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P2Y11 Impairs Cell Proliferation by Induction of Cell Cycle Arrest and Sensitizes Endothelial Cells to Cisplatin-Induced Cell Death

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ABSTRACT

Extracellular ATP mediates a wide range of physiological effects, including cell proliferation, differentiation, maturation, and migration. However, the effect of ATP on cell proliferation has been contradictory, and the mechanism is not fully understood. In the current study, we found that extracellular ATP significantly inhibited the proliferation of human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs). Treatment with ATP did not induce cell apoptosis but instead induced cell cycle arrest in S phase. ATP induced the phosphorylation of ERK1/2, but the ERK inhibitors, U0126 and PD9809, did not regulate the inhibition of cell proliferation induced by ATP. However, ATP-induced inhibition of cell proliferation was blocked by suramin, a nonspecific antagonist of the P2Y receptors, and endothelial cells expressed P2Y11, a P2Y receptor that specifically binds ATP. Moreover, the down-regulation of P2Y11 by RNA interference not only reversed the inhibition of cell proliferation but also ameliorated cell cycle arrest in S phase. In addition, P2Y11 sensitized endothelial cells to cisplatin-induced cell death by down-regulation of the expression of Bcl-2. Taken together, these results suggest that extracellular ATP impairs cell proliferation by triggering signaling to induce cell cycle arrest and sensitizes cell to death via P2Y11 in endothelial cells. J. Cell. Biochem. 112: 2257–2265, 2011.

KEY WORDS: P2Y11; ATP; ENDOTHELIAL CELLS; CELL PROLIFERATION; CELL CYCLE; CELL DEATH; CISPLATIN

Nucleotides serve as the substrates for nucleic acid synthesis and are also a class of ubiquitous and potent extracellular signaling molecules [Abbracchio et al., 2006]. In the cardiovascular system, nucleotides are released during ischemia, trauma, inflammation, cell necrosis, and shear stress from endothelial cells, smooth muscle cells, aggregating platelets, and inflammatory cells, and they function to regulate proliferation, differentiation, chemotaxis, cytokine production, and reactive oxygen generation [Di Virgilio et al., 2001]. These effects are mediated through a specific class of plasma membrane receptors called purinergic P2 receptors, which are subdivided into two distinct categories, the metabotropic G protein-coupled (P2Y) receptors and the ionotropic ligand-gated channel (P2X) receptors [Di Virgilio et al., 2001; Rong et al., 2003; Abbracchio et al., 2006]. P2Y receptors are seven-transmembrane-spanning G protein-coupled receptors (GPCRs). There are eight mammalian metabotropic P2Y receptors, including P2Y1, 2, 4, 6, 11, 12, 13, and 14 [Abbracchio et al., 2006], that differ pharmacologically in their selectivity for nucleotide ligands. For example, P2Y2 binds both ATP and UTP; P2Y4 preferentially binds UTP, P2Y11 binds ATP and P2Y6 prefers UDP; P2Y1, P2Y12, and P2Y13 all bind ADP, whereas P2Y14 binds UDP-glucose [Abbracchio et al., 2006].

It has been reported that endothelial cells express several subtypes of functional P2Y receptors that regulate endothelial cell migration and proliferation [Van Daele et al., 1992; Ehring et al., 2000; Shen and DiCorleto, 2008]. P2Y receptors have also been reported to be expressed on other cell types, but their effects on cell proliferation are contradictory. In mouse embryonic stem cells, ATP has been shown to stimulate cell proliferation via protein kinase C, PI3K, and ERK [Heo and Han, 2006]. However, in bone marrow-derived human mesenchymal stem cells, ATP is spontaneously

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released at early stages of culture and decreases cell proliferation [Coppi et al., 2007]. P2Y receptors have also been demonstrated to stimulate proliferation of vascular smooth muscle cells [Taurin et al., 2008], Caco-2 human colonic cancer cells [Buzzi et al., 2010] and human breast cancer MCF-7 cells [Bilbao et al., 2010]. In contrast, P2Y receptors have been shown to inhibit cell proliferation in the human lung adenocarcinoma cell line LXF-289 [Schafer et al., 2006], and in primary neurospheres from the adult mouse subventricular zone [Stafford et al., 2007]. In bovine aortic endothelial cells, ATP induced the proliferation of cells cultured in the absence of serum but did not enhance the proliferation of cells cultured in the presence of serum [Van Daele et al., 1992].

In the current study, we found that human endothelial cells expressed P2Y receptors and the activation of P2Y11 by ATP inhibited cell proliferation, which was due to the induction of cell cycle arrest. Moreover, ATP enhanced the sensitivity of endothelial cells to cisplatin via down-regulation of Bcl-2. These results suggested that extracellular ATP is an important regulator of endothelial cell regeneration and may play an important role in angiogenesis.

MATERIALS AND METHODS

ANTIBODIES AND REAGENTS

ATP and UTP were purchased from Sigma–Aldrich (St. Louis, MO). Rabbit anti-human P2Y2 and P2Y11 antibodies and shRNA plasmids containing sequences targeting human P2Y2 and P2Y11 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human total ERK and phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA).

CELL CULTURE

Primary human aortic endothelial cells (HAECs, ScienCell) were plated on culture dishes pre-coated with 10 ng/ml fibronectin (Millipore) and cultured in endothelial cell medium (ECM, ScienCell) supplemented with 5% fetal calf serum (FCS), 1% endothelial cell growth supplement (ECGS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were used from passages three to six in all experiments. Immortalized human umbilical vein endothelial cells (HUVECs) were purchased from ATCC (Manassas, VA) and cultured in DMEM containing 10% FCS and antibiotics. All cells were cultured in a humidified atmosphere with 5% CO_2 at 37°C.

CCK-8 CELL PROLIFERATION ASSAY

Cell proliferation was determined using a cell counting kit-8 (CCK-8) (Dojido, Kumamoto, Japan) assay according to the manufacturer's instructions. Briefly, 2,000–5,000 cells in 100 μl of medium were plated on a 96-well plate and cultured with or without nucleotides for 1–6 days. After the incubation period, 10 μl of CCK-8 was added to each well, and cells were further incubated for 1 h at 37°C. Absorbance was then measured at 450 nm using a microplate reader (PerkinElmer, USA).

