# ADP Stimulates Human Endothelial Cell Migration via $P2Y_1$ Nucleotide Receptor–Mediated Mitogen-Activated Protein Kinase Pathways

Jianzhong Shen, Paul E. DiCorleto

***Abstract***—Extensive research on the role of ADP in platelet activation led to the design of new anti-thrombotic drugs, such as clopidogrel (Plavix; sanofi-aventis); however, very little is known about the ADP-preferring nucleotide receptors ($P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$) in endothelium. Here, we show that ADP stimulates migration of cultured human umbilical vein endothelial cells (HUVECs) in both Boyden chamber and in vitro wound repair assays. This promigratory effect was mimicked by 2-MeSADP, but not by AMP, and was inhibited by MRS2179 ($P2Y_1$ receptor antagonist) but not by AR-C69931MX ($P2Y_{12/13}$ receptor antagonist). RT-PCR revealed abundant $P2Y_1$, barely detectable $P2Y_{12}$, and absent $P2Y_{13}$ receptor message in these cells. In addition, both ADP and 2-MeSADP, but not AMP, activated the mitogen-activated protein kinase pathways as evidenced by increased phosphorylation of extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK), and p38 kinase. ADP also stimulated phosphorylation of p90RSK, a downstream substrate of phosphorylated ERK1/2, and induced phosphorylation of such transcription factors downstream of the JNK and p38 pathways as c-Jun and activating transcription factor-2. These signaling events were inhibited by MRS2179 but not by AR-C69931MX. Furthermore, blockade of the ERK or JNK pathways by U0126 and SP600125, respectively, abolished ADP- and 2-MeSADP–stimulated HUVEC migration. However, inhibition of the p38 pathway by SB203580 partially suppressed ADP- and 2-MeSADP–induced HUVEC migration. We conclude that ADP promotes human endothelial cell migration by activating $P2Y_1$ receptor–mediated MAPK pathways, possibly contributing to reendothelialization and angiogenesis after vascular injury. **(*Circ Res*. 2008;102:448-456.)**

**Key Words:** nucleotide receptors ■ vascular endothelial cells ■ protein kinases ■ cell migration



Vascular endothelial cell migration and proliferation are vital in many physiological and pathological processes, including organ development, angiogenesis, and healing of the injured endothelium.[1,2] Numerous factors produced within the vascular wall or from the circulating blood can stimulate migration and/or proliferation of endothelial cells through their cognate receptors, which are either receptor tyrosine kinases, such as vascular endothelial growth factor (VEGF), or G protein–coupled receptors, such as thrombin.[2,3] In addition to these well-established factors, extracellular nucleotides are relatively newly defined vasoactive substances in the vasculature.[4–6] ATP and UTP stimulate vascular smooth muscle constriction and proliferation[5,6]; however, in intact arteries, nucleotides usually cause endothelium-dependent relaxation,[5] suggesting that endothelium has 1 or more functional nucleotide receptors. Indeed, among the 8 cloned P2Y nucleotide receptors ($P2Y_{1,\ 2,\ 4,\ 6,\ 11,\ 12,\ 13,\ 14}$),[5] a previous study in human vascular endothelial cells showed significant level of $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ receptor mRNAs. The expression levels of the newly cloned $P2Y_{12,\ 13,\ 14}$ receptors were not investigated.[7] It also has been shown that stimulation of the $P2Y_2$ receptor by ATP or UTP in cultured vascular endothelial cells or smooth muscle cells leads to cell migration.[8,9]

Although much information has been gained regarding the roles of ATP/UTP-sensitive $P2Y_2$ receptor in the vasculature, relatively little is known about the roles for ADP-preferring receptors ($P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$), especially in the endothelium.[5] Given the fact that ADP is the major nucleotide released from activated platelets[5,10] and because the ADP receptor antagonist clopidogrel (Plavix; sanofi-aventis) has been widely used in different cardiovascular and surgical events as a new antithrombotic drug,[11] it is important to understand how ADP affects cellular functions of the endothelium via 1 or more of its receptors. We hypothesized that in vascular endothelial cells, ADP stimulates cell migration via specific P2Y receptor–mediated cell signaling pathways.

Mitogen-activated protein kinases (MAPKs) have been implicated in cell migration in response to various extracellular stimuli.[12] Three major subfamilies of structurally related

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MAPKs have been identified in mammalian cells, which are termed p44/42 MAPK (extracellular-signal regulated kinase [ERK]1/2), p38 MAPK, and c-Jun N-terminal kinase/stress-activated protein kinases (JNK/SAPKs). The relative contribution of these 3 MAPK pathways in cell migration is variable, depending on the type of cell and the applied stimulus.[12] In coronary artery endothelial cells, ATP and UTP stimulate the ERK and p38 pathways via the $P2Y_2$ receptor.[13] However, nothing is known about ADP stimulation of these signaling pathways and their potential linkage to ADP receptor–mediated regulation of endothelial cell migration.

The principle objective of the current study was to determine whether ADP can stimulate endothelial cell migration, and if so, which P2Y nucleotide receptor(s) is responsible for ADP-promoted cell migration. Secondly, we sought to determine whether the MAPK pathways are involved in ADP receptor–mediated cell migration. Our findings demonstrate that in human umbilical vein endothelial cells (HUVECs), ADP stimulates cell migration predominantly through the $P2Y_1$ nucleotide receptor. Further, all 3 MAPK pathways are involved in ADP-stimulated HUVEC migration.

## Materials and Methods

### Cell Isolation and Culture

HUVECs were isolated by trypsin digestion of human umbilical veins as previously described.[14] Isolated HUVECs were maintained in MCDB medium containing 15% fetal bovine serum (FBS), 0.009% heparin, 0.015% endothelial cell growth supplement (Sigma) and used between passages 3 and 5.

