P2Y Receptors Activate MAPK/ERK Through a Pathway Involving PI3K/PDK1/PKC- ζ in Human Vein Endothelial Cells

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Key Words

Endothelial cells • P2 receptors • G-proteins • MAP kinases • Signal transduction

Abstract

Aims: In this study we investigated the effects of P2 receptors in the regulation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) in human umbilical vein endothelial cells (HUVEC). **Methods:** Cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured using fura-2/AM, and MAPK/ERK phosphorylation using Western blot analysis. **Results:** ATP, 2-meSATP, UTP and UDP cause a rapid and transitory increase in the phosphorylation of MAPK/ERK. In contrast, negligible response was seen for α, β -meATP, a general P2X receptors agonist. ATP-dependent activation of MAPK/ERK was prevented by pretreatment of HUVEC with pertussis toxin or MEK inhibitor PD98059. In addition, activation of the MAPK/

ERK cascade by ATP was blocked in cells pretreated with wortmannin and LY294002, but not by U73122, BAPTA or a Ca^{2+} -free medium. Furthermore, an inhibition of ATP-dependent MAPK/ERK phosphorylation was observed in HUVEC pretreated with high doses of GF109203X or myristoylated PKC- ζ pseudosubstrate. Similar results were observed when cells were pretreated with the Src tyrosine kinase inhibitor PP2. However, ATP-stimulated MAPK/ERK activation was unaffected in cells pretreated with AG1478 or perillid acid. We also found that ATP stimulates both the phosphorylation of 3-phosphoinositide-dependent protein kinase-1 (PDK1) and its translocation to plasma membrane in a time-dependent manner. **Conclusion:** These observations suggest that the effects mediated by ATP in HUVEC occur via PTX-sensitive G-protein-coupled P2Y receptors through PI3K-dependent mechanisms, in which PDK1 and PKC- ζ are two key molecules within signal cascade leading to MAPK/ERK activation.

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Introduction

Nucleotides which may be present in the extracellular fluids as a consequence of cell lysis, selective plasma membrane permeabilization or exocytosis of secretory granules [1], have been recognized as key molecules that exert a range of physiological functions in diverse tissues and cell types. These actions of nucleotides are mediated through the activation of two families of plasma membrane receptors, identified by pharmacological and molecular cloning studies, which differ in their molecular structures and signal transduction pathways. P2X receptors are ligand-gated intrinsic ion channels, whereas P2Y receptors belong to the superfamily of G-protein coupled receptors (GPCR) [2, 3].

Early reports showed that various P2X and P2Y receptor subtypes were expressed on different types of cells in the cardiovascular system, such as cardiomyocytes, vascular smooth muscle and endothelial cells [4, 5], indicating that extracellular nucleotides play an essential role in the modulation of vascular homeostasis and can be involved in the pathophysiology of cardiovascular diseases. Thus, in the course of a vascular injury, nucleotides play an important role in haemostasis through activation of platelets, modulation of vascular tone, recruitment of neutrophils and monocytes to the injured site, and facilitation of adhesion of leucocytes to the endothelium [6]. In addition, extracellular nucleotides released during vascular damage contribute to endothelial function through the production and release of PGI₂ and NO [7-9], two potent vasodilators and inhibitors of platelet function [10].

Mitogen-activated protein kinases (MAPK), a family of serine-threonine protein kinases that transduces signals from the cell membrane to nucleus in response to a wide variety of stimuli, including environmental stresses, growth factors and GPCR, are closely involved in the proliferation, migration or morphogenesis of vascular cells [11-13]. Extracellular nucleotides have been demonstrated to activate the MAPK/extracellular signal-regulate kinases (MAPK/ERK) cascade in an extensive range of cell types, including the vascular endothelial cells [7, 14, 15]. However, the P2 receptor signaling pathways for endothelial cells response to nucleotides are poorly known. In this study we demonstrate that ATP-induced MAPK/ERK activation in HUVEC occurs via PTX-sensitive G-protein-coupled P2Y receptors through PI3K-dependent mechanisms, in which PDK1 and PKC- ζ are two key

molecules within signal cascade leading to MAPK/ERK activation.

