UTP for 15 min (*A*). Group data averages were used for construction of UTP dose-response curves (*B*). UTP-induced TF protein expression (4 h) and MAPK phosphorylations (15 min) were analyzed after HCAEC were pretreated for 1 h with the individual inhibitor for ERK1/2 (*C*), p38 (*D*), or JNK (*E*) pathway. Differential roles of suramin (*F*) and NF-157 (*G*) in UTP-induced p38 phosphorylation in HCAEC are shown. ADP was used as control for the selectivity of NF-157. #, $p < 0.05$ *versus* control; *, $p < 0.05$ *versus* UTP. *H*, shown is the effect of VX745 (10 μM) and TCS-JNK6o (10 μM) on UTP-induced TF protein expression in HCAEC.

$P2Y_2$, not $P2Y_{11}$, in UTP signaling. These data indicate that p38 and JNK, but not ERK1/2, are involved in $P2Y_2$ receptor-mediated TF expression in HCAEC.

Differential Roles of PLC, Src, and Rho Kinase in P2Y$_2$ Receptor Signaling and TF Induction—Because $P2Y_2$ receptor activates PLC via the G_q protein, leading to an increase of intracel-

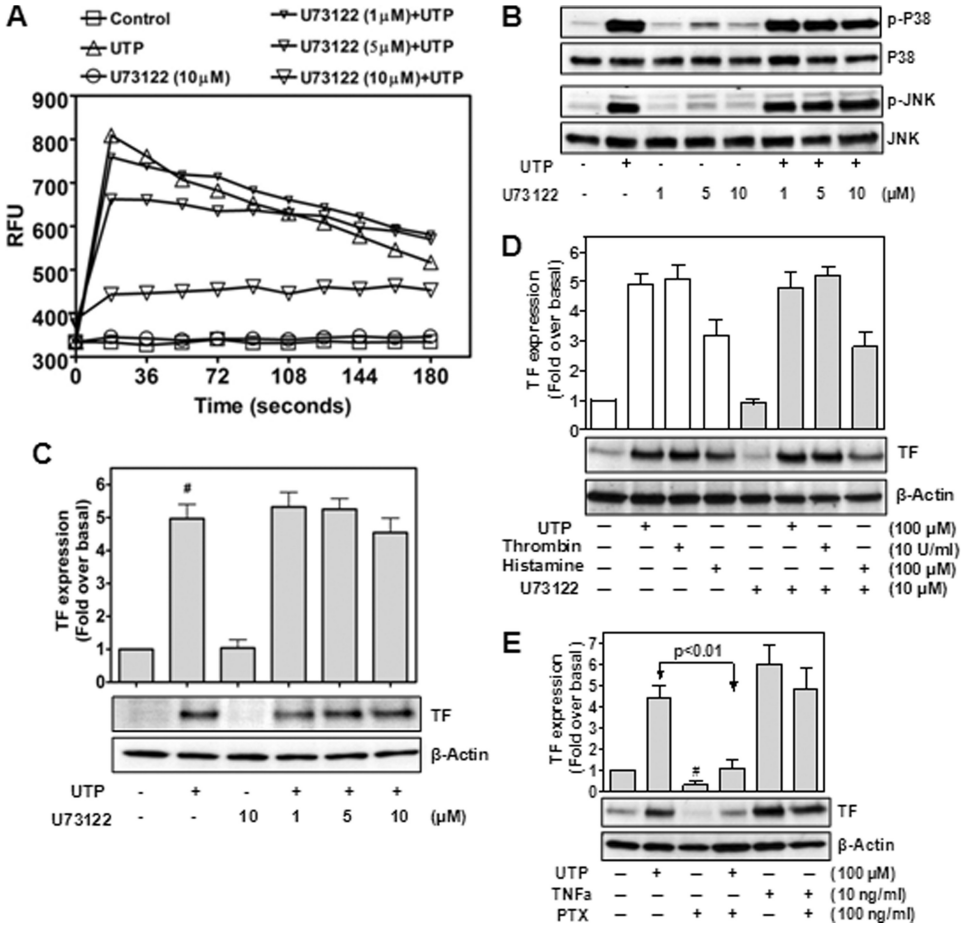

FIGURE 7. **Effect of PLC inhibition on P2Y$_2$ receptor signaling and TF induction.** UTP-induced intracellular $[Ca^{2+}]_i$ increase was determined after HCAEC were stimulated by UTP (100 μM) with or without the presence of U73122 at different doses for 1 h (*A*). UTP-induced p38 and JNK phosphorylation (100 μM, 15 min) in the absence or presence of U73122 for 1 h (*B*) is shown. UTP-induced TF protein expression was determined after HCAEC were pretreated with or without U73122 for 1 h at the indicated doses. #, $p < 0.05$ *versus* control (*C*). shown is the effect of U73122 on thrombin- and histamine-induced TF protein expression (*D*). Shown are differential roles of pertussis toxin (*PTX*, overnight treatment) on UTP- and TNFα (*TNFa*)-induced TF protein expression in HCAEC (*E*). #, $p < 0.05$ *versus* control.

lular Ca^{2+}, we postulated that PLC might be the potential upstream regulator of p38 and JNK in P2Y$_2$ receptor signaling and TF induction. As expected, U73122, a specific inhibitor of PLC, dose-dependently suppressed intracellular Ca^{2+} elevation caused by UTP (Fig. 7*A*). However, neither the phosphorylation of p38 nor the phosphorylation of JNK was affected by U73122 (Fig. 7*B*). Similarly, UTP-induced TF expression