### Boyden Chamber Cell Migration Assay

Cell migration was performed with the modified Boyden chamber trans-well system (BD Biosciences). Briefly, pre-coated cell culture inserts having 8 $\mu$m pore size membranes were placed into 24-well plates. Confluent HUVECs were detached by trypsin-EDTA, washed once, and re-suspended in MCDB medium containing 0.2% FBS. Cell suspensions (150 $\mu$L, 10,000 cells) were added to the upper chambers (the inserts) of the Boyden chamber system. The lower chamber was filled with 500 $\mu$L MCDB medium containing 0.2% FBS with or without nucleotides in different concentrations. For checkerboard analysis, ADP or 2-MeSADP was added in either the lower, upper, or both chambers. Cells were stimulated overnight by nucleotides or VEGF, after which the cells remaining on the upper surface of the membrane were removed by cotton swab. Inserts were then washed 3 times with PBS and cells on the underside of the membrane were fixed with cold methanol for 15 minutes and stained with Accustain (hematoxylin solution, Sigma) for 1 hour. The membranes were washed again, removed and mounted on glass slides. Cells were counted in a blinded manner. The assays were performed in triplicate wells for each condition, and each experiment was repeated at least 3 times. For experiments using MAPK pathway inhibitors or P2Y receptor antagonists, cells were seeded into the upper chamber 2 hour before adding the inhibitors or antagonists to both chambers to minimize potential interference of these pharmacological agents with the cellular adhesion process.

### Monolayer Wound-Healing Experiments

To study the effect of ADP on HUVEC chemokinesis (random migration), a wound healing assay was performed following a published method.[15] Briefly, HUVECs were seeded in 6-well plates in MCDB medium containing 15% FBS and grown to confluence. Cells were then serum-starved (0.2% FBS) for 2 hour, and a linear wounding zone was created using a 200 $\mu$L pipette tip. Cells were then washed twice and maintained in MCDB medium (0.2% FBS) for 1 hour, after which the cells were stimulated overnight with nucleotides, VEGF, or vehicle. For the evaluation of "wound closure" in different experimental conditions, the cells were fixed and stained as described for the Boyden chamber assay. The migrated cells were counted in the wounding zone determined by a predefined frame. The experiments were performed in triplicate and repeated at least 3 times.

### Western Blot Analysis of Protein Phosphorylation

HUVECs were seeded in 6-well plates for 48 hours in MCDB medium containing 15% FBS, after which the cells were serum-starved (0.2% FBS in MCDB) for 3 hours at 100% confluence. The quiescent HUVECs were pretreated with kinase inhibitors or receptor antagonists for 30 minutes before stimulation with different agonists at the indicated concentration and time. After stimulation, cells were lysed and a standard western blotting was performed as previously described.[6,16,17] The individual primary antibodies used were anti-p-ERK1/2, anti-p-JNK, and anti-p-p38 (1:1000 dilutions, Cell Signaling). Equal protein loading was verified by stripping off the original antibodies, and the membranes were reprobed with the primary antibody anti-tubulin (1:1000, Sigma) or anti–total ERK, JNK, or p38 (1:1000, Cell Signaling).

### RT-PCR Analysis

Total RNA and DNA were extracted from cultured HUVECs using the RNeasy and DNeasy kits, respectively (Qiagen). For the synthesis of the first strand of cDNA, 1 $\mu$g 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 U *Taq* DNA polymerase (Roche). Sets of primers used for detecting mRNAs for individual ADP-preferring P2Y receptors were: $P2Y_1$ forward, 5′-GTGCTGGTGTGGCTCATTG-TGGT-3′; $P2Y_1$ reverse, 5′-GGCTTGTATCTCCATTCTGCT-TGA-3′ (yielding a 600 bp product); $P2Y_{12}$ forward, 5′-CAAGCCGTCGACAACCTCACCTC-3′; $P2Y_{12}$ reverse, 5′-TCTCGGCTGCCTGTTGGTCAGAA-3′ (yielding a 500 bp product); $P2Y_{13}$ forward, 5′-TGACCGGCATCCTGCTGAATA-CTT-3′; and $P2Y_{13}$ reverse, 5′-CGATGGTGTTGCTTCCTTGTT-GCT-3′ (yielding a 400 bp product). The PCR condition was for 40 cycles of the following: jump start for 2 minutes at 95°C, denaturation for 1 minute at 95°C, annealing for 1 minute at 56°C, and extension at 72°C for 1 minute. The resulting PCR products were resolved on a 1.5% agarose ethidium bromide gel, and the amplified bands were visualized with ultraviolet light, after which the PCR products were purified,[6,16] and sequence identity was confirmed by sequencing.

### Materials

The following compounds were purchased from TOCRIS: 2-methylthioadenosine diphosphate (2-MeSADP) and MRS2179. ADP, 5′-AMP, and adenosine were purchased from Sigma. U0126 was from Cell Signaling. SP600125 and SB203580 were from Calbiochem. AR-C69931MX was from The Medicines Company.

### Data Analysis

Data are expressed as means±SEM. Means of 2 groups were compared using Student's *t* test (unpaired, 2-tailed), and 1-way ANOVA was used for comparison of more than 2 groups, with $P<0.05$ considered to be statistically significant. Unless indicated in the figure legends, all the experiments were performed at least 3 times with similar results.

## Results

### Effect of ADP and 2-MeSADP on HUVEC Migration

To determine whether ADP is a pro-migratory factor in human vascular endothelial cells, we stimulated cultured HUVECs in a Boyden chamber system as illustrated in Figure 1A. By counting the number of cells that migrated beneath