Materials and Methods

Materials

Pertussis toxin (PTX), myristoylated PKC- ζ pseudosubstrate peptide, 1,2-bis (o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM) and U73122 were purchased from Calbiochem-Novabiochem (San Diego, CA). GF109203X, wortmannin, LY294002, phorbol 12-myristate 13-acetate (PMA), genistein, PP2, and PD98059 were obtained from Tocris (Bristol, UK). Perillic acid was purchased from Alexis Biochemicals (San Diego, CA, USA). ATP, UTP, UDP, α,β -methylene-ATP (α,β -meATP), 2-methylthio-ATP (2-meSATP), collagenase A, M199 medium, fetal bovine serum (FBS), heparin, endothelial cell growth supplemented (ECGS), fura 2-AM, pluronic F-127, protease inhibitors, antibiotics and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal p-ERK and polyclonal ERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal AKT antibody was from Transduction Laboratories (Lexington, KY, USA), and polyclonal p-AKT and p-PDK1 antibodies were from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-mouse IgG and goat anti-rabbit IgG-horseradish peroxidase conjugated, calibrated pre-stained standards and the chemiluminescence kits were obtained from Pierce (Rockford, IL, USA). Poly(vinylidene difluoride) membranes were obtained from Millipore (Bedford, MA, USA), and Hyperfilm ECL was purchased from Amersham International plc (Buckinghamshire, UK).

Cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords as previously described [16]. Briefly, the cords were cleaned with PBS and incubated with collagenase A (0.5 mg/ml) for 15 min at 37°C. Endothelial cells were collected by centrifugation and resuspended in M199 medium with 20% FBS. Cells were seeded on a gelatin-coated flask and grown in M199 medium supplemented with 20% FBS, 30 μ g/ml ECGS and 100 μ g/ml heparin, containing gentamicin (50 μ g/ml) and amphotericin B (0.25 μ g/ml). The cells were maintained in a water-saturated atmosphere of 5% CO₂ and 95% air at 37°C, and used at passages 2-4. Before the experiments, cells were harvested with 0.05% trypsin-EDTA solution, plated onto plastic culture dishes and incubated in serum-free fresh medium.

Determination of cytosolic Ca²⁺ concentration ([Ca²⁺]_i)

The medium was aspirated, and the cells were then harvested with a 0.05% trypsin-EDTA solution. After washing the cells three times by pelting (50 g for 3 min), the cells were incubated with 5 μ M fura 2-AM for 45 min at 37°C with continuous shaking. Following the loading period, the cells

Fig. 1. Effect of ATP on MAPK/ERK phosphorylation in HUVEC. Cells were stimulated with 100 μ M ATP for different times (0.2–20 min) (A) or with various ATP concentrations (1–500 μ M) for 3 min (B), and cell lysates were analyzed by Western blotting using anti-pERK or anti-ERK antibodies. The figure is representative of three independent experiments.

were washed twice with a modified Krebs-Ringer buffer in which the bicarbonate was replaced by 20 mM HEPES, pH 7.4, incubated again for at least 10 min at room temperature to facilitate hydrolysis of the esterified probe, and washed once again. The cells were resuspended in the same buffer containing 0.1% BSA, and 2 ml of the cell suspension was added to a fluorescence cuvette kept at 37°C, and stirred throughout the experiment. The fluorescence intensity was measured with a Hitachi F2000 fluorimeter and $[Ca^{2+}]_i$ was calculated using software of the spectrofluorometer.