FLOW CYTOMETRIC ANALYSIS

Cell death was detected by fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) staining. Briefly, $1–2 \times 10^6$ cells were washed twice with PBS, then labeled with FITC-annexin V and PI in binding buffer according to manufacturer's instructions. Fluorescence signals were detected on a FACScan (BD Bioscience, San Jose, CA). The log of FITC-annexin V-fluorescence was displayed on the x-axis, and the log of PI fluorescence was displayed on the y-axis. For each analysis, 10,000 events were recorded.

Cell cycle status was analyzed using propidium iodide (PI) staining. Briefly, $1–2 \times 10^6$ cells were synchronized in serum-free medium and incubated with or without various concentrations of nucleotides. After two washes with ice-cold PBS, the cells were fixed in ethanol overnight at 4°C and incubated with a mixture of 50 μg/ml PI (Sigma–Aldrich) and 25 μg/ml RNase A (Sigma–Aldrich) at 37°C for 30 min. The level of PI fluorescence was measured with a FACScan, and the proportion of cells in Go/G_1, S, and G_2/M phases was measured.

SEMI-QUANTITATIVE RT-PCR

Total RNA was extracted from 1 to 5×10^6 cells using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. mRNA was reverse transcribed with RevertAid (MBI Fermemtas, Burlington, ON, Canada) at 42°C for 60 min, and the resulting cDNA was subjected to PCR (94°C for 1 min followed by 25–30 cycles at 94°C for 30 s, 60°C for 30 s, 68°C for 90 s and an extension cycle for 10 min at 68°C). PCR products were separated on 1.0% agarose gels and visualized with ethidium bromide. Forward and reverse primer pairs are listed (5′–3′) as follows:

A1-F: TATTTACAGGCTGGCTCAGG
A1-R: ATTTTCCCAGCCTCCGTTTT
Bcl-2-F: CGTTTGGCAGTGCAATGGT
Bcl-2-R: TTCTTGATTGAGCGAGCCTT
Bcl-w-F: AAGCTGAGGCAGAAGGGTTAT
Bcl-w-R: TGTCCTCACTGATGCCCAGTT
Bcl-xl-F: CCTACCTGATTGGTGCAACC
Bcl-xl-R: TAGGGTGGTGCTGAACTGCAA
GAPDH-F: AATCCCATCACCATCTTCCA
GAPDH-R: CCTGCTTCACCACCTTCTTG
Mcl-1-F: TGTCAAAAGTCCCCTCAGGAA
Mcl-1-R: TAATGAATTGGGAAGTGGGGC
P2Y1-F: ATGTGTGCTTTCAATGACAGGGTTT
P2Y1-R: TGTGGATGTGGGCATTTCTACTTCT
P2Y2-F: GTGTGCATTCATGAGTGAGGAACC
P2Y2-R: ATCAGACACAGCCAGGTGGAACATA
P2Y4-F: CCAGTACAGAGTCCTCCCTGTTGAG
P2Y4-R: CCAATAGAAAAGAAAGCGGACGAAC
P2Y6-F: GCTTATTTCCCATCAAGGATCAAGG
P2Y6-R: ATGGTTCTTCTGGCAGAGGTCTTG
P2Y11-F: CTACAGAGCGTATAGCCTGGTGCTG
P2Y11-R: CCATGTAGAGTAGAGGGTGGACACA
P2Y12-F: CATTCAAACCCTCCAGAATCAACAG
P2Y12-R: CGATCGATAGTTATCAGTCCCAGGAA
P2Y13-F: GGTGTTTGTTCACATCCCCAG
P2Y13-R: CTTTAAGGAAGCACACTTTTTCAC
P2Y14-F: AGGAAAAGCTGACACCCAGA
P2Y14-R: CTTTCCCATTCGCCAGTAGA.

WESTERN BLOTTING

Cells ($1–2 \times 10^6$) were lysed in 200 μl lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin). The cell lysate was centrifuged at 12,000*g* at 4°C for 5 min. Equivalent amounts of protein were electrophoresed on 10% SDS–PAGE gels and transferred onto Immobilon P membranes (Millipore). The membranes were blocked by incubation with 3% nonfat dry milk for 1 h at room temperature and then incubated with primary antibodies (1:200–1,000) in PBS containing 0.01% Tween 20 overnight at 4°C. After incubation with a horseradish peroxidase-conjugated secondary antibody (1:2,000), the protein bands were detected with SuperSigna Chemiluminescent Substrate Stable Peroxide Solution (Pierce) and BIOMAX-MR film (Eastman Kodak). When necessary, the membranes were stripped with Restore Western Blot Stripping Buffer (Pierce) and re-probed with antibodies against various cellular proteins.

shRNA TRANSFECTION

Cells cultured in six-well plates were transfected with 1 μg of the shRNA plasmid containing sequences targeting human P2Y2 or P2Y11 using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions. Expression of P2Y2 and P2Y11 in the transfected cells was examined by RT-PCR 48 h after transfection. For stable transfection, puromycin-resistant cells were selected after incubation with 5 μg/ml puromycin for 3 weeks.

QUANTITATIVE REAL-TIME RT-PCR (qRT-PCR)

The qRT-PCR was performed as described by Sun et al. [2011]. Briefly, total RNA from cells was isolated and reverse transcribed, as above. The cDNA was amplified using TaqMan Universal PCR master mix (Applied Biosystems) and an ABI Prism 7500 sequence detection system (Applied Biosystems). Amplification of Bcl-2 gene was normalized using the amplification levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. The efficiency of the PCR was tested by amplification of the target from serially diluted cDNA generated from the reverse transcription of a stock set of human RNA. Data analysis and calculations were performed using the $2^{-\Delta\Delta CT}$ comparative method, as described by the manufacturer. Gene expression is shown as the fold inductions of a gene measured in ATP-treated samples, relative to samples cultured with medium.

STATISTICAL ANALYSIS

All experiments were performed at least three times, and the representative results were shown. Results are expressed as the mean plus or minus the standard deviation (SD). Differences between groups were examined for statistical significance using Student's *t*-test, and *P* values equal to or less than 0.05 were considered statistically significant.