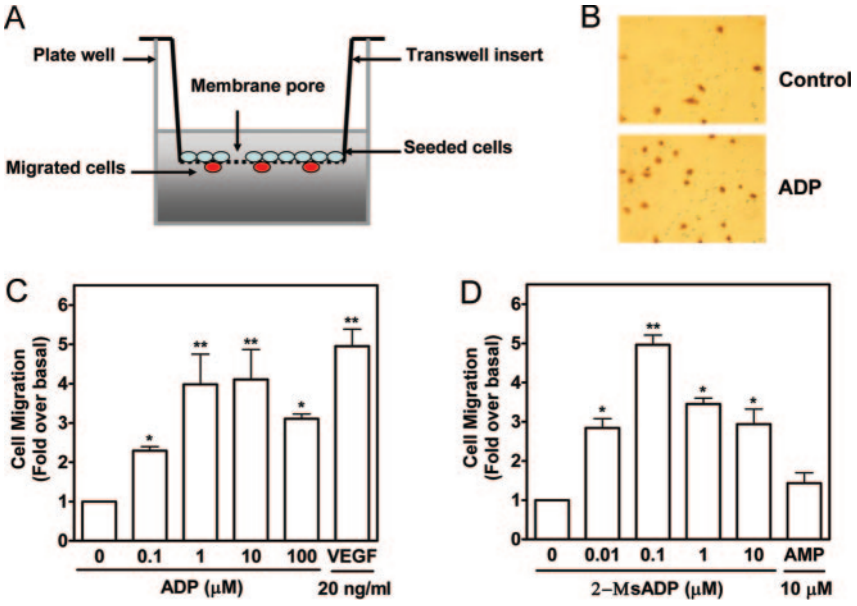


**Figure 1.** ADP and 2-MeSADP stimulation of HUVEC migration. A, Cell migration was determined in the Boyden chamber system after seeding HUVECs in the upper insert and addition of the agonists in the lower chamber. B, The cells migrated beneath the membrane were fixed and stained. C and D, The effects of ADP and VEGF (C) and 2-MeSADP and AMP (D) on cell migration, at the indicated concentrations, were determined after overnight incubation. Number of migrated cells in control: 148±21 (C); 118±18 (D). *$P<0.05$, **$P<0.01$ relative to the respective control.

the membrane (Figure 1A and 1B), we observed that ADP stimulated HUVEC migration in a concentration-dependent manner, with a threshold concentration at 0.1 μmol/L and peaking at 1 to 10 μmol/L (Figure 1C). Further increase of the concentration up to 100 μmol/L decreased the level of cell migration (Figure 1C). Notably, the efficacy of ADP was comparable to that induced by a maximal concentration of VEGF (Figure 1C). Because ADP is known to be degraded to AMP by CD39 expressed by vascular endothelial cells,[5] we further tested whether the promigratory effect of ADP was mediated by ADP itself or by its degradation products. Figure 1D shows that 2-MeSADP stimulated HUVEC migration more potently than ADP itself and AMP did not mimic the effect of ADP up to 10 μmol/L. These results indicate that ADP is a stimulatory factor for HUVEC migration.

## Chemotactic Versus Chemokinetic Effects of ADP and 2-MeSADP on HUVECs

To examine whether the promigratory effect of ADP was attributable to chemotaxis and/or chemokinesis, we performed a checkerboard study using the Boyden chamber system. As shown in Figure 2A, when ADP and 2-MeSADP were added to both the upper and the lower chambers to eliminate the concentration gradient, the promigratory effects of ADP and 2-MeSADP were significantly decreased but not abolished as compared with when only the lower chambers had the agonists. This result suggests that ADP- and 2-MeSADP–stimulated HUVEC migration involves both chemotaxis and chemokinesis. Interestingly, 2-MeSADP added into the upper chamber also stimulated HUVEC migration, which was not mimicked by ADP (Figure 2A). To

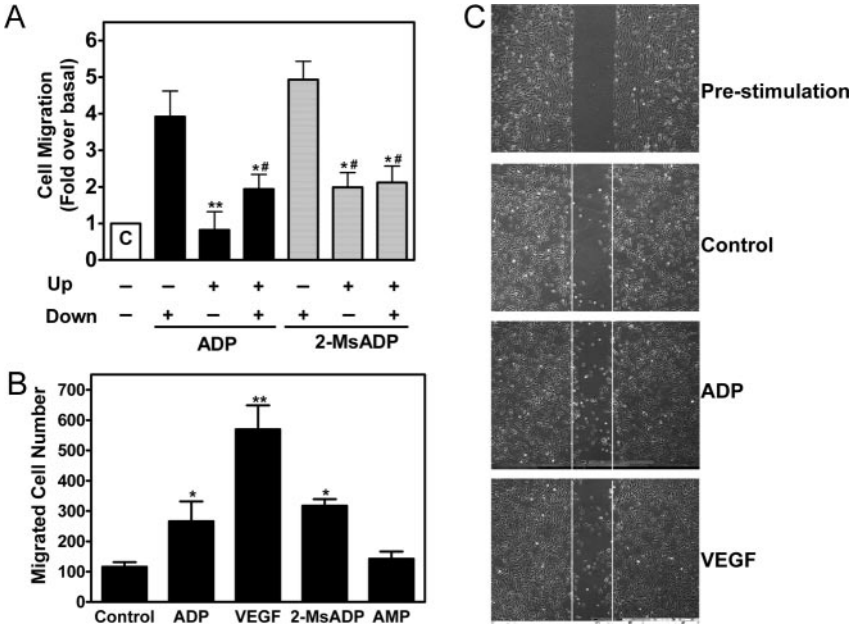


**Figure 2.** Chemotactic vs chemokinetic effects of ADP and 2-MeSADP on HUVECs. A, Checkerboard analysis in the Boyden chamber system in which ADP (10 μmol/L) and 2-MeSADP (0.1 μmol/L) were placed in the bottom, top, or both chambers as indicated. The effects of ADP and 2-MeSADP on cell migration were determined after overnight incubation. Number of migrated cells in control: 144±32. *$P<0.05$, **$P<0.01$ relative to the agonists placed in the bottom; #$P<0.05$ relative to the control without agonist. B, Representative microphotographs of wound repair assay. C, After wounding of the HUVEC monolayer, cells were stimulated with vehicle (control), ADP, AMP (both 10 μmol/L), 2-MeSADP (0.1 μmol/L), or VEGF (20 ng/mL) overnight, after which cells migrated into the denuded area were counted. *$P<0.05$, **$P<0.01$ relative to control.