Western blot analysis

HUVEC were incubated at 37°C with vehicle, agents or nucleotides as indicated. Cells were rinsed with ice-cold phosphate-buffered saline (PBS) containing 0.5 mM Na_3VO_4 and then collected in 0.2 ml lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.2 mM Na_3VO_4 , 1 mM EGTA, 0.4 mM EDTA, 1 μ g/ml of aprotinin and leupeptin, and 0.1 μ g/ml of phenylmethylsulphonyl fluoride). Lysates were centrifuged at 10,000 g for 15 min at 4°C to remove insoluble material and normalized for protein content. For analysis of PDK1 translocation to plasma membrane HUVEC were scraped in 20 mM Tris-HCl, pH 7.5, containing 250 mM sucrose, 2 mM EGTA, 2 mM EDTA, 10 mM $MgCl_2$, 2 mM DTT and a mixture of protease inhibitors, and after two freeze and thaw cycles, cells were centrifuged at 900 g for 15 min at 4°C. Pellet was re-suspended

Fig. 2. Effect of α,β -meATP, 2-meSATP, UTP and UDP on MAPK/ERK phosphorylation in HUVEC. Cells were stimulated with 100 μ M of nucleotides for different times (0.5–10 min), and cell lysates were analyzed by Western blotting using anti-pERK or anti-ERK antibodies. The figure is representative of three independent experiments.

and centrifuged once more, and the pooled supernatants were centrifuged at 100,000 g for 60 min to obtain membrane and cytosolic fractions.

Equal amounts of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred electrophoretically to poly(vinylidene difluoride) membranes. Membranes were incubated for 1 h in blocking buffer (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 5% non-fat dry milk, and 0.1% Tween-20) and then incubated for 90 min with p-ERK, p-AKT or p-PDK1 antibodies. Membranes were washed twice with blocking buffer and incubated for 45 min with IgG-horseradish peroxidase conjugated as a secondary antibody. Membranes were finally washed twice with washing buffer (10 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and 0.1% Tween-20), incubated with enhanced chemiluminescence detection reagents and exposed to Hyperfilm ECL. Approximate molecular masses were estimated using calibrated prestained standards. To ensure that there were similar amounts of protein in each sample the same membranes were stripped off, reprobed with polyclonal ERK or AKT antibodies and developed with anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody by ECL.

Protein analysis

Protein contents were measured using the Coomassie blue binding method of Bradford [17].

Fig. 3. Effect of pertussis toxin (PTX) and MEK inhibitor PD98059 (PD) on ATP-mediated MAPK/ERK phosphorylation in HUVEC. Cells were pretreated overnight with 100 ng/ml PTX or with 30 μ M PD98059 for 30 min, and then stimulated with 100 μ M ATP. Cell lysates were analyzed by Western blotting using anti-pERK or anti-ERK antibodies. Results shown in the bottom part are from a representative experiment. Data from three independent experiments are expressed as a percentage of unstimulated cells. * P <0.05 versus control. ^a P <0.05 versus ATP.

Data analysis

Results were expressed as mean $\pm$ SEM. Data were compared by ANOVA followed by the Newman-Keuls multiple comparison test using GraphPad Prism, version 3.01 (San Diego, CA).

Results

Effect of extracellular nucleotides in MAPK/ERK phosphorylation

In order to examine the role of extracellular nucleotides in MAPK/ERK activation, we first checked whether ATP and other P2 receptor agonists were able to regulate MAPK/ERK phosphorylation in HUVEC. As shown in Figure 1, ATP stimulated the phosphorylation of

Fig. 4. Effect of ATP-induced intracellular-free Ca^{2+} mobilization on MAPK/ERK phosphorylation in HUVEC. Cells were incubated without (Trace 1) or with 2 mM EGTA (Trace 2) for 1 min or 5 μ M U73122 (Trace 3) for 30 min before stimulation with 100 μ M ATP (A). Cells were pretreated with 5 μ M U73122 for 30 min, 2 mM EGTA for 1 min or 10 μ M BAPTA for 20 min before stimulation with 100 μ M ATP, and cell lysates were analyzed by Western blotting with anti-pERK or anti-ERK antibodies (B). Results shown in the bottom part are from a representative experiment. Data from three independent experiments are expressed as a percentage of unstimulated cells. * P <0.05 versus control. ^a P <0.05 versus ATP.

