Published in final edited form as:

Thromb Haemost. 2005 April ; 93(4): 735–742. doi:10.1267/THRO05040735.

Modulation of endothelial cell migration by extracellular nucleotides. Involvement of focal adhesion kinase and phosphatidylinositol 3-kinase - mediated pathways

Elzbieta Kaczmarek¹, Laurie Erb², Katarzyna Koziak¹, Robert Jarzyna¹, Marcia R. Wink¹, Olaf Guckelberger¹, J. Krzysztof Blusztajn³, Vickery Trinkaus-Randall³, Gary A. Weisman², and Simon C. Robson¹

¹Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

²University of Missouri-Columbia, Columbia, MO 65212

³Boston University School of Medicine, Boston MA 02118

SUMMARY

Extracellular nucleotides bind to type-2 purinergic/pyrimidinergic (P2) receptors that mediate various responses, such as cell activation, proliferation and apoptosis, implicated in inflammatory processes. The role of P2 receptors and their associated signal transduction pathways in endothelial cell responses has not been fully investigated. Here, it is shown that stimulation of human umbilical vein endothelial cells (HUVEC) with extracellular ATP or UTP increased intracellular free calcium ion concentrations ($[Ca^{2+}]_i$), induced phosphorylation of focal adhesion kinase (FAK), p130^{cas} and paxillin, and caused cytoskeletal rearrangements with consequent cell migration. Furthermore, UTP increased migration of HUVEC in a phosphatidylinositol 3-kinase (PI3-K)-dependent manner. BAPTA or thapsigargin inhibited the extracellular nucleotide-induced increase in $[Ca^{2+}]_i$, a response crucial for both FAK phosphorylation and cell migration. Furthermore, long-term exposure of HUVEC to ATP and UTP, agonists of the G protein-coupled P2Y2 and P2Y4 receptor subtypes, caused upregulation of α_v integrin expression, a cell adhesion molecule known to directly interact with P2Y2 receptors. Our results suggest that extracellular nucleotides modulate signaling pathways in HUVEC influencing cell functions, such as cytoskeletal changes, cellular adhesion and motility, typically associated with integrin-activation and the action of growth factors. We propose that P2Y2 and possibly P2Y4 receptors mediate those responses that are important in vascular inflammation, atherosclerosis and angiogenesis.

Keywords

endothelial cells; extracellular nucleotides; migration; P2 receptors; FAK

INTRODUCTION

Extracellular nucleoside di- and triphosphates appear in tissue fluids and plasma as a consequence of cellular responses to pro-inflammatory mediators such as cytokines (1), and in tissue damage with cell death. Nucleotides are secreted or released from aggregating platelets and activated vascular cells, including endothelial cells (EC) (2). These extracellular mediators

generate a variety of responses within the vasculature, such as platelet activation or aggregation, changes in smooth muscle contractility and vasodilatation (2,3). Extracellular nucleotides play an important role in both type I and type II activation of EC, inducing secretion of von Willebrand factor, and upregulation of VCAM-1 (4,5). They also have been implicated in vascular inflammation, atherosclerosis and angiogenesis (6–8).

Extracellular nucleotides serve as signaling molecules via P2 receptors (3). Pharmacological, functional, and molecular cloning data have facilitated the classification of P2 receptors into two main groups: P2X that form ligand-gated ion channels, and P2Y that are heptahelical G-protein-linked receptors. EC are known to express multiple P2 receptor subtypes. Analyses of P2 receptors at the RNA and protein levels, and evaluation of their pharmacological characteristics, indicate that EC express P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2X1, P2X2, P2X4, P2X5, P2X6, and P2X7 receptors (9–11). Among these P2 receptor subtypes, the P2X receptors, in contrast to P2Y2, P2Y4 and P2Y6 receptors, are not activated by uridine nucleotides, whereas P2Y1 receptors are preferentially activated by ADP.