was also not affected by U73122, even at its highest concentration (Fig. 7*C*), suggesting that PLC plays no role in P2Y$_2$ receptor signaling to p38 or JNK or TF induction in HCAEC. To assess whether the canonical PI-PLC pathway is dispensable for other G-protein-coupled receptor-induced TF expression, HCAEC were pretreated with U73122 and then stimulated with either thrombin or histamine. Fig. 7*D* clearly shows that U73122 had no significant effect on thrombin- and histamine-induced TF up-regulation. To further explore a potential role of $G_{i/o}$ protein, we employed pertussis toxin. Fig. 7*E* shows that pertussis toxin treatment (overnight, 100 ng/ml) abolished UTP-induced TF expression, with no significant impact on TNFα-induced TF up-regulation.

Because P2Y$_2$ receptor directly interacts with and activates Src kinase (15), we sought to determine a potential role of Src kinase. It is recommended that a combination of Src-I and PP2, two structurally different Src kinase inhibitors, be used to evaluate a biological function of Src kinase (16). Fig. 8*A* shows that both Src-I and PP2 reduced phosphorylation of p38, but not JNK, implying Src might be the upstream regulator of p38. In addition, Src kinase was activated by UTP as evidenced by rapid phosphorylation of Src and its substrate FAK (Fig. 8*B*). In parallel, UTP-induced TF expression was abolished by Src-I and was greatly reduced by PP2 (Fig. 8*C*). Furthermore, we confirmed the effective inhibition of Src and FAK by PP2 (Fig. 8*D*). Taken together, these data indicate Src is the upstream regulator of p38 in mediating P2Y$_2$ receptor induction of TF expression.

Because the Rho pathway is implicated in thrombin-induced TF expression (17) and the P2Y$_2$ receptor has been shown to activate the small GTPase RhoA (18), we therefore examined whether Rho kinase might be the upstream regulator of p38 or JNK in response to P2Y$_2$ receptor activation. Pretreatment of HCAEC with a selective Rho kinase inhibitor Y27632 partially inhibited phosphorylation of JNK but not of p38 (Fig. 8*E*). In addition, UTP-induced TF expression was significantly reduced by Y27632 (Fig. 8*F*), suggesting that Rho A kinase is the upstream regulator of JNK, contributing to P2Y$_2$ receptor induction of TF expression in HCAEC.