RESULTS

ATP INHIBITED CELL PROLIFERATION IN A TIME-DEPENDENT MANNER

The effects of ATP on cell proliferation have been contradictory [Van Daele et al., 1992; Heo and Han, 2006; Schafer et al., 2006; Coppi et al., 2007; Stafford et al., 2007; Taurin et al., 2008; Bilbao et al., 2010; Buzzi et al., 2010]. Here, we first tested the effect of ATP and UTP on endothelial cell proliferation. Cells were left untreated or stimulated with 1–100 μM concentrations of ATP or UTP, and cell numbers were determined by CCK-8 assay. The data show that at lower concentrations (1, 5, and 10 μM), ATP did not inhibit cell proliferation, but at higher concentrations (50 and 100 μM), ATP induced significant inhibition of cell proliferation (Fig. 1A). When the cells were treated with 1–100 μM UTP, no inhibition of cell proliferation was observed (Fig. 1B). The HUVECs were then treated with 100 μM ATP for 6 days, and cell proliferation was assayed every day. The data show that the inhibition of cell proliferation by ATP was time-dependent, whereas UTP did not affect cell proliferation at any of the time points measured (Fig. 1C).

ATP DID NOT INDUCE CELL APOPTOSIS

To determine whether cell apoptosis was involved in ATP-mediated inhibition of cell proliferation, HUVECs were treated with 50 or 100 μM ATP, and cell death was measured by PI/Annexin V staining. Unexpectedly, no cell apoptosis was detected in ATP-treated endothelial cells (Fig. 2A,B). UTP also did not induce apoptosis (Fig. 2A,B). These data indicate that cell apoptosis is not involved in ATP-induced inhibition of cell proliferation.

ATP PROMOTED CELL CYCLE ARREST IN S PHASE

Cell cycle arrest is another mechanism by which cell proliferation is inhibited. HUVECs were treated with 1–100 μM ATP, and cell cycle stage was detected by PI staining. The data show that ATP did not regulate the proportion of cells in G0/G1 phase, but at 50 and 100 μM, ATP significantly decreased the proportion of cells in G2/M phase and significantly increased the proportion of cells in S phase (Fig. 2C,D). These data suggest that cell cycle arrest in S phase is induced by ATP, which correlates with the inhibition of cell proliferation.

ERK WAS NOT INVOLVED IN ATP-INDUCED INHIBITION OF CELL PROLIFERATION

Nucleotides and P2Y receptors have been reported to activate extracellular signal-regulated kinase (ERK) signaling [Chang et al., 2008]. In our study, the phosphorylation of ERK was examined by Western blot, and the results show that 100 μM ATP activated ERK in a time-dependent manner (Fig. 3A,B). The phosphorylation of ERK lasted from 1 to 8 min after ATP treatment (Fig. 3A,B). In addition, an ERK inhibitor, U0126 inhibited the phosphorylation of ERK induced by ATP (Fig. 3C). Moreover U0126, or another ERK inhibitor, PD98095, down-regulated cell proliferation (Fig. 3D,E), but they did not reverse ATP-mediated inhibition of cell proliferation (Fig. 3D,E). These results indicate that ERK is not involved in ATP-induced inhibition of cell proliferation.

P2Y RECEPTOR EXPRESSION IN ENDOTHELIAL CELLS

To determine which receptor is involved in the ATP-mediated inhibition of cell proliferation, HUVECs were pre-treated with 100 μM suramin, a nonspecific P2Y receptor inhibitor, for 1 h and then stimulated with indicated concentrations of ATP. CCK-8

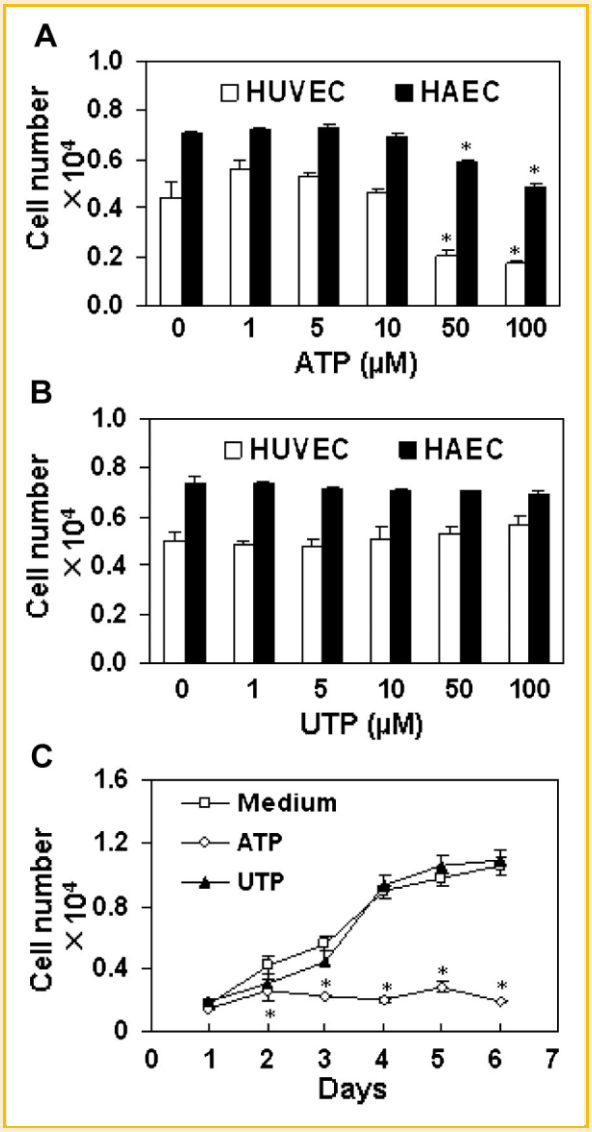

Fig. 1. The effect of nucleotides on endothelial cell proliferation. A,B: Endothelial cell proliferation in respond to various concentrations of nucleotides. HUVECs and HAECs were cultured with indicated concentrations of ATP (A) or UTP (B) for 48 h. Medium was replaced with fresh medium containing nucleotides in the second day. Cell number was measured with CCK-8 assay. C: The time course of HUVECs growth in response to nucleotides. Cells were cultured with 100 μM nucleotides for indicated days. Medium was replaced with fresh medium containing 100 μM nucleotides every day. Cell number was measured with CCK-8 assay. $^{*}P < 0.05$ compared with the control groups.

assay results reveal that suramin alone did not regulate cell proliferation, but it blocked ATP-mediated inhibition of cell proliferation (Fig. 4A), suggesting the involvement of P2Y receptors. We then analyzed gene expression of P2Y receptors in endothelial cells by RT-PCR. The data show that HUVECs expressed P2Y1, 2, 4, 11, 13, and 14 (Fig. 4B), and HAECs expressed P2Y2, 4, 11, 13, and 14 (Fig. 4B). Moreover, ATP up-regulated protein expression of P2Y2 and P2Y11 in HAECs in a dose-dependent manner (Fig. 4C).