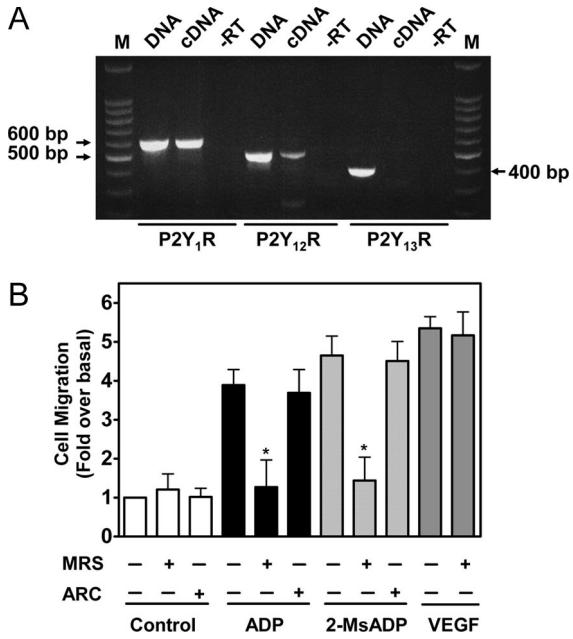


**Figure 3.** ADP receptor expression and function in HUVEC migration. A, mRNA expression for the 3 ADP-sensitive P2Y receptors was determined by RT-PCR analysis in HUVECs. -RT indicates RT-PCR performed without reverse transcriptase. DNA was used as a positive control. B, Effects of MRS2179 (MRS) (10 μmol/L) and AR-C69931MX (ARC) (10 μmol/L) on basal (control), 10 μmol/L ADP, 0.1 μmol/L 2-MeSADP, and 20 ng/mL VEGF-stimulated HUVEC migration. Number of migrated cells in control: 162±20. *$P<0.01$ relative to the respective controls.

confirm the involvement of chemokinesis, we performed a wound repair assay by scratching the confluent monolayer HUVECs (Figure 2B). Stimulation of the cells with either ADP or VEGF after "injury" significantly increased cell migration into the "wounded" zone (Figure 2B and 2C). Consistent with the Boyden chamber assay, the effect of ADP was mimicked by 2-MeSADP but not by AMP (Figure 2C).

## Role for the $P2Y_1$ Receptor in ADP- and 2-MeSADP–Stimulated HUVEC Migration

To characterize the receptor that mediates ADP-stimulated cell migration, we performed RT-PCR analysis and found that HUVECs expressed high levels of the ADP-preferring $P2Y_1$ nucleotide receptor (Figure 3A). A very low level of the $P2Y_{12}$, but not the $P2Y_{13}$, ADP-selective receptor message was also detectable after a 40-cycle amplification (Figure 3A). This result suggests that HUVECs may express predominantly the $P2Y_1$ receptor. To assess whether the $P2Y_1$ receptor plays a role in ADP-stimulated cell migration, HUVECs were pretreated with MRS2179 or AR-C69931MX, $P2Y_1$- and $P2Y_{12/13}$-selective antagonists,[5] respectively, before cell stimulation. Figure 3B shows that MRS2179 abolished ADP- and 2-MeSADP–stimulated, but not VEGF-stimulated, cell migration. In contrast, the effects of ADP and 2-MeSADP were not affected by AR-C69931MX. Together, these results indicate a key role of the $P2Y_1$ receptor.

## Effects of ADP and 2-MeSADP on ERK1/2, JNK, and p38 MAPK Pathways in HUVECs

To define the potential cell signaling pathways relevant to ADP-stimulated HUVEC migration, we stimulated the cells with ADP and observed that ADP dose-dependently increased the phosphorylation levels of ERK1/2 and JNK, with similar potency and efficacy (Figure 4B). Specifically, significant phosphorylation of both ERK1/2 and JNK were induced by 0.1 μmol/L ADP, and maximal phosphorylation (≈3.5-fold) was observed at 100 μmol/L (Figure 4B). ADP also increased the phosphorylation level of p38 with similar potency (apparent EC50: ≈1 μmol/L) but less efficaciously

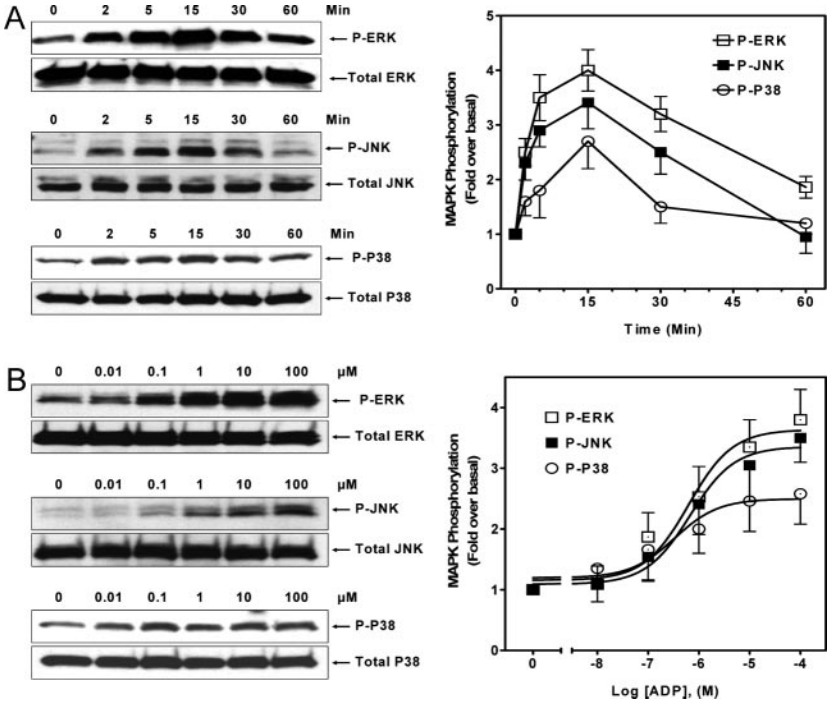


**Figure 4.** ADP stimulation of ERK1/2, JNK, and p38. The time course (A) and dose–response curves (B) of ERK1/2, JNK, and p38 phosphorylations were determined by Western blot analysis after HUVECs were stimulated with ADP at the indicated times and concentrations. Representative blots are shown on the left, and group data averages are shown on the right. Equal loading was confirmed by reprobing the membranes with anti–total ERK, JNK, or p38 antibodies.