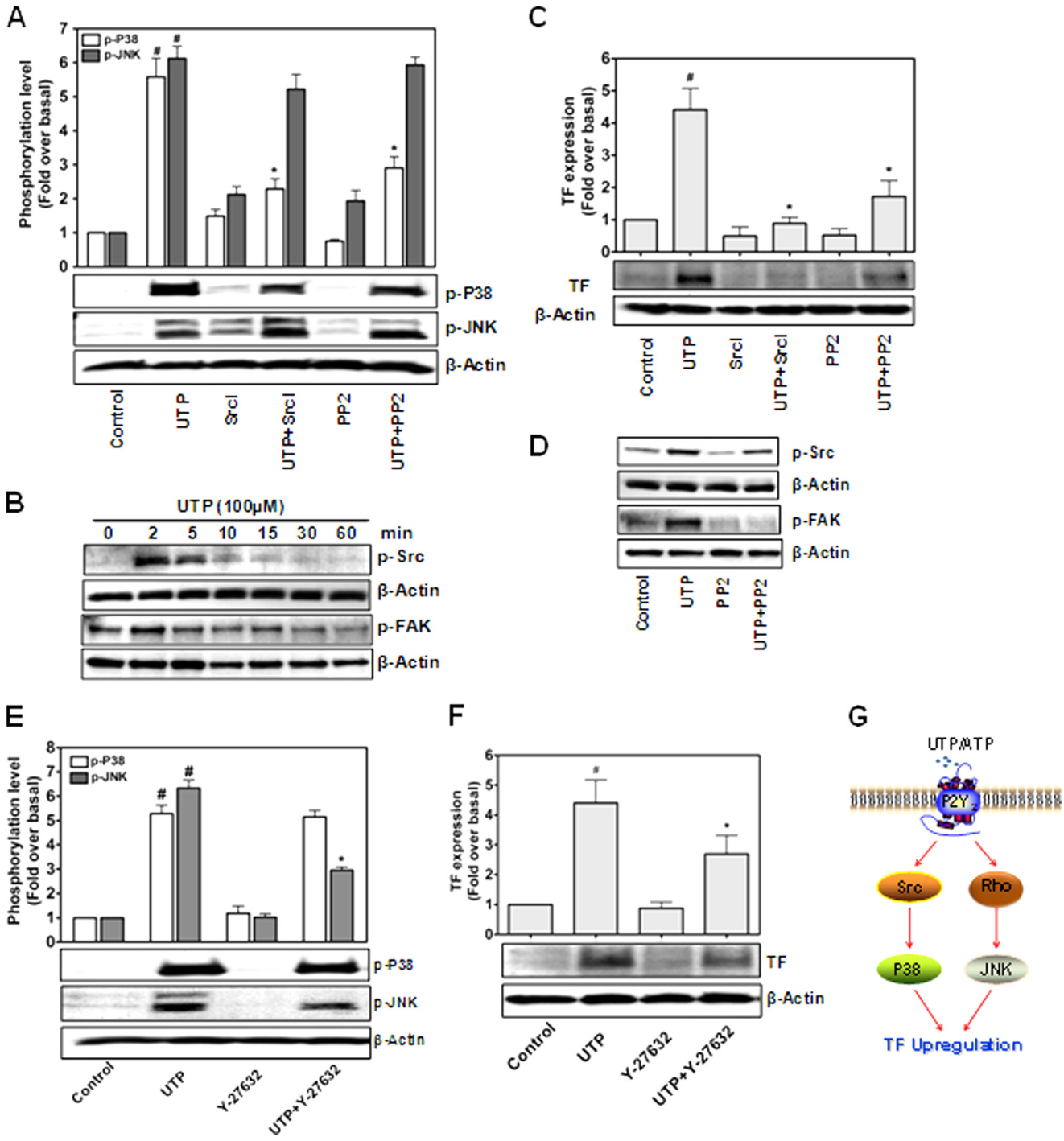

FIGURE 8. **Roles of Src and Rho in P2Y$_2$ receptor signaling and TF induction.** UTP-induced p38 and JNK phosphorylation were analyzed after HCAEC were pretreated with Src inhibitors (Src I and PP2, 20 μM) for 1 h before agonist stimulation (*A*). Phosphorylation of Src and its substrate FAK was determined after stimulation of the cells with UTP for the indicated times (*B*). TF protein induction was evaluated after the cells were pretreated with Src I or PP2 for 1 h before UTP incubation for 4 h (*C*). Shown is PP2 suppression of Src and FAK phosphorylation (*D*). UTP-induced p38 and JNK phosphorylation were analyzed after HCAEC were pretreated with Rho kinase inhibitor Y-27632 (10 μM) for 1 h before UTP stimulation (*E*). TF protein induction was determined after the cells were pretreated with Y-27632 for 1 h before UTP stimulation for 4 h (*F*). A schematic diagram shows the mechanisms of nucleotide-induced TF up-regulation in HCAEC (*G*). #, $p < 0.05$ *versus* control; *, $p < 0.05$ *versus* UTP.

DISCUSSION

In the present study we show for the first time that UTP or ATP stimulates TF expression both in protein and mRNA levels in HCAEC. We also show that Src/p38 and Rho/JNK pathways are involved in nucleotide induction of TF expression. Furthermore, we have demonstrated that the P2Y$_2$ receptor is responsible for UTP/ATP signaling and TF induction in HCAEC. These findings provide a new link between non-platelet P2Y receptor and the induction of TF, a master initiator of coagulation cascade, leading to thrombosis.

Although extensive prior studies have focused on platelet P2Y$_{12}$ receptor and thrombosis, virtually nothing is known

about the roles of non-platelet P2Y receptors in the initiation and/or propagation of thrombosis. However, accumulating evidence indicates pathological roles of non-platelet P2Y receptors in other vascular diseases. This is particularly true for the P2Y$_2$ receptor, which is up-regulated in smooth muscle cells of injured rat aorta (19), rabbit carotid artery (20), and porcine coronary artery after angioplasty stenting (21). Studies in these different animal models consistently showed that activation of