ATP INHIBITED CELL PROLIFERATION AND INDUCED CELL CYCLE ARREST VIA P2Y11

ATP is recognized by P2Y2 and P2Y11, and UTP is recognized by P2Y2 and P2Y4 [Abbracchio et al., 2006]. In the current study, ATP but not UTP inhibited cell proliferation in endothelial cells (Fig. 1A), so we hypothesized that P2Y11 contributes to ATP-mediated inhibition of cell proliferation. To confirm this assumption, HUVECs were transfected with shRNA plasmids targeting human P2Y2 or P2Y11 mRNA, respectively. RT-PCR results show that the gene expression of P2Y2 and P2Y11 was effectively blocked by RNA interference (Fig. 5A). The inhibition of cell proliferation was observed in wild type cells (WT), a nonspecific sequence transfected cells (Nssi) and P2Y2 shRNA-transfected cells (P2Y2si), but proliferation was restored in P2Y11 shRNA-transfected cells (P2Y11si) (Fig. 5B), suggesting that P2Y11 but not P2Y2 contributes to ATP-induced inhibition of cell proliferation. Furthermore, in P2Y11 shRNA-transfected HUVECs, the proportion of cells in S phase was decreased, and the proportion of cells in G2/M phase was increased (Fig. 5C), suggesting that P2Y11 also participates in ATP-mediated cell cycle arrest in S phase.

ATP SENSITIZES ENDOTHELIAL CELLS TO CISPLATIN-INDUCED CELL DEATH

It has been reported that the inhibition of S phase progression enhances the cisplatin sensitivity in budding yeast and human cancer cells [Jain et al., 2010]. In the current study, ATP and cisplatin were administered to the cell culture to test the effect of ATP on cisplatin-induced cell death. The results show that 5 μg/ml cisplatin alone induced 19.3% of endothelial cells to cell death (Fig. 6A). However, 50 or 100 μM ATP enhanced the cell death induced by 5 μg/ml cisplatin to 49.7% and 50.3%, respectively (Fig. 6A). As Bcl-2 confers to the resistance against cisplatin-induced cell death [Michaud et al., 2009; Villar et al., 2009], we analyzed the effect of ATP on Bcl-2 expression. The RT-PCR results show that ATP alone down-regulated the gene expression of Bcl-2 (Fig. 6B). ATP also down-regulated the gene expression of Bcl-2 family members, such as Bcl-w, A1, and Mcl-1 slightly, but did not regulate the gene expression of Bcl-xl, another Bcl-2 family member (Fig. 6B). As a control, UTP had no effect on the gene expression of Bcl-2 (Fig. 6C). Western blot results show that ATP and cisplatin synergized to decrease the protein expression of Bcl-2 (Fig. 6D). The down-regulation of Bcl-2 is P2Y11-dependent, because the P2Y11 RNA interference partially reversed the down-regulation of Bcl-2 induced by ATP (Fig. 6E). Moreover, P2Y11 RNA interference decreased the sensitivity of HUVECs to cisplatin (Fig. 6F). In addition, ATP enhanced the cell death induced by 0.1 μM staurosporine (Fig. 6G), which is also a Bcl-2 related toxic compound [Kfir et al., 2007; Rommelaere et al., 2011]. Meanwhile, we found that ATP promoted the cell death induced by cisplatin in nasopharyngeal cancer cell line 5-8F, a malignant cell line with potential to metastasize easily [Hu et al., 2005; Zhang et al., 2009]. These results suggest that ATP enhanced the sensitivity of cells to cisplatin-induced death via P2Y11.