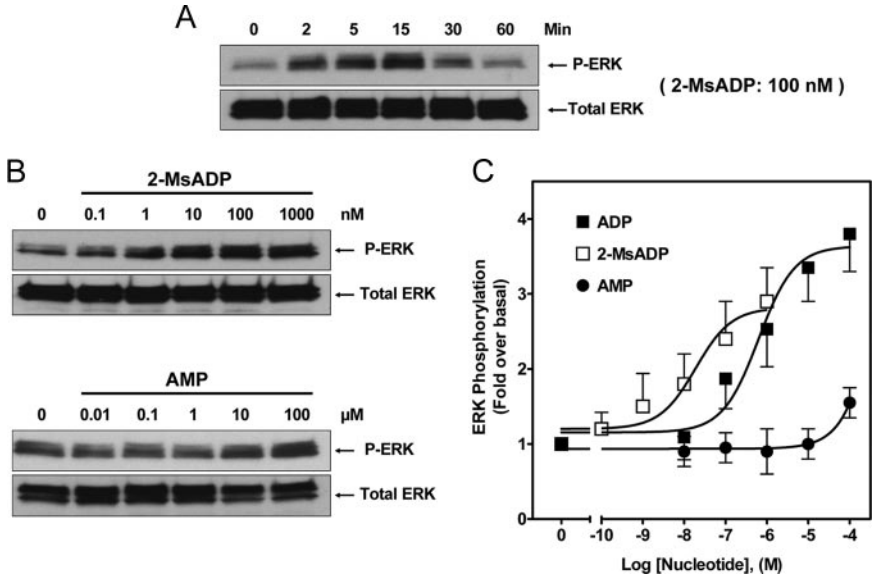


**Figure 5.** Differential effects of 2-MeSADP and AMP on ERK phosphorylation. A and B, The cellular phosphorylation levels of ERK1/2 were determined by Western blot analysis after HUVECs were stimulated with 2-MeSADP for the indicated times (A) or with 2-MeSADP and AMP at the indicated concentrations (B). C, Group data averages from B were shown on C. For comparison, data points for ADP in C were copied from Figure 4B. Equal loading was confirmed by reprobing the membranes with anti–total ERK.

(Figure 4B). Figure 4A shows that the effect of ADP on MAPK phosphorylation was time-dependent, with peak activation at 15 minutes. The same time course was observed when the cells were stimulated by 2-MeSADP (Figure 5A). In addition, 2-MeSADP largely mimicked the effect of ADP on ERK1/2 activation (Figure 5B and 5C). In contrast, AMP, up to 10 $\mu$mol/L, did not significantly increase ERK1/2 phosphorylation (Figure 5B and 5C). Because phosphorylation of ERK, JNK, and p38 strongly correlates with their kinase activity, these results indicate that ADP can activate all 3 MAPK pathways in HUVECs.

## Effects of ADP on the Downstream Targets of MAPK Pathways in HUVECs

To further confirm activation of the MAPK pathways by ADP, we determined the phosphorylation status of p90RSK, a downstream substrate of activated ERK1/2, and the 3 transcription factors (Elk1, c-Jun and activating transcription factor [ATF]-2) known to be phosphorylated by activated ERK1/2, JNK, and p38. Figure 6A through 6C shows that stimulation of the cells with ADP caused transient increases in the phosphorylation levels of p90RSK, ElK1, and c-Jun, with a time course consistent with MAPK activation. Interestingly, ATF-2 was persistently activated by ADP up to 60 minutes (Figure 6D). These results provide further evidence that ADP activates all 3 MAPK pathways in HUVECs.

## Role for the $P2Y_1$ Receptor in ADP- and 2-MeSADP–Stimulated MAPK Pathways

To investigate the role of $P2Y_1$ receptor in ADP signaling, HUVECs were pretreated with the receptor antagonists MRS2179 or AR-C69931MX before stimulation by the agonists. Figure 7A shows that ADP-induced ERK1/2 phosphorylation was completely blocked by MRS2179 but not by AR-C69931MX. In addition, MRS2179 also concentration-dependently inhibited 2-MeSADP–induced ERK1/2 phosphorylation (Figure 7B). Similarly, ADP-induced JNK and p38 phosphorylations were blocked by MRS2179, but not by AR-C69931MX (Figure 7C and 7E), and 2-MeSADP–induced JNK and p38 phosphorylations were suppressed by MRS2179 in a does-dependent manner (Figure 7D and 7F). Together, these results clearly indicate that the $P2Y_1$ receptor plays a central role for ADP signaling via the MAPK pathways.

## Role of MAPK Pathways in ADP- and 2-MeSADP–Stimulated HUVEC Migration

Finally, we investigated whether the 3 MAPK pathways were involved in ADP-promoted cell migration. As shown in

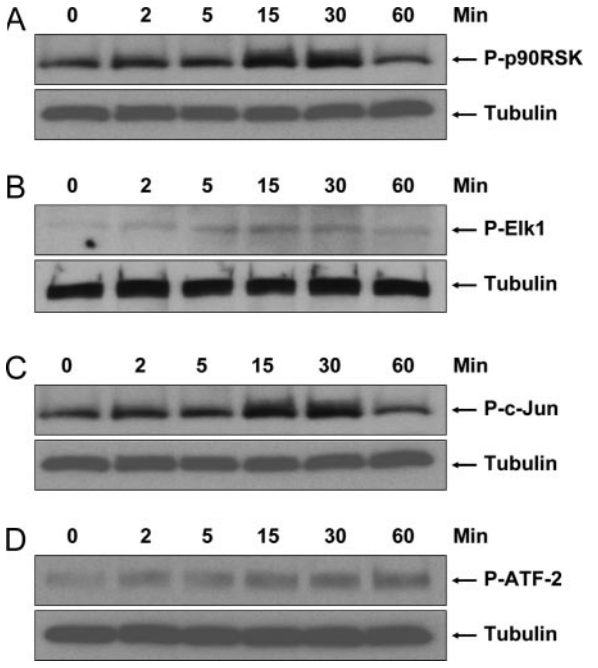


**Figure 6.** Effects of ADP on the downstream targets of MAPK pathways in HUVECs. The cellular phosphorylation levels of p90RSK (A), Elk1 (B), c-Jun (C), and ATF-2 (D) were determined by Western blot analysis after HUVECs were stimulated with 10 $\mu$mol/L ADP for the indicated times. Equal loading was confirmed by reprobing the membranes with anti-tubulin. Data shown are the representative of results from 3 independent experiments.