the up-regulated P2Y$_2$ receptor promotes smooth muscle cell proliferation, contributing to neointima formation. In addition, it has also been proposed that the endothelial P2Y$_2$ receptor exerts a proinflammatory effect on the development of atherosclerosis (6). For example, UTP/ATP stimulates expression of VCAM-1 through activation of P2Y$_2$ receptor and increases the adherence of monocytic cells to human endothelial cells (18). Here, we show that ATP and UTP are able to induce TF expression in HCAEC through P2Y$_2$ receptor activation, suggesting that P2Y$_2$ receptor not only plays an important role in the pathogenesis of atherosclerosis but may also be implicated in thrombosis through up-regulation of TF expression. In addition to endothelial cells, monocytes/macrophages are alternatives that express inducible TF, which trigger increased cellular pro-coagulant activity (22). Because the P2Y$_2$ receptor is also highly expressed in human monocytes, it is plausible that UTP/ATP-induced TF expression is not limited to HCAEC but also applies to monocytes.[3] Thus, it is tempting to propose that the process of nucleotide release, P2Y$_2$ receptor activation, and TF induction may be one of the mechanistic explanations for the higher risk of thrombosis noticed in various diseases, such as sepsis, atherosclerosis, and cancer.

Endothelial TF is induced by various factors such as TNFα, histamine, thrombin, and VEGF (14, 23–25). It should be noted that some of these stimuli also promote ATP release. For instance, receptor-mediated ATP release has been observed in aortic endothelial and smooth muscle cells treated with thrombin (26). In addition, TNFα is known to up-regulate P2Y$_2$ receptor in vascular cells (27, 28) and to down-regulate endothelial CD39, an ecto-ATPase that degrades ATP/UTP (29). Therefore, it is conceivable that P2Y$_2$ receptor activation by released and accumulated ATP/UTP due to inflammation and/or cell death may be a significant component of total TF induction triggered by many other stimuli. In this perspective, identification of a novel role for the P2Y$_2$ receptor in TF induction may have unique importance in advancing our knowledge on pathological thrombosis.