Fig. 5. Effect of PI3K on ATP-stimulated MAPK/ERK phosphorylation in HUVEC. Cells were pretreated with 0.5 μ M wortmannin (Wm) or 20 μ M LY294002 (LY) for 30 min before stimulation with 100 μ M ATP, or incubated with PI3K inhibitors for 30 min. Cell lysates were analyzed by Western blotting using anti-pERK or anti-ERK antibodies. Results shown in the bottom part are from a representative experiment. Data from three independent experiments are expressed as a percentage of unstimulated cells. * P <0.05 versus control. ^a P <0.05 versus ATP.

MAPK/ERK in time- and dose-dependent manner. Maximum stimulation was observed with a 3-min ATP treatment, and rapidly declined thereafter (Fig. 1A). Phosphorylation of MAPK/ERK became detectable at 1 μ M ATP and reached a maximum of over 3.2-fold at 100 μ M ATP (Fig. 1B). In addition, 2-meSATP, UTP and UDP also transiently enhanced MAPK/ERK phosphorylation, while P2X receptors agonist α,β -meATP was ineffective (Fig. 2), suggesting that P2Y receptors play a critical role in the regulation of MAPK/ERK cascade.

The next experiments were designed to determine what ATP-mediated signaling events were important for the MAPK/ERK activation. Overnight pretreatment of HUVEC with 100 ng/ml PTX markedly reduced MAPK/ERK phosphorylation (Fig. 3), suggesting that PXT-sensitive heterotrimeric G-proteins were involved in ATP-induced MAPK/ERK activation. We then checked the effect of a specific mitogen-activated protein kinase kinases (MEK) inhibitor on MAPK/ERK activation. As expected, pretreatment of cells with 30 μ M PD98059 for 30 min abolished ATP-induced MAPK/ERK phosphorylation (Fig. 3), showing that MAPK/ERK activation depends completely on the upstream phosphorylation of MEK.

Influence of intracellular-free Ca^{2+} in ATP-induced MAPK/ERK phosphorylation

Exposure of HUVEC to 100 μ M ATP induced a rapid and transitory increase in intracellular-free Ca^{2+} concentration ($[Ca^{2+}]_i$), which was completely blocked by pretreatment of cells with 5 μ M U73122 for 30 min, a selective inhibitor of PLC, and substantially reduced by adding 2 mM EGTA to the cellular suspension to chelate extracellular Ca^{2+} prior to ATP stimulation (Fig. 4A).

Further studies were performed to determine if changes $[Ca^{2+}]_i$ were necessary for ATP-induced MAPK/ERK phosphorylation. As shown in Figure 4B, neither preincubation of HUVEC in the presence of PLC inhibitor U73122, nor removal of extracellular Ca^{2+} with

2 mM EGTA or intracellular Ca^{2+} with 10 μ M BAPTA prevented MAPK/ERK phosphorylation. Taken together, these results suggest that ATP-induced MAPK/ERK activation in HUVEC occurs through downstream mechanisms that does not involve intracellular-free Ca^{2+} mobilization.

Role of PI3K in ATP-induced MAPK/ERK phosphorylation

To test whether phosphorylation of MAPK/ERK occurred subsequently to ATP-stimulated PI3K activation, we treated the cells with the selective PI3K inhibitors wortmannin and LY294002. As shown in Figure 5, pretreatment of HUVEC with 0.5 μ M wortmannin or 20 μ M LY294002 markedly decreased both the ATP-induced MAPK/ERK phosphorylation and the basal phosphorylation status of MAPK/ERK, indicating that PI3K was required for ATP-evoked activation of MAPK/ERK pathway in these cells.