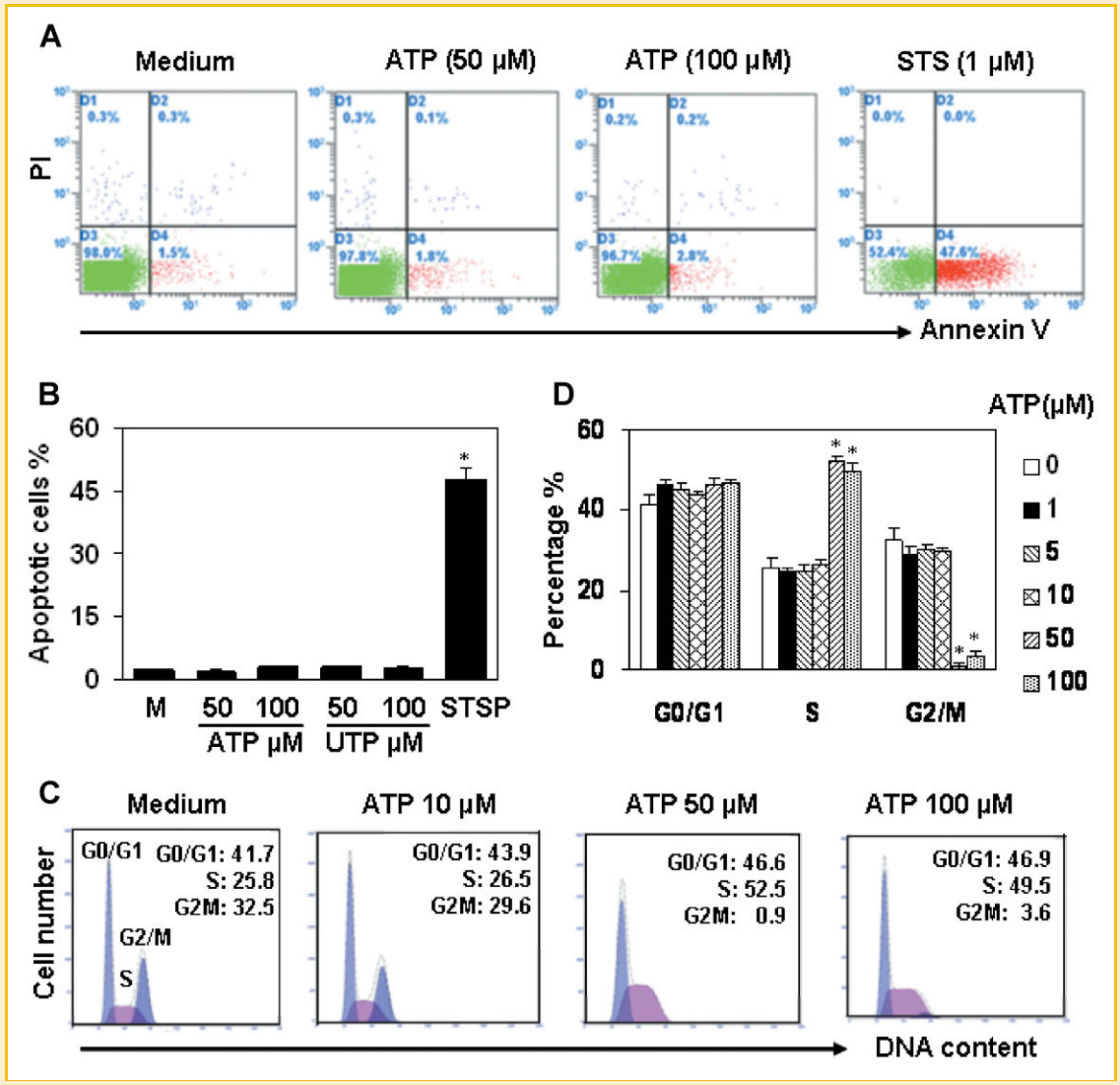

Fig. 2. ATP induces cell cycle arrest but not cell apoptosis. A: ATP did not induce cell apoptosis in HUVECs. Cells were cultured with indicated concentrations of ATP for 48 h. Medium was replaced with fresh medium containing nucleotides in the second day. Cell apoptosis were measured by PI/Annexin V staining. Cells, treated with 1 μM staurosporine (STSP), were used as positive control. B: Quantitation of cell apoptosis induced by nucleotides in (A). $^{*}P < 0.05$ compared with the control group. C: ATP induced cell cycle arrest in S phase. HUVECs were cultured with indicated concentrations of ATP for 48 h. Medium was replaced with fresh medium containing nucleotides in the second day. Cell cycles were measured by PI staining. D: Quantitation of cell cycles induced by ATP in (C). $^{*}P < 0.05$ compared with the control groups.

DISCUSSION

P2Y receptors are expressed on several cell types, including neurons, glial cells, muscle cells, immune cells, epithelial cells, endothelial cells, mesangial cells, and osteoblasts [Abbracchio et al., 2006]. Endothelial cells have been reported to express P2Y1 [Shen and DiCorleto, 2008], P2Y2 [Kukulski et al., 2010], P2Y4 [da Silva et al., 2009], P2Y6 [Bar et al., 2008], P2Y11 [Gorini et al., 2010], and P2Y12 [Bonello et al., 2010]. In the current study, high levels of mRNA of P2Y2 and P2Y11 were detected in HUVECs. In HAECs, high levels of mRNA of P2Y11, and low levels of mRNA of P2Y2 were detected. Moreover, the treatment of endothelial cells with ATP dose-dependently up-regulated the expression of P2Y2 and P2Y11, suggesting that ATP may play important roles in the regulation of angiogenesis and wound healing, because ATP can be released from injured cells and tissues [Smith et al., 2005; Yin et al., 2007].

Nucleotides were reported to regulate cell proliferation [Heo and Han, 2006; Schafer et al., 2006; Coppi et al., 2007; Stafford et al., 2007; Taurin et al., 2008; Bilbao et al., 2010; Buzzi et al., 2010], cell differentiation [Atarashi et al., 2008], cell maturation [Wilkin et al., 2001], cell apoptosis [Di Iorio et al., 2002; Vaughan et al., 2007], cell migration [Shen and DiCorleto, 2008; Gorini et al., 2010; Kukulski et al., 2010], to regulate the expression of VCAM-1 [Seye et al., 2003], to regulate the clearance of apoptotic cells as a find-me signal [Elliott et al., 2009], and to regulate the triggering and maintain of inflammation [Idzko et al., 2007]. However, several studies have shown conflicting results on the effect of nucleotides on cell proliferation. Nucleotides have been reported to promote cell