The extent of total TF protein induction does not always correlate well with cell surface TF activity. The possible reason is the spread distribution of TF into several cellular compartments, including Golgi (30). Biologically active TF is indeed located at the cell surface, whereas intracellular TF constitutes a pool that is only released on cell damage. In this study we found that UTP-induced TF surface activity is comparable with total TF protein induction, which is not always the case for other TF inducers. For instance, VEGF greatly increases TF expression yet induces less impressive TF activity in intact human endothelial cells (31). On the other hand, it is known that cellular TF activity is counterbalanced by TFPI, which is mainly synthesized by vascular endothelial cells (32). Several TF inducers have been investigated for their ability to affect TFPI expression; indeed, an increase, a decrease, or no alteration in TFPI expression has been observed after endothelial stimulation with TNFα, LPS, and 5-hydroxytryptamine (33–35). In the current study, stimulation of HCAEC with UTP did not affect TFPI expression; thus, P2Y$_2$ receptor-mediated TF up-regulation may lead to a distinctive change in endothelial pro-coagulative status because it is not paralleled by an increase in TFPI expression.

Previous studies showed that in addition to transcriptional regulation, TF expression is also controlled at the post-transcriptional level by mRNA degradation. Here we found that the protein synthesis inhibitor cycloheximide induced TF mRNA, which is consistent with a previous report showing that cycloheximide "superinduces" TF mRNA expression and substantially increases the half-life of TF transcript (36, 37). Additional post-transcriptional mechanisms were postulated in control of TF expression; indeed, LPS induces TF up-regulation also via an increased TF mRNA stability (37). In either case, the exact molecular mechanism(s) responsible for increased TF mRNA stability remains unknown. In line with this, we could not exclude the possibility that UTP/ATP-induced TF mRNA up-regulation may be partially due to mRNA stabilization. We wish to dissect the transcriptional and post-transcriptional components in a follow-up study. However, it is obvious that P2Y$_2$ receptor-mediated TF mRNA up-regulation does not require *de novo* protein synthesis, as cycloheximide did not suppress UTP-induced TF mRNA expression in HCAEC.

The MAPK pathways are implicated in TF induction in several systems, but each member of the MAPK family contributes to TF induction in an extracellular stimulus- and/or cell type-dependent manner (17, 23, 25, 38, 39). Consistent with this notion, we observed differential roles of p38, ERK1/2, and JNK in UTP-induced TF expression in HCAEC. Our results indicate that UTP activates individual MAPK members to different extents, and UTP-stimulated TF expression displays differential sensitivity to the inhibition of individual MAPKs, with p38 absolutely required and JNK partially involved and ERK1/2 being dispensable. It has been known that activation of MAPKs stimulates the TF promoter by activating downstream transcription factors such as AP-1, NF-κB, and EGR-1, ultimately resulting in up-regulation of TF mRNA. Unexpectedly, NF-κB pathway, which is the major pathway responsible for TNFα-induced TF expression, is not involved in UTP-stimulated TF expression. It has been known that human TF promoter contains two AP-1 sites and one NF-κB site that mediates TF induction. An EMSA study demonstrated that human endothelial cells contain constitutive AP-1 binding activity, whereas the NF-κB site binds an inducible nuclear complex composed of c-Rel-p65 heterodimers (40). Integrity of both AP-1 sites and the NF-κB site was required for optimal TF induction by TNFα (41). Therefore, a lack of NF-κB pathway activation may account for a less impressive TF induction by UTP/ATP as compared with the effect of TNFα.

[3] L. Ding, W. Ma, and J. Shen, unpublished observation.