Role of PKC in ATP-induced MAPK/ERK phosphorylation

In order to determine whether PKC might be involved in ATP-stimulated MAPK/ERK activation, we evaluated the effect of phorbol ester PMA and

Fig. 6. Effect of PKC on ATP-stimulated MAPK/ERK phosphorylation in HUVEC. Cells were treated with 100 μ M ATP, 1 μ M PMA for 3 min, or pretreated overnight with 1 μ M PMA or 5 μ M GF109203X (GFX) for 30 min before stimulation with 100 μ M of ATP (A), or preincubated with different GFX concentrations (1-10 μ M) for 30 min or 25 μ M PKC- ζ pseudosubstrate peptide (ζ -PS) for 60 min before stimulation with 100 μ M ATP (B). Cell lysates were analyzed by Western blotting using anti-pERK or anti-ERK antibodies. Results shown in the bottom part are from a representative experiment. Data from three independent experiments are expressed as a percentage of unstimulated cells. *P<0.05 versus control. ^aP<0.05 versus ATP.

GFX109203X, a selective activator and inhibitor of PKC respectively. Treatment of cells with 1 μ M PMA for 3 min caused a marked phosphorylation of MAPK/ERK similar to that observed with 100 μ M ATP (Fig. 6A). However, in cells incubated overnight with 1 μ M PMA, to down-regulate conventional and novel PKC isoforms [18], ATP-induced MAPK/ERK phosphorylation was unaltered (Fig. 6A). In addition, the effect of ATP on MAPK/ERK cascade activation was significantly reduced by 10 μ M GF109203X but not by 1 μ M of PKC inhibitor (Fig. 6B), a concentration at which most PKC isoforms should be blocked substantially [19]. Since exposition of cells to cell-permeable PKC- ζ pseudosubstrate peptide prevented the ATP-provoked MAPK/ERK phosphorylation (Fig. 6B), the results suggest that atypical PKC- ζ participates in the downstream signaling events of ATP-induced MAPK/ERK activation.

Fig. 7. Effect of ATP on AKT phosphorylation in HUVEC. Cells were stimulated with 100 μ M of ATP for different times (0.2-3 min) (A) or incubated with 0.5 μ M wortmannin (Wm) or 20 μ M LY294002 (LY) for 30 min (B). Cell lysates were analyzed by Western blotting using anti-pAKT or anti-AKT antibodies. The figure is representative of three independent experiments.

Fig. 8. Effect of ATP on PDK1 phosphorylation in HUVEC. Cells were stimulated with 100 μ M of ATP for different times (0.2–3 min) or treated with 0.5 μ M wortmannin (Wm) or 20 μ M LY294002 (LY) for 30 min before stimulation with 100 μ M ATP. Proteins from membrane (●) or cytosolic (○) fractions were analyzed by Western blotting using anti-pPDK1 antibody. Results shown in the bottom part are from a representative experiment. Data from three experiments are expressed as a percentage of unstimulated cells. * P <0.05 versus control.

Fig. 9. Effect of protein-tyrosine kinases and small GTPases on ATP-mediated MAPK/ERK phosphorylation in HUVEC. Cells were pretreated with 100 μ M genistein (Gen), 20 μ M PP2 or 10 μ M AG1478 (AG) for 30 min, or with 100 μ M perillallic acid (Per) for 24 h before stimulation with 100 μ M ATP. Cell lysates were analyzed by Western blotting using anti-pERK or anti-ERK antibodies. Results shown in the bottom part are from a representative experiment. Data from three independent experiments are expressed as a percentage of unstimulated cells. * P <0.05 versus control. ^a P <0.05 versus ATP.

Role of AKT in ATP-induced MAPK/ERK phosphorylation

To investigate the possibility that ATP activates the MAPK/ERK cascade through AKT activation, Ser⁴⁷³ phosphorylation of AKT in cells treated with ATP was analyzed by Western blot using a specific antibody. As shown in Figure 7A, 100 μ M ATP did not affect AKT phosphorylation for the time period of maximum stimulation of MAPK/ERK, suggesting that AKT did not regulate MAPK/ERK activation pathway. Our data also indicate that PI3K activity was required for the basal AKT phosphorylation observed in this cell type. Treatment of HUVEC with PI3K inhibitors wortmannin and LY294002 completely blocked the endogenous phosphorylated form of AKT (Fig. 7B).