Studies of nucleotide-activated signal transduction pathways in EC have been limited. In general, early cellular responses to extracellular nucleotides include elevation of $[Ca^{2+}]_i$ due to calcium influx or release from intracellular stores (12). Calcium ions function as second messengers, regulating numerous cellular processes. Brief spikes and waves in the $[Ca^{2+}]_i$ result in the regulation of the expression of selected genes (13).

FAK and p130^{cas} appear to be key factors in those signaling pathways implicated in vascular cell migration and differentiation (14,15). It has been reported that $\alpha_v\beta_3$ integrin plays a further role in mediating cell adhesion to the extracellular matrix, cell migration and angiogenesis (16). Integrins are involved in activation of the FAK signal transduction pathway (17), while increases in $[Ca^{2+}]_i$ are necessary to induce the interaction of integrins with the cytoskeleton (18). The integrin-binding motif RGD in P2Y2 receptors has been shown to promote colocalization with $\alpha_v\beta_3$ integrin, enabling nucleotides to functionally activate integrin signaling pathways (19). Hence, interactions among P2 receptors, integrins and certain signaling pathways appear to be of major importance in generating responses of EC to (patho) physiological stimuli.

Our data presented here indicate that extracellular nucleotides induce phosphorylation of proteins involved in focal adhesion: FAK, paxillin and p130^{cas}, leading to EC spreading and migration. Increases in $[Ca^{2+}]_i$ mediated by P2Y receptors are essential for activation of FAK and cell migration. Activation of PI3-K also is crucial for UTP-induced migration of HUVEC. These data suggest that extracellular nucleotides and P2 receptors are important factors that modulate vascular inflammation, wound healing and angiogenesis.

MATERIALS AND METHODS

Materials

Antibodies to phosphotyrosine, FAK, paxillin, and p130^{cas} were purchased from Transduction Laboratories (San Diego, CA). Anti-phospho-Tyr₃₉₇-FAK was from Biosource International (Camarillo, CA). Antibodies to human α_v and α_5 integrins were from Santa Cruz (Santa Cruz, CA). Protein A/G Agarose, 1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), thapsigargin, wortmannin, and LY294002 were from Gibco-BRL (Grand Island, NY). Type I collagen (1% stock solution) was from Sigma (St. Louis, MO).

Cell culture

Human umbilical vein endothelial cells (HUVEC) (Cambrex, Walkersville, MD), were cultured in EGM-2 BulletKit medium (Cambrex, Walkersville, MD) containing human epidermal growth factor, hydrocortisone, human fibroblast growth factor, vascular endothelial growth factor, ascorbic acid, gentamicin, amphotericin-B, human insulin-like growth factor, heparin and 2% (v/v) fetal bovine serum. Cells were used for experiments at fourth passage, and at full confluence.

Measurement of $[Ca^{2+}]_i$

HUVEC were grown to confluency on 22×22 mm glass coverslips. Cells were incubated in 10 mM HEPES containing 137 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 3 mM $CaCl_2$, and 25 mM glucose, pre-loaded with 5 μ M fluo-3-AM for 20 min, rinsed, and exposed to extracellular nucleotides. Changes in $[Ca^{2+}]_i$ were monitored using a Zeiss LSM 510 Axiovert confocal laser scanning microscope equipped with an Argon laser. Cells were perfused with HEPES-buffered saline (60 sec), nucleotide (120 sec), HEPES-buffered saline (100 sec) and a second dose of nucleotide (120 sec). The LSM 510 Imaging Software was used to determine the percent change in average fluorescence versus time.

Cell lysate preparation

Cells were washed three times with ice cold Tris-saline buffer (20 mM Tris, pH 7.5, 150 mM NaCl) and lysed in cell lysis buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 1% (v/v) Nonidet P40, 1 mM sodium orthovanadate, 100 mM sodium fluoride, 2 μ g/ml aprotinin, 1 μ g/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride), incubated on ice for 20 min and centrifuged at ~14,000×g for 5 min at 4°C. Supernatants were kept at -80°C until used for analysis. Protein concentrations were measured using a Bio-Rad (Hercules, CA) detergent compatible protein assay, a modification of Lowry's method (20), according to the manufacturer's protocol.