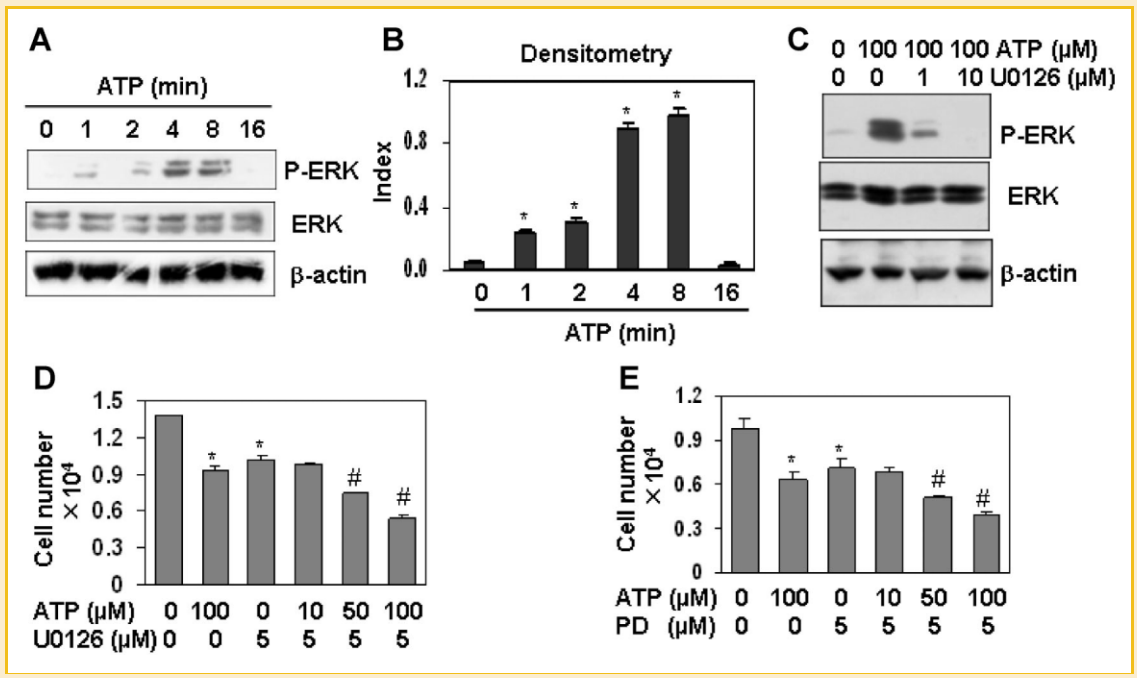

Fig. 3. ERK is not involved in ATP-induced inhibition of cell proliferation. A: ATP induced the phosphorylation of ERK. HUVECs, starved overnight with serum-free medium, were treated with 100 μM ATP at 37°C, and lysed at the indicated time points. Western blot was performed for the detection of phospho-ERK1/2 and total ERK1/2, respectively. β-actin protein was detected as loading control. B: Quantitation of phosphorylated ERK normalized to total ERK in (A). $^{*}P < 0.05$ compared with the control group. C: The effect of U0126 on ATP-induced ERK activation. HUVECs, starved overnight with serum-free medium, were pre-treated with indicated concentrations of U0126 for 1 h, followed by the treatment of indicated concentrations of ATP for 5 min for the detection of phospho-ERK1/2 and total ERK1/2, respectively. D: The effect of U0126 on ATP-induced cell proliferation inhibition. Cells, pre-treated with ERK inhibitor, U0126 (5 μM at 37°C for 1 h), were re-treated with indicated concentrations of ATP for 24 h, followed with the same treatment in the next 24 h. Cell number was measured by CCK-8 assay. $^{*}P < 0.05$ compared with the nontreated group. $^{\#}P < 0.05$ compared with the U0126-treated group. E: The effect of ERK inhibitor, PD98095 (PD), on ATP-induced cell proliferation inhibition. $^{*}P < 0.05$ compared with the nontreated group. $^{\#}P < 0.05$ compared with the PD98095-treated group.

proliferation in some cases [Heo and Han, 2006; Taurin et al., 2008; Bilbao et al., 2010; Buzzi et al., 2010], but in other cases to inhibit cell proliferation [Schafer et al., 2006; Coppi et al., 2007; Stafford et al., 2007]. The reason for the discrepant results may be due to differences in cell type, culture conditions, nucleotide concentrations and the intracellular signaling. In our study, we found that ATP at lower concentrations (1–10 μM) did not inhibit cell proliferation, but at higher concentrations (50–100 μM), ATP significantly inhibited cell proliferation.

Cell cycle arrest is a mechanism to inhibit cell proliferation. Many factors inhibit cell proliferation by modulating cell cycle progression [Kulkarni et al., 2009; Pichichero et al., 2010; Ray et al., 2010; Zhang et al., 2011]. Extracellular nucleotides, including ATP, UTP, ADP, and UDP, inhibit growth of oesophageal cancer cells via P2Y

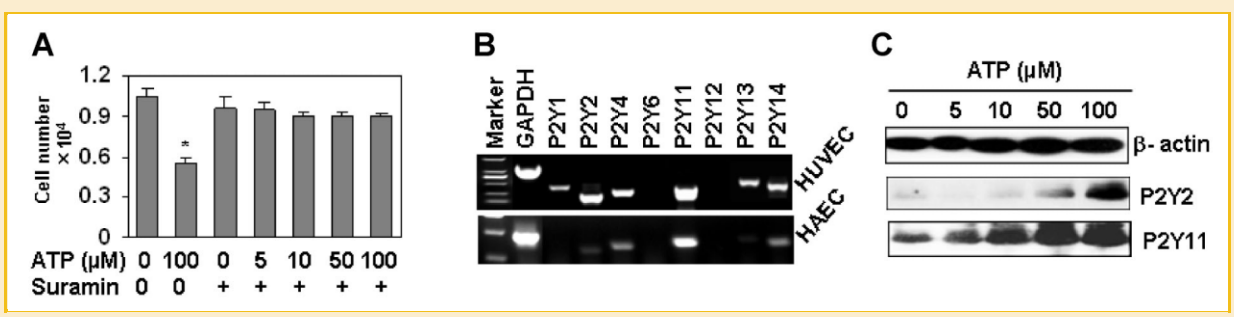

Fig. 4. The involvement of P2Ys in ATP-induced cell proliferation inhibition. A: Suramin blocked ATP-induced cell proliferation inhibition. HUVECs, nontreated or pre-treated with 100 μM suramin for 1 h, were re-treated with indicated concentrations of ATP for 24 h, followed with the same treatment in the next 24 h. Cell number was measured with CCK-8 assay. $^{*}P < 0.05$ compared with the nontreated group. B: Endothelial cells expressed gene transcripts coding for P2Y receptors. HUVECs and HAECs were culture in six-well plates for 24 h. Cells were harvested for the detection of the gene expression of P2Y receptors by RT-PCR. C: ATP up-regulated protein expression of P2Y2 and P2Y11 in HAECs. Cells were treated with indicated concentrations of ATP for 24 h. Protein expression of P2Y2 and P2Y11 was detected by Western blot.