Role of PDK1 in ATP-induced MAPK/ERK phosphorylation

Further studies were conducted in order to investigate if PDK1 is a downstream target of P2Y receptors mediating signal to the MAPK/ERK pathway. ATP-induced phosphorylation of PDK1 was analyzed by Western blot using a specific anti-phospho-Ser²⁴¹ antibody. As shown in Figure 8, time-course experiments revealed that PDK1 phosphorylation in cytosolic fraction from HUVEC was maximal after 1 min of stimulation and declined thereafter. Moreover, ATP also increased PDK1 phosphorylation in membrane fraction from these cells, being inhibited by PI3K inhibitors wortmannin and LY294002. Collectively, these results suggested that ATP-induced MAPK/ERK activation in HUVEC occurs partially through PDK1-dependent mechanisms.

Fig. 10. Schematic model for activation of MAPK/ERK by P2Y receptors in HUVEC. Activation of PI3K, possibly via PTX-sensitive G_{α} and $G_{\beta\gamma}$ subunits or Src-dependent interaction, initiates phosphorylation of PDK1, and further PKC- ζ activation leading to MAPK/ERK cascade. For more details, see "Discussion".

Role of protein tyrosine kinases and small GTPase in ATP-induced MAPK/ERK phosphorylation

Receptor tyrosine kinases (RTK) and non-receptor tyrosine kinases have been implicated in the activation of MAPK/ERK by GPCR via Shc/Grb-SOS-Ras pathway. We have also investigated the possible involvement of protein tyrosine kinases and small GTPase in ATP-induced MAPK/ERK phosphorylation. Pretreatment of cells with 100 μ M genistein, a non-selective inhibitor of protein tyrosine kinases, or 20 μ M PP2, a selective inhibitor of Src tyrosine kinases, markedly reduced MAPK/ERK phosphorylation by ATP (Fig. 9). However, 10 μ M tyrphostin AG1478, a potent inhibitor of EGFR kinase, failed to inhibit ATP-induced MAPK/ERK phosphorylation. Moreover, pretreatment of HUVEC for 24 h with 100 μ M perillidic acid, a post-translational inhibitor of isoprenylation of small G-proteins, did not alter the ATP effect on MAPK/ERK cascade (Fig. 9). Our finding reveals that Src family of tyrosine kinases, but not EGFR transactivation and small GTPase activation, was required for ATP-induced MAPK/ERK activation in HUVEC.

Discussion

Previous studies have shown that stimulation of P2 receptors activates the MAPK/ERK cascade in endothelial cells from several origins, such as bovine aorta endothelial cells [7], EAhy 926 endothelial cells [14] and brain capillary endothelial cells [15], although the precise mechanisms by which the extracellular nucleotides

modulates this process remain poorly understood. In accordance with these findings, we demonstrate that ATP, 2-meSATP, UTP and UDP cause a rapid and transitory increase in the phosphorylation of MAPK/ERK, whereas negligible response was found for P2X receptors agonist α,β -meATP, indicating that P2Y receptors play a crucial role in activation of MAPK/ERK cascade in HUVEC.

Recently, using real-time PCR and Western blotting analysis, it has been demonstrated that P2Y₁, P2Y₂, P2Y₄, P2Y₆ and P2Y₁₁ are the most expressed P2 receptors in HUVEC [5]. At present, P2Y receptor subtypes are difficult to differentiate pharmacologically due to the lack of selective ligands, but our data suggest that the observed responses might be mediated by any of these P2Y receptors. In this way, 2-meSATP and UDP are the most potent agonists for the P2Y₁ and P2Y₆ subtypes respectively [20, 21], and although UTP is not an agonist at the P2Y₁ and P2Y₁₁ subtypes [22] we cannot yet rule out the possibility that UTP and ATP act at separate sites.