Immunoprecipitation

Protein aliquots obtained from cells harvested at each time-point (200 μ g of total cell lysate; final concentration ~1 mg/ml) were incubated with constant mixing with 1.5 μ g of anti-phosphotyrosine or anti-paxillin antibody at 4°C for 2 h. Twenty five μ l of Protein A/G plus Agarose suspension were added and samples were mixed for 1 h at 4°C. Agarose gel was washed 4 times with cell lysis buffer and suspended in 15 μ l of reducing electrophoresis sample buffer, boiled for 5 min and centrifuged at ~8,000×g for 1 min. Supernatants were analyzed by gel electrophoresis followed by Western analysis.

Western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (21) using a 4–12% polyacrylamide gel under reducing conditions followed by transfer to PVDF membrane (Immobilon P, Millipore, Bedford, MA) by semi-dry electroblotting. After blocking with either 5% (w/v) milk or 1% (w/v) BSA in Tris-saline buffer containing 0.1% (v/v) Tween 20, membranes were probed with an appropriate antibody: anti-human FAK (diluted 1000x), anti-human p130^{cas} (diluted 1000x), anti-human paxillin (diluted 10000x), anti-phospho-Tyr₃₉₇-FAK (diluted 2000x), and anti-phosphoTyr (diluted 2500x), and antibodies were visualized using horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (Pierce, Rockford, IL) (diluted 5000x), followed by Enhanced Chemiluminescence assay (New England Nuclear, Beverly, MA), according to the manufacturer's instructions.

Cytoskeletal rearrangements

HUVEC, untreated or incubated with UTP or ATP, were fixed with 3.7% (v/v) paraformaldehyde for 10 min, permeabilized with 0.1% (v/v) Triton X-100 for 3–5 min, blocked with 1% (w/v) BSA for 20 min, stained with Alexa Fluor 488 Phalloidin (Molecular Probes, Eugene, OR; 20 μ l of methanol stock in 250 μ l of 1% (w/v) BSA in PBS) for 20 min, and examined by fluorescence microscopy according to manufacturer's instructions.

Cell migration assay

Cell migration was detected with the Transwell (Costar) system (6.5 mm wells; 8 μ m pore size) that allows cells to migrate through pores in polycarbonate membranes. Briefly, HUVEC were trypsinized, washed, resuspended in EGM-2 basal medium containing 0.1% (w/v) BSA, and seeded (10^5 cells in 100 μ l) into the top chamber of Transwells that were precoated on the undersurface with 0.05% (w/v) type I collagen. Cells were incubated for 5 h with agonist (ATP or UTP; added to the lower chamber) and/or additional agents (wortmannin, 200 nM; LY294002, 10 μ M; or BAPTA-AM, 10 μ M; 20 min preincubation with cells). Cells that migrated to the bottom part of the filter were fixed in methanol for 10 min, stained with 0.2% (w/v) crystal violet in 2% (v/v) ethanol, and counted in 5–6 fields from each well. In some experiments, crystal violet was eluted from cells with 10% (v/v) acetic acid and migration was estimated by measurement of absorbance at 590 nm. Experiments were run in triplicate and repeated at least 3 times.