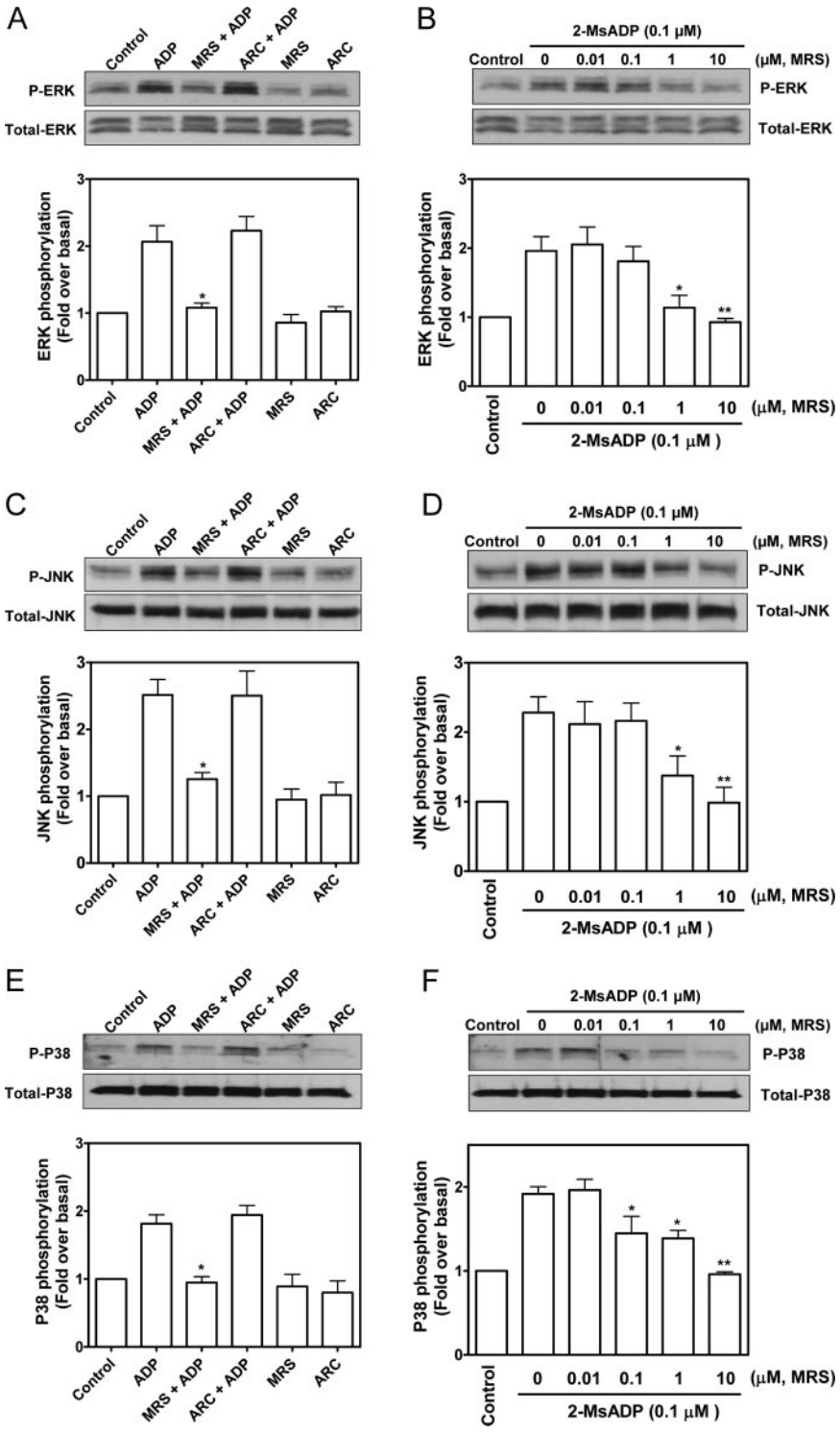


**Figure 7.** Role of $P2Y_1$ receptor in ADP and 2-MeSADP stimulation of MAPK pathways. The cellular phosphorylation levels of ERK1/2 (A and B), JNK (C and D), and p38 (E and F) were determined by Western blot analysis after HUVECs were stimulated with ADP (10 $\mu$mol/L) or 2-MeSADP (0.1 $\mu$mol/L) for 15 minutes in the absence or presence of MRS2179 (MRS) or AR-C69931MX (ARC) (each 10 $\mu$mol/L in A; pretreatment time, 30 minutes). Representative blots are shown above the group data averages. Equal loading was confirmed by reprobing the membranes with anti–total ERK1/2, JNK, or p38. *$P<0.05$, **$P<0.01$ relative to the respective controls.

Figure 8A through 8C, we first confirmed that ADP- and 2-MeSADP–induced phosphorylation of ERK1/2, JNK, and p38 was effectively inhibited by the established specific kinase inhibitors U0126, SP600125, and SB203580, respectively. At the same concentration, U0126 and SB203580 did not inhibit basal cell migration; however, SP600125 inhibited basal cell migration by ≈50% (Figure 8D). In addition, both ADP- and 2-MeSADP–stimulated cell migration was eliminated by U0126 or by SP600125 (Figure 8D). However, suppression of the p38 pathway by SB203580 only partially inhibited ADP- or 2-MeSADP–stimulated cell migration. These results suggest differential roles of the 3 MAPK pathways in ADP-promoted HUVEC migration.