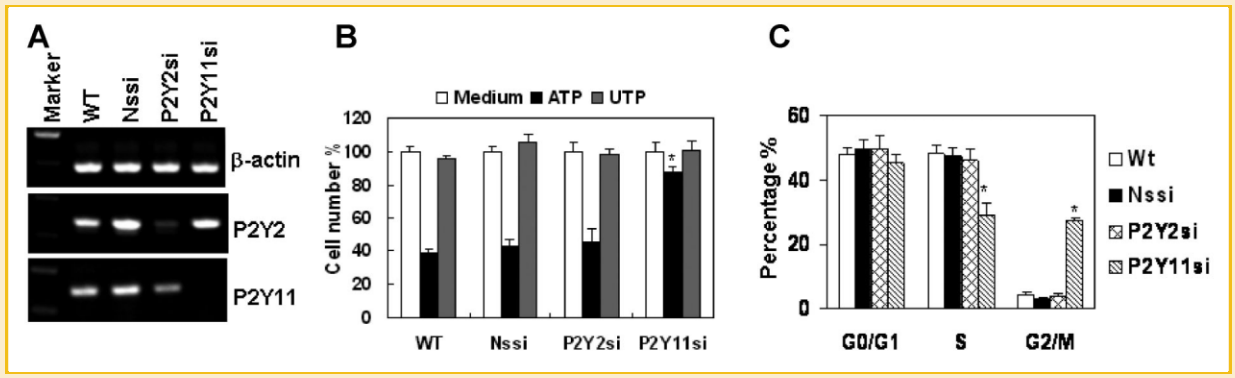

Fig. 5. The down-regulation of P2Y11 blocks ATP-induced cell proliferation inhibition. A: The down-regulation of P2Y2 and P2Y11 by RNA interference. HUVECs cultured in six-well plate were transfected with control shRNA plasmid (scbt, sc-108060), P2Y2 shRNA plasmid (scbt, sc-425790-SH) and P2Y11 shRNA plasmid (scbt, sc-76026-SH), respectively. Puromycin resistant clones were selected. Gene expression of P2Y2 and P2Y11 in wild type cells (WT), nonspecific shRNA transfectants (Nssi), P2Y2 shRNA transfectants (P2Y2si), and P2Y11 shRNA transfectants (P2Y11si) were detected by RT-PCR. B: The effect of P2Y2 and P2Y11 down-regulation on the cell proliferation induced by ATP. WT, Nssi, P2Y2si, and P2Y11si cells were cultured in 96-well plates and treated with medium, 100 μM ATP or 100 μM UTP for 48 h. Cell numbers were measured by CCK-8 assay and normalized to control groups. $^{*}P < 0.05$ compared with the control group. C: The effect of P2Y2 and P2Y11 down-regulation on ATP-induced cell cycle arrest in S phase. WT, Nssi, P2Y2si, and P2Y11si cells were cultured with 100 μM ATP for 48 h. Cell cycles were measured by PI staining. $^{*}P < 0.05$ compared with the WT group.