RESULTS

Calcium flux in HUVEC in response to ATP or UTP

It has been shown on many occasions that various cells exposed to extracellular nucleotides respond with an increase in $[Ca^{2+}]_i$. Similarly, treatment of HUVEC with 100 μ M ATP or UTP caused a rapid increase in $[Ca^{2+}]_i$ (Fig. 1). To determine the source of the increase in $[Ca^{2+}]_i$, (extracellular versus intracellular stores) cells were preincubated for 20 min in HEPES buffer containing either 10 μ M BAPTA-AM (10 μ M), an intracellular chelator of calcium ions, or thapsigargin (1 μ M), a selective Ca^{2+} -ATPase inhibitor that depletes intracellular calcium stores. HUVEC calcium responses were also investigated in buffer with no calcium that additionally contained 3 mM EGTA. Pretreatment of HUVEC with either BAPTA or thapsigargin suppressed the increase in $[Ca^{2+}]_i$ caused by ATP or UTP while EGTA had minor effects on levels of $[Ca^{2+}]_i$ (Fig. 1). These results indicate that ATP and UTP modulate $[Ca^{2+}]_i$ in HUVEC primarily by inducing calcium ion release from intracellular stores, and suggest that select P2Y receptors for uridine nucleotides are responsible for this response. However, some minor effect of P2X receptor, especially P2X4 that is highly expressed in HUVEC, is still possible. UDP (10 and 100 μ M) did not induced changes in $[Ca^{2+}]_i$ while responses to ADP (and 2-methylthio-ADP) were significantly altered although changes were less prominent than for similar concentration of ATP and UTP (data not shown). In HUVEC stimulated with α,β -methylene-ATP, an potent agonist of P2X1 and P2X3 receptors, an increase in $[Ca^{2+}]_i$ was not detected (data not shown), suggesting that the observed changes in $[Ca^{2+}]_i$ were not associated with these P2X receptor activation responses.

ATP/UTP-induced phosphorylation of FAK, paxillin and p130^{cas}

Changes in the $[Ca^{2+}]_i$ initiate various signal transduction pathways. We investigated the specific signaling pathways activated by extracellular nucleotides in HUVEC. Treatment of HUVEC with 100 μ M ATP for 0 to 10 min caused tyrosine phosphorylation of several proteins including these with approximate molecular masses of 68, 125, and 130 kDa. By immunoprecipitation with anti-phospho-Tyr antibody and Western blot analysis we identified those proteins as FAK, p130^{cas}, and paxillin (Figs. 2A, B and C, respectively). Consistent with

these results, anti-paxillin antibody immunoprecipitated tyrosine-phosphorylated paxillin (detected with anti-phospho-Tyr antibody) from cell lysates of HUVEC treated with ATP (Figs. 2D and E). Tyrosine phosphorylation of FAK, p130^{cas} and paxillin also was detected in HUVEC treated with 100 μ M UTP (Fig. 3) with a maximal stimulation seen within 1–2 min. Induction of phosphorylation of FAK at Tyr₃₉₇ by ATP and UTP was substantially increased even for lower concentrations of these nucleotides (1 and 10 μ M; data not shown).

FAK activation induced by nucleotides in HUVEC can result from integrin activation and/or an increase in $[Ca^{2+}]_i$, since FAK activation has been shown to occur by these pathways (22, 23). HUVEC preincubated for 20 min with BAPTA-AM (10 μ M) and then stimulated with UTP (100 μ M) did not exhibit Tyr₃₉₇-FAK phosphorylation as compared to UTP-treated control cells (Fig. 4A). Similar inhibitory effects of BAPTA were observed for ATP-induced FAK phosphorylation (data not shown). In parallel experiments, BAPTA also inhibited FAK phosphorylation in HUVEC stimulated with 100 μ M UTP, as indicated by immunoprecipitation of cell lysates with anti-phospho-Tyr antibody and immunodetection with anti-FAK antibody (Fig. 4B). These results suggest that UTP/ATP-induced FAK activation in HUVEC is calcium-dependent.

Cytoskeletal rearrangements

Activation of FAK can result in cell spreading and migration (24). In HUVEC under conditions that activate FAK (Figs. 2–4), ATP or UTP (100 μ M) affected cellular architecture, and increased the degree of cell spreading and stress fiber formation as shown in cells stained with Alexa Fluor 488 Phalloidin to visualize actin filaments by fluorescence microscopy in comparison to untreated cells (Fig. 5). This effect was diminished in cells treated for 20 min with 10 μ M BAPTA-AM (data not shown).