## Discussion

In the present study, we show for the first time that ADP, the major nucleotide released from activated platelets, stimulates HUVEC migration involving both chemotaxis and chemoki-

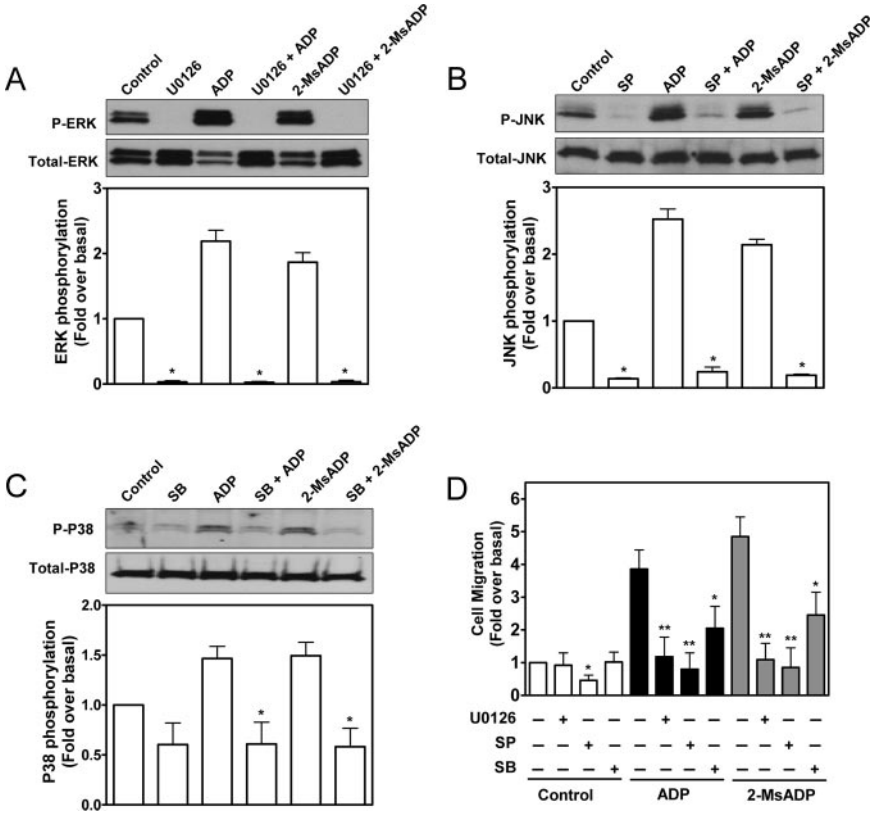


**Figure 8.** Effects of protein kinase inhibitors on ADP and 2-MeSADP stimulation of MAPK pathways and cell migration. A through C, The cellular phosphorylation levels of ERK1/2 (A), JNK (B), and p38 (C) were determined by Western blot analysis after HUVECs were stimulated with ADP (10 $\mu$mol/L) or 2-MeSADP (0.1 $\mu$mol/L) for 15 minutes in the absence (control) or presence of 10 $\mu$mol/L U0126, SP600125 (SP), or SB203580 (SB) (pretreatment time, 30 minutes). Representative blots are shown above the group data averages. *$P<0.01$ relative to the respective controls. D, Effects of U0126, SP600125 (SP), and SB203580 (SB) (each 10 $\mu$mol/L) on basal (control), 10 $\mu$mol/L ADP-stimulated, and 0.1 $\mu$mol/L 2-MeSADP–stimulated HUVEC migration. Number of migrated cells in control: 135±25. *$P<0.05$, **$P<0.01$ relative to the respective controls.

nesis. We also show that ADP activates MAPK signaling pathways including ERK, JNK, and p38. ERK and JNK are required for and p38 contributes to ADP-stimulated HUVEC migration. Furthermore, we have demonstrated that the $P2Y_1$ receptor mediates ADP stimulation of MAPK pathways and endothelial cell migration.

The role of ADP in platelet activation has been known for decades. Briefly, it has been demonstrated that the dense granules of platelets store ADP at a concentration as high as 653 mmol/L.[10] On stimulation by an agonist such as thrombin, platelets release ADP, which binds to 2 types of P2Y nucleotide receptors, $P2Y_1$ and $P2Y_{12}$, causing a shape change and full aggregation of the platelets, respectively.[18] Characterization of the ADP receptors and their functions in platelets led to the design of new anti-thrombotic drugs targeting the $P2Y_{12}$ receptor, such as clopidogrel. Despite extensive research in platelets, relatively little is known about the role of ADP receptors in vascular endothelium. Well before cloning any of the purinergic or nucleotide receptors, Van Coevorden and Boeynaems showed that micromolar concentrations of ADP stimulated the release of prostaglandin $(PG)I_2$ from cultured bovine aortic endothelial cells.[19] Recently, it has been reported that ADP induces endothelium-dependent relaxation of several arteries, including rat mesenteric artery,[20] guinea pig, and mouse aorta[21,22] via NO release. These studies suggested that ADP could exert 2 opposite actions on platelet aggregation in vivo: a direct stimulation and an indirect inhibition mediated by $PGI_2$ and NO released from the endothelium. In the present study, we have found using a Boyden chamber system that ADP stimulates HUVEC migration in a concentration range that is consistent with ADP stimulation of $PGI_2$ and NO release. In addition, this promigratory effect was fully mimicked by 2-MeSADP but not by AMP, suggesting that ADP acts as a promigratory factor in endothelial cells, a novel cellular function for ADP. We also observed that when the agonist concentration gradient was eliminated in the Boyden chamber system, ADP- and 2-MeSADP–stimulated HUVEC migration was decreased but not abolished, indicating that ADP stimulation of HUVEC migration may involve both chemotaxis and chemokinesis. This view was strongly supported by our in vitro wound repair assay showing that addition of ADP or 2-MeSADP, but not AMP, increased cell migration into the "wound." Thus, in addition to regulating vascular contractility and platelet activation, our study has identified a new role for ADP in vascular endothelial cells: promoting cell migration.

Extracellular nucleotide stimulation of cell migration has received increasing attention; however, previous studies in this field have focused on the ATP- and UTP-sensitive $P2Y_2$ receptor in different cells, including vascular smooth muscle cells,[8] endothelial cells,[9] astrocytes,[23] and more recently neutrophils.[24] Because ADP shares different receptors with ATP/UTP,[5] it remained to be determined whether ADP receptors are equally important in cell migration. In the present study, we have shown that ADP-stimulated HUVEC migration is mimicked by 2-MeSADP, a stable analog specifically targeting ADP-sensitive receptors, and is totally blocked by MRS2179, a highly selective $P2Y_1$ receptor antagonist.[5] These results indicate that the $P2Y_1$ receptor plays an essential role in ADP stimulation of HUVEC migration. Other evidence in support of this view is from our RT-PCR analysis showing that HUVECs express abundant

$P2Y_1$, barely detectable $P2Y_{12}$, and absent $P2Y_{13}$ receptor messages. In addition, we found that AR-C69931MX, a selective $P2Y_{12/13}$ receptor antagonist,[5] did not affect ADP-stimulated cell migration. We believe that this is the first report showing a stimulatory role for the $P2Y_1$ receptor in cell migration.