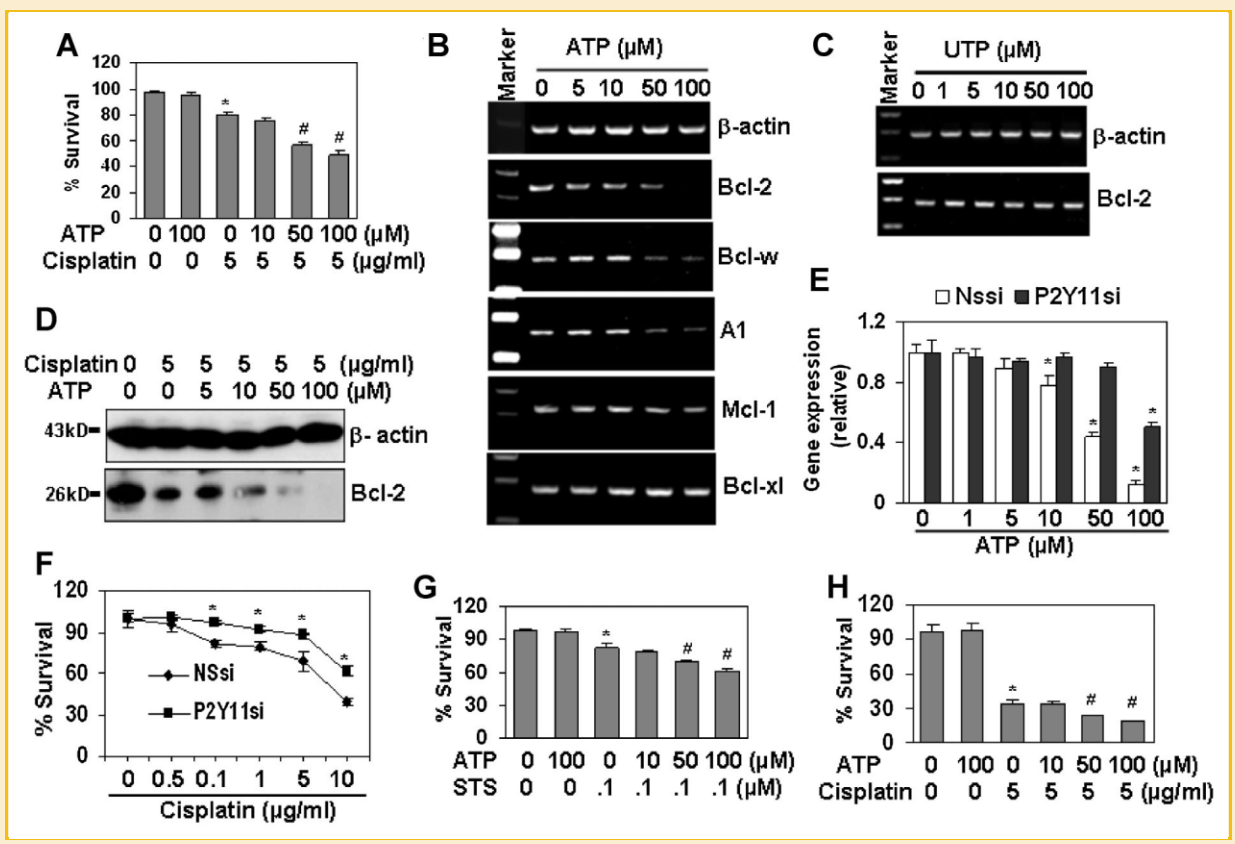

Fig. 6. The effect of ATP on cisplatin-induced cell death. A: ATP augmented cisplatin-induced cell death. HUVECs were cultured with indicated concentrations of cisplatin and ATP for 24h. Cell death was measured by Annexin V/PI staining. $^{*}P < 0.05$ compared with the nontreated group. $^{\#}P < 0.05$ compared with the cisplatin alone treated group. B: The effect of ATP on the gene expression of Bcl-2 family members. HUVECs were cultured with indicated concentrations of ATP for 24 h. The gene expression of Bcl-2 family members was detected by RT-PCR. C: The effect of UTP on the gene expression of Bcl-2. D: ATP and cisplatin synergized to decrease the protein expression of Bcl-2. HUVECs were treated with indicated concentrations of ATP and cisplatin for 24 h. The protein expression of Bcl-2 was detected by Western blot. E: qRT-PCR results show the effect of ATP on the gene expression of Bcl-2 in Nonspecific shRNA transfectants (Nssi), and P2Y11 shRNA transfectants (P2Y11si). $^{*}P < 0.05$ compared with the nontreated group. F: P2Y11 RNA interference decreased the cisplatin sensitivity in HUVECs. Cells were treated with indicated concentrations of cisplatin for 24 h. Survival cells were detected by CCK-8 assay and normalized to nontreated group. $^{*}P < 0.05$ compared with the Nssi group. G: ATP augmented staurosporine (STS)-induced cell death in HUVECs. $^{*}P < 0.05$ compared with the nontreated group. $^{\#}P < 0.05$ compared with the staurosporine alone treated group. H: ATP augmented cisplatin-induced cell death in 5-8F cells. $^{*}P < 0.05$ compared with the nontreated group. $^{\#}P < 0.05$ compared with the cisplatin alone treated group.

receptors, which is due to the induction of cell apoptosis and cell cycle arrest [Maaser et al., 2002]. Adenine nucleotides, including ATP and ADP, have been reported to inhibit proliferation of the human lung adenocarcinoma cells via the induction of massive accumulation of cells in the S phase [Schafer et al., 2006]. However, ATP has also been reported to induce cell proliferation by promoting the cell cycle from G1 to S phase in the mouse developing retina [Sholl-Franco et al., 2010]. In our study, we found that extracellular ATP, but not UTP, inhibited proliferation of human endothelial cells at the concentrations of 50–100 μM, which was due the induction of cell cycle arrest in S phase. The reason for these discrepant results might be due to the differences in cell types and the differences in cell developing phases.

The biological effects of extracellular nucleotides are mediated through the activation of P2 purinergic receptors, which are subdivided into two distinct categories, the metabotropic G protein-coupled (P2Y) receptors [Abbracchio et al., 2006] and the ionotropic ligand-gated channel (P2X) receptors [Khakh and North, 2006]. We found that human endothelial cells express P2X4 (data not shown) and P2Y1, 2, 4, 11, 13, and 14. To confirm which receptor is involved in ATP-induced inhibition of cell proliferation, we detected the effect of suramin, a P2Y nonspecific antagonist, on ATP-induced inhibition of cell proliferation. The results show that the pre-treatment with suramin significantly inhibited ATP-induced inhibition of cell proliferation, suggesting the involvement of P2Y receptor. As ATP is recognized by P2Y2 and P2Y11, and UTP is recognized by P2Y2 and P2Y4 [Abbracchio et al., 2006], we hypothesized that P2Y11 may contribute to the ATP-mediated inhibition of cell proliferation. This assumption was further confirmed by the observation that the down-regulation of P2Y11, but not P2Y2, by RNA interference abrogated the inhibition of cell proliferation induced by ATP. Furthermore, P2Y11 down-regulation abrogated the cell cycle arrest in S phase induced by ATP. These results suggest that P2Y11 contributed to ATP-induced inhibition of cell proliferation and cell cycle arrest.

Stimulation of the P2Y receptors is known to activate a variety of signaling molecules, including the mitogen-activated protein kinases, ERK1/2, p38, and JNK, the small GTPase RhoA, and the release of the second messenger Ca^{2+} into the cytoplasm. Adenine nucleotides have been reported to inhibit proliferation of the human lung adenocarcinoma cells by activation of ERK1/2, P38, PI3K, and NF-κB pathways [Schafer et al., 2006]. The activation of P2Y2 by UTP mediates the up-regulation of vascular cell adhesion molecule-1 in coronary artery endothelial cells, which is dependent on p38 signaling pathway, but not on ERK1/2 signaling pathway [Seye et al., 2003]. In our study, we found that ATP induced the phosphorylation of ERK1/2. However, the pre-treatment of HUVECs with U0126 or PD98095, two ERK1/2 inhibitors, did not abrogate the inhibition of cell proliferation induced by ATP. Thus, we concluded that ATP-induced inhibition of cell proliferation is not dependent on the activation of ERK1/2 signaling.

Cell cycle arrest is related to the sensitivity to the DNA damage reagents. Cell cycle arrest in S phase sensitizes human cancer cells to cisplatin [Jain et al., 2010]. The induction of cell cycle arrest in S phase by ATP suggests that ATP might increase the sensitivity of vascular endothelial cells to chemotherapy. This assumption was confirmed by the observation that ATP significantly increased cisplatin-induced cell death in HUVECs, which was P2Y11 dependent, because P2Y11 RNA interference down-regulated the cisplatin sensitivity. The up-regulation of the sensitivity to cisplatin induced by P2Y11 activation in human vascular endothelial cells may be beneficial to the therapy of the clinical cancer patients via the inhibition of tumor angiogenesis, but may also be deleterious via the increase of blood vessel damage in normal tissues. Furthermore, we found that ATP down-regulated the expression of Bcl-2, an important molecule to regulate cisplatin-induced cell death [Michaud et al., 2009; Villar et al., 2009]. As Bcl-2 is a targeted gene of the tumor suppressor, p53 [Esposito et al., 2010], the down-regulation of Bcl-2 by ATP suggests that p53 or related p63 or p73 might be involved in the regulation of the functions of P2Y11. Meanwhile, we found that ATP significantly increased cisplatin-induced cell death in cancer cells. All together, these results suggest that P2Y11 may be target for the development of anti-cancer reagents. More detailed studies will be necessary and significant in the future.

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