Cell migration

To determine the role of UTP and ATP in EC migration, modified Boyden chambers were used. Both UTP and ATP substantially increased cell migration when compared to control, untreated cells (Fig. 6A and B). However, ADP (1, 10 and 100 μ M) had no effects on HUVEC migration (Fig. 6C), indicating that the specific P2Y receptors responding to this ligand appear not to be involved in HUVEC migration. Wortmannin (200 nM), and LY294002 (10 μ M), inhibitors of PI3-K, significantly attenuated UTP-induced HUVEC migration (Figs. 6D and 6E, respectively). These results suggest that PI3-K activation by extracellular nucleotides regulates cell migration, consistent with the reported involvement of this kinase in chemokine-stimulated cell migration (25). BAPTA-AM (10 μ M, 20 min preincubation) also significantly reduced migration of HUVEC induced by UTP (Fig. 6F), in agreement with the known role of calcium-dependent FAK activation in cell migration (26).

Upregulation of α_v integrin expression by UTP

The involvement of $\alpha_v\beta_3$ integrin in extracellular matrix-mediated cell adhesion, cell migration, and angiogenesis has been well documented (27,28), and P2Y₂ receptors are known to colocalize with $\alpha_v\beta_3$ integrin, causing maximal UTP-induced stimulation of FAK (19). We have determined that treatment of HUVEC with UTP (Fig. 7) or ATP (data not shown) increased α_v integrin expression in a time-dependent manner, without affecting α_5 integrin expression, suggesting novel mechanisms by which nucleotide-mediated upregulation of integrins modulates cell migration on different extracellular substrata.

DISCUSSION

Our data demonstrate that in HUVEC extracellular nucleotides increase $[Ca^{2+}]_i$, and induce activation of FAK and PI3-K, cytoskeletal rearrangements and cell migration, with the associated upregulation of α_v integrins.

While much is known about the activation of EC by pro-inflammatory cytokines, it has become clear only recently that nucleotides are capable of activating EC (4,5,29,30). Mechanisms of extracellular nucleotide action on endothelium, and factors involved in signal transduction downstream of P2 receptors, have remained largely unexplored.

Extracellular nucleotides induce calcium fluxes in many cell systems (31,32). This nucleotide-induced increase in $[Ca^{2+}]_i$ may originate either from calcium influx mediated by P2X receptors acting as ion channels, or from intracellular stores due to G-protein-coupled P2Y receptor-mediated activation of phospholipase C and hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate to generate inositol 1,4,5-triphosphate (IP_3), an intracellular messenger for the release of stored calcium ions from endoplasmic reticulum.

In the experiments performed with BAPTA, we do not observe complete inhibition of $[Ca^{2+}]_i$ flux. However, the inhibition of nucleotide-induced increases in $[Ca^{2+}]_i$ by thapsigargin and the lesser effects of EGTA, imply that P2Y receptors are involved in this process. We cannot entirely eliminate the role of P2X receptors, especially P2X4 receptor that is highly expressed in HUVEC (11) (data not shown). Furthermore, α,β -methylene-ATP did not induce changes in $[Ca^{2+}]_i$, excluding any major role for P2X1 and P2X3 receptors in the mediation of calcium flux. However, we presume that these minimal increases in $[Ca^{2+}]_i$ in HUVEC treated with ATP or UTP in the presence of BAPTA-AM are not directly linked to P2X receptor activation. One of the possibilities is that of capacitative calcium entry via receptor-activated calcium entry channels (33).

ADP triggers increases in $[Ca^{2+}]_i$ but does not affect HUVEC migration. Therefore P2Y1, P2Y12 and P2Y13 receptors can be excluded as important receptors mediating migration. Additionally, P2Y12 receptor is not expressed on HUVEC (11) (data not shown). The possible role of ATP-stimulated P2Y11 receptors in EC migration cannot be fully discounted because of a current lack of specific agonists and antagonists for this receptor.

The ability of both UTP and ATP to activate responses in HUVEC is characteristic of the pharmacological profile of P2Y2 receptors that are activated equipotently by either ATP or UTP (34). UTP is the selective agonist of P2Y4 receptors (35); hence this receptor might also be involved in the responses we have observed. Both P2Y2 and P2Y4 receptors are expressed in HUVEC (9,11) (data not shown). Although the metabolism of extracellular nucleotides could potentially generate agonists for P2Y6 receptors, i.e., nucleoside diphosphates, the involvement of this uridine nucleotide receptor is less likely since expression of this receptor in HUVEC is relatively low and cells do not respond to 100 μ M UDP with increase in $[Ca^{2+}]_i$ (data not shown). UTP does not activate P2X receptors, and therefore it is unlikely that these receptors are involved in the reported responses in HUVEC, although effects of ATP may be mediated, at least in part, by the activation of P2X receptors.