Little is known about ADP receptor signaling in vascular endothelial cells. Luckhoff and Busse showed that adenine nucleotides including ADP induce an increase in intracellular $Ca^{2+}$ in bovine aortic endothelial cells.[25] This has been confirmed by Boeynaems and colleagues, who showed that both ATP and ADP increase intracellular inositol trisphosphate ($IP_3$) in the same type of endothelial cell,[26] suggesting the involvement of Gq-coupled nucleotide receptors, which have been characterized as $P2Y_1$ for ADP and $P2Y_2$ for ATP.[27] Recent studies have revealed that like many other G protein–coupled receptors, activation of the $P2Y_2$ receptor by ATP or UTP in coronary artery endothelial cells activates the MAPK pathways, including ERK1/2 and p38.[13] Whether the $P2Y_1$ receptor also signals via MAPK pathways in endothelial cells had not been previously addressed. In the current study, we found that stimulation of HUVECs by ADP increased phosphorylation levels of ERK1/2, JNK, and p38 in a time- and dose-dependent manner, suggesting that all 3 MAPK pathways can be activated by the $P2Y_1$ receptor. Several lines of evidence support this notion. (1) ADP stimulated the phosphorylation of p90RSK, a downstream target of activated ERK1/2 and also activated several transcription factors known to be phosphorylated by MAPK pathways, including Elk1, c-Jun, and ATF-2. (2) The effect of ADP was fully mimicked by 2-MeSADP but not by AMP. (3) ADP-induced activation of ERK1/2, JNK, and p38 was inhibited by $P2Y_1$-selectve antagonist (MRS2179) but not by AR-C69931MX ($P2Y_{12/13}$ antagonist). (4) MRS2179 also concentration-dependently suppressed 2-MeSADP–induced MAPK phosphorylation. Thus, we have provided compelling evidence supporting a new role for the $P2Y_1$ receptor in signaling to the MAPK pathways in endothelial cells. Cloned recombinant $P2Y_1$ receptor expressed in human 1321N1 astrocytoma cells coupled to the ERK1/2 and JNK, but not the p38, pathways.[28] The reason for this apparent discrepancy is unclear, but it is possible that different cell types may exhibit different specificity of $P2Y_1$ receptor signaling. Alternatively, the native $P2Y_1$ receptor in HUVECs may have intrinsic properties not shared by the recombinant receptor, such as dimerization with another endogenous receptor, which has been reported in other system for the $P2Y_1$ receptor.[29] It should be noted that the ATP/UTP-sensitive $P2Y_2$ receptor also mediates HUVEC migration, which has been shown to be associated with increased phosphorylation of FAK and paxillin and dependent on phosphatidylinositol 3-kinase.[9] It remains to be determined whether the $P2Y_1$ receptor also activates these pathways to promote cell migration.

The MAPK pathways have been implicated in cellular migration in a number of systems, but each member of the MAPK family contributes to cell motility in an extracellular stimulus- and/or cell type–dependent manner.[12] Consistent with this notion, we observed differential roles of ERK1/2, JNK, and p38 in HUVEC migration. Under basal conditions, suppression of JNK, but not ERK1/2 and p38, caused a significant decrease of cell migration. This is not attributable to a nonspecific toxicity of the JNK inhibitor SP600125, as evaluated by lactate dehydrogenase release assay (data not shown). Indeed, this result is consistent with the recent report showing that JNK phosphorylation of paxillin and c-Jun is essential for basal migration of some cell types.[30,31] Interestingly, we also found that inhibition of either ERK1/2 or JNK pathways abolished ADP- and 2-MeSADP–stimulated cell migration. However, the promigratory effect of ADP and 2-MeSADP was only partially inhibited by blocking the p38 pathway. Thus, our results indicate that ADP coordinately activates individual MAPK members to different extents, and ADP-stimulated cell migration displays differential sensitivity to inhibition of individual MAPKs, with ERK1/2 and JNK being absolutely required and p38 being partial involved. This pattern of inhibition is consistent with a recent study showing that endothelin-1–induced brain endothelial cell migration also requires ERK1/2 and JNK with partial contribution from p38.[32] Whether this is a general mechanism for endothelial cell migration merits further investigation. It is also intriguing to know how the $P2Y_1$ receptor regulates the complex machinery of cell motility through the individual MAPK pathways.

In summary, we report the first evidence that in human vascular endothelial cells ADP functions as a chemotactic and chemokinetic factor through activation of the MAPK pathways, of which ERK1/2 and JNK are required and p38 contributes for ADP-stimulated cell migration. Also, the $P2Y_1$ receptor is primarily responsible for ADP stimulation of promigratory signaling and cell migration. These findings suggest that ADP released from activated platelets may be a beneficial factor in the process of reendothelialization and angiogenesis after vascular injury. This concept was supported by a recent study showing that activated platelets stimulate chemotaxis of endothelial progenitor cells, although the role of ADP was not addressed.[33] In addition, identification of $P2Y_1$ as the key receptor responsible for ADP signaling and promigration in endothelial cells may also have significant implications for translational research in this field. Given the fact that activation of the $P2Y_1$ receptor by ADP causes release of $PGI_2$ and NO and promotes endothelial cell migration, the use of a $P2Y_1$ receptor blocker as an antithrombotic drug, as recently proposed,[34] may have its drawbacks. However, future studies using $P2Y_1$-null mice may provide a better understanding of the roles of this receptor in endothelium. It will also be important to know the relative expression levels of $P2Y_1$ versus $P2Y_{12/13}$ receptor in normal and regenerating endothelium. Local delivery of a $P2Y_1$-selective agonist may be a viable approach to promoting reendothelialization and angiogenesis.

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## Disclosures

None.

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