Previous reports have shown that sensitivity to PTX is variable from one P2Y receptor subtype to the other, suggesting the involvement of distinct G-proteins in the signaling transduction pathways. The inositol phosphate response mediated by P2Y₁, P2Y₆ and P2Y₁₁ receptors was not inhibited by PTX, while P2Y₂ and P2Y₄ receptors exhibited a partial sensitivity to PTX [3]. As a first step to elucidate the intracellular mechanisms of MAPK/ERK activation in response to ATP, we examined MAPK/ERK phosphorylation in cells pretreated with PTX. Our results indicated that in HUVEC the P2Y receptors are coupled to PTX-sensitive G-proteins, and suggested that probably P2Y₂ and/or P2Y₄ receptors are mainly involved in ATP-

evoked activation of MAPK/ERK. In addition, our findings demonstrated that MAPK/ERK activation depends completely on the upstream phosphorylation of MEK.

One of the main objectives of this study was to analyze the signaling pathways that link the ATP-induced P2Y receptors activation to MAPK/ERK signaling in HUVEC. Different cell systems seem to display different responses with respect to P2Y receptors. Thus, activation of P2Y receptors may stimulate MAPK/ERK cascade in a way partially mediated through RTK transactivation [23] or without cross-activation of RTK in a way that is dependent on integrin-mediated cell anchorage, intracellular-free Ca^{2+} elevation and activation of PKC [24]. Since, classical activators of MAPK/ERK pathway include various upstream mediators, further studies were carried out to identify the target molecules that may be involved in signaling events from P2Y receptors to MAPK/ERK activation.

Intracellular-free Ca^{2+} mobilization has been recognized as a possible regulator of MAPK/ERK cascade by GPCR activation. It is well established that activation of PLC, with the subsequent increase in levels of inositol-1,4,5-trisphosphate (IP_3) and intracellular-free Ca^{2+} , can activate the MAPK/ERK pathway through stimulation of different Ca^{2+} -dependent kinases, such as the proline-rich tyrosine kinase Pyk2, conventional PKC isoforms, and Ca^{2+} /calmodulin (CaM) kinases [25-27]. We have investigated whether changes in intracellular-free Ca^{2+} were necessary for the mechanism of MAPK/ERK activation. Exposure of HUVEC to ATP mobilizes intracellular-free Ca^{2+} in a PLC-dependent manner, but ATP-induced MAPK/ERK phosphorylation was not affected by Ca^{2+} chelation or PLC inhibition, indicating that MAPK/ERK activation in HUVEC occurs via Ca^{2+} -independent mechanisms.

Although our findings suggest that conventional PKC did not participate in MAPK/ERK activation, novel or atypical PKC have been associated with the activation of MAPK/ERK in response to GPCR stimulation [28]. To determine whether P2Y receptors activation of MAPK/ERK was PKC-dependent or not, HUVEC were treated overnight with phorbol ester to down-regulate novel PKC [18], or with low concentrations of PKC inhibitor GF109203X before ATP stimulation. Under these conditions, ATP-induced MAPK/ERK phosphorylation was unaffected, indicating that novel PKC stimulation was not necessary for MAPK/ERK activation by ATP. However, ATP-induced MAPK/ERK activation in HUVEC was inhibited by high concentration of

GF109203X or myristoylated PKC- ζ pseudosubstrate, suggesting that atypical PKC- ζ lies upstream in the signal transduction pathway linking P2Y receptors to MAPK/ERK activation.

PI3K has also been identified as a potential candidate that contributes to the activation of MAPK/ERK by GPCR, including P2Y receptors [23, 29]. Activation of PI3K catalytic subunits $\text{p110}\beta$ and $\text{p110}\gamma$ through the α -subunit or $\beta\gamma$ -subunits of the G-proteins [30] produce phosphatidylinositol-3,4,5-trisphosphate (PIP_3), which directly binds to pleckstrin homology (PH) domains of several proteins that might act as mediators of MAPK/ERK pathway. To investigate the possible role of PI3K in ATP-induced MAPK/ERK activation, we analyzed the effect of two selective inhibitors of PI3K, LY294002 and wortmannin. Our findings that ATP-stimulated MAPK/ERK activation was sensitive to both PI3K inhibitors indicated that PI3K activity is necessary to activate MAPK/ERK cascade in HUVEC.