Rho A is another important pathway that positively regulates inducible expression of TF in thrombin stimulation of human endothelial cells (42), but the downstream target of Rho A remains unclear. The P2Y$_2$ receptor has been shown to activate Rho A, leading to VCAM-1 induction (18). In the present study, we found that Rho A is also involved in UTP-induced TF expression. More importantly, for the first time we found that inhibition of Rho kinase specifically inhibited activation of JNK, but not p38, suggesting that JNK is a potential new downstream target of Rho A in HCAEC. On the contrary, the PI3K/AKT pathway negatively regulates endothelial TF expression as inhibition of PI3K or its downstream mediators increased TF expression in response to TNFα, histamine, thrombin, and VEGF (14). However, we did not find an involvement of the AKT pathway in P2Y$_2$ receptor-mediated TF induction.

Studies have indicated that P2Y$_2$ receptor-mediated MAPK activation is dependent upon transactivation of the EGF receptor via a Src/Pyk2-dependent pathway (43, 44). However, a study on embryonic fibroblasts derived from Src$^{-/-}$, Pyk2$^{-/-}$, or Src$^{-/-}$Pyk2$^{-/-}$ mice showed that Src and Pyk2 are essential for GPCR-mediated transactivation of EGF receptor but not for GPCR-mediated MAPKs activation (45). Whether the Src kinase is involved in TF induction has not been addressed. In the current study the inhibitor Src-I was used in parallel with PP2 to assess the role of the Src family of protein-tyrosine kinases as recommended (16). We found Src kinase to be the upstream regulator of p38 in control of UTP-induced TF expression. Several lines of evidence support this notion; 1) UTP stimulated phosphorylation of Src and its known substrate FAK, 2) blockade of Src by Src-I or PP2 reduced phosphorylation of p38 but not JNK, and 3) inhibition of Src by Src-I or PP2 completely or greatly reduced TF induction. Thus, we provide strong evidence supporting a new signaling pathway for inducible expression of TF in HCAEC. However, which member of the Src family proteins is responsible for UTP-induced TF expression remains in need of further investigation.

The P2Y$_2$ receptor activates PLC via G_q protein to mediate the production of inositol 1,4,5-trisphosphate, leading to an increase of intracellular Ca^{2+}. We speculated that this classical pathway would have a role in P2Y$_2$ receptor-mediated TF induction. However, we found that U73122, a well established PLC inhibitor, did not affect UTP/ATP-induced activation of p38 and JNK and had no impact on TF induction, although it almost eliminated UTP-induced Ca^{2+} signals. These results indicate that the P2Y$_2$ receptor does not rely on a PLC-dependent mechanism to up-regulate TF in HCAEC. This seems to be a general mechanism for GPCR, as we found that inhibition of PLC by U73122 did not affect thrombin- or histamine-induced TF up-regulation either. Overall, our finding supports a prior study showing that the P2Y$_2$ receptor can signal in a G_q/PLC-independent mechanism in a transfected cell line (35). However, we cannot rule out the contribution of other G proteins, especially for $G_{i/o}$ proteins, as our result clearly showed that pertussis toxin treatment selectively inhibited UTP- but not TNFα-induced TF up-regulation. The interrelationships among $G_{i/o}$ proteins, Src, and Rho kinase will be the focus of our follow-up study.

In summary, we report the first evidence that activation of P2Y$_2$ receptor by UTP/ATP induces TF expression in HCAEC, in which Src/p38 is required for and Rho/JNK, contributes to UTP/ATP-induced TF expression. Our finding suggests that in addition to the platelet P2Y$_{12}$ receptor, non-platelet P2Y receptor(s), *e.g.* the endothelial P2Y$_2$ receptor, is a potential new drug target in prevention and/or treatment of various TF-related diseases such as sepsis, atherothrombosis, and cancers.

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The $P2Y_2$ Nucleotide Receptor Mediates Tissue Factor Expression in Human Coronary Artery Endothelial Cells

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