Two main PH-domain-containing effector proteins have been proposed for interaction with PIP_3 in response to extracellular stimuli linking GPCR to MAPK/ERK cascade: AKT, also called protein kinase B (PKB), and PDK1 [31]. Modulation of AKT activity has been reported for P2Y in renal mesangial cells [32] and C6 glioma cells [33], but as far as we know there is not information regarding activation of AKT and PDK1 in HUVEC by P2Y stimulation. Since phosphorylation of both AKT and PDK1 is necessary for their catalytic activity [34, 35], we have investigated the possibility that these kinases activate the MAPK/ERK cascade analyzing Ser⁴⁷³ phosphorylation of AKT and Ser²⁴¹ phosphorylation of PDK1. Although our data revealed that basal AKT phosphorylation was dependent on PI3K activity, ATP did not affect Ser⁴⁷³ phosphorylation of AKT within the time period in which maximum MAPK/ERK activation was observed. However, time-course experiments revealed a rapid Ser²⁴¹ phosphorylation of PDK1 recruited to plasma membrane, as well as in cytosol preparations from HUVEC, which was markedly inhibited by PI3K inhibitors. Collectively, these findings suggest that PDK1, which possesses higher affinity for PIP_3 than AKT [36], acts downstream of PI3K playing a crucial role in ATP-induced MAPK/ERK activation. According to earlier studies, PDK1 could phosphorylate a number of protein serine/threonine kinases of the AGC kinase superfamily, including the activation loop sites of atypical PKC- ζ [37-38].

Activation of MAPK/ERK cascade by GPCR often involves cross-talk with classical RTK, such as the

epidermal growth factor receptor (EGFR). In general, phosphorylation of specific tyrosine residues of RTK leads to recruitment of several adaptor molecules that might cause activation of MAPK/ERK cascade through downstream signaling events from Ras [39, 40]. To explore the possible role of EGFR transactivation we have analyzed the effect of tyrphostin AG1478, a selective inhibitor of EGFR tyrosine kinase, on ATP-induced MAPK/ERK activation in HUVEC. Our finding that MAPK/ERK phosphorylation evoked by ATP was clearly insensitive to AG1478 suggests that activation of MAPK/ERK cascade takes place via EGFR-independent signaling mechanisms, although other RTK could be implicated in MAPK/ERK activation.

Recently, it has been demonstrated that G-proteins $G_{\alpha s}$ and $G_{\alpha i}$, but neither $G_{\alpha q}$, $G_{\alpha 12}$ nor $G_{\beta\gamma}$, stimulate the kinase activity of Src family of tyrosine kinase [41]. On the other hand, there is evidence that PI3K is also involved in the activation of Src tyrosine kinases [42], as well as in the stimulation of small GTPase Ras [43]. In addition, a direct interaction between the p85 regulatory subunit of PI3K and Src tyrosine kinases has been demonstrated [44, 45]. In this study we examined the involvement of Src family kinases and Ras in ATP-mediated MAPK/ERK activation in HUVEC. In accordance with previous findings [46], phosphorylation of MAPK/ERK by P2Y receptors stimulation was partially inhibited by pretreatment of cells with PP2, a

selective Src tyrosine kinases inhibitor. However, inhibition of isoprenylation, which plays a critical role in promoting the association of Ras with the cell membrane [47], by treatment of HUVEC with perillidic acid was ineffective in preventing ATP-induced MAPK/ERK phosphorylation. These findings indicate that MAPK/ERK activation induced by ATP was independent of Ras, but that some member of Src family was required for activation of MAPK/ERK cascade in HUVEC.

In summary, the findings of the present study indicate that extracellular nucleotides induce activation of MAPK/ERK which could be important for regulation endothelial cell functions, such as proliferation, migration or death cellular. Analysis of P2Y downstream effectors (see proposed in Fig. 10) reveal that the effects mediated by ATP occurs via PTX-sensitive G-proteins through PI3K-dependent mechanisms, in which PDK1 and PKC- ζ are two key molecules leading to MAPK/ERK activation in HUVEC.

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