We have determined that FAK, paxillin and p130^{cas}, molecules that regulate cell adhesion, spreading and motility, undergo phosphorylation following stimulation of HUVEC with ATP (Fig. 2) or UTP (Fig. 3). These protein phosphorylations are transient, with maximum levels observed within 1–2 min of agonist addition. Phosphorylation of FAK and paxillin has been observed previously in EC treated with VEGF (36), and it is intriguing to note that P2Y2 receptors have been recently reported to transactivate growth factor receptors including VEGF receptors (37,38).

Our data showing that treatment of HUVEC with ATP or UTP stimulates FAK phosphorylation are consistent with the hypothesis that FAK plays an important role in the P2Y receptor-activated pathway(s) leading to cell spreading and migration. Whether integrin-mediated pathways (39), calcium-dependent pathways (acting via PLC and PKC) (23) or both (17) are responsible for the activation of FAK, remain to be determined. However, our experiments with BAPTA do indicate that increases in $[Ca^{2+}]_i$ are critical for FAK phosphorylation in HUVEC (Fig. 4).

The formation of actin stress fibers has been described in EC exposed to mechanical stress or pro-inflammatory agonists, such as histamine or thrombin (40). Our current results demonstrate that extracellular nucleotides exert comparable effects and can induce actin filament formation, and cytoskeletal rearrangements resulting in spreading of HUVEC (Fig. 5). Similar actin stress fiber formation has been described previously for aortic smooth muscle cells treated with ATP or UTP (41). UTP-induced stress fiber formation was decreased by the presence of BAPTA (data not shown).

Activation of FAK is known to play a crucial role in cell migration (42). FAK^{-/-} cells plated on fibronectin fail to spread as effectively as wild type cells (43). Additionally, FAK-null fibroblasts exhibit a rounded morphology and show defects in cell migration (44). Other studies have shown that inhibition of FAK with the dominant negative mutant FRNK causes decreased cell motility (36).

Our data indicate that exposure of HUVEC to extracellular UTP or ATP enhances cell migration with the maximum effect observed at 10 μ M nucleotide (Figs. 6A and B). At this concentration of nucleotides, the phosphorylation of FAK was substantially increased (data not shown). Since chelation of intracellular calcium with BAPTA inhibited UTP-induced FAK phosphorylation (Fig. 4) and migration (Fig. 6E) in HUVEC, these responses are likely linked to activation of P2Y2 and P2Y4 receptors.

We observed similar effects of augmented cell migration with lower nucleotide concentrations in epithelial cells (45). We assume that one of the possibilities of decreased HUVEC migration with higher agonist concentration (100 μ M ATP and UTP versus 10 μ M) relates to the desensitization of these receptors. Desensitization and possible receptor internalization may be less crucial in fast (seconds or minutes after addition of agonist) responses, such as increase in $[Ca^{2+}]_i$ and protein phosphorylation. However, migration is longer process (hours) and receptor desensitization can influence this process.

Additionally, levels of extracellular nucleotides are modulated by nucleoside triphosphate diphosphohydrolases (NTPDases), ecto-enzymes of the CD39 family that hydrolyze tri- and diphosphate nucleosides to the respective di- and monophosphate nucleosides, by ecto-nucleotide kinases as well as ecto-ATP pyrophosphatase, and ATP synthase. In the vasculature, NTPDase 1 (CD39) is the dominant ecto-enzyme influencing levels of extracellular nucleotides (30,46).

The mechanism of UTP-induced migration of HUVEC is dependent upon PI3-K activation since the PI3-K inhibitors wortmannin and LY294002 both attenuate migration of HUVEC (Figs. 6D and E). Previous reports suggest that FAK phosphorylation depends on the activity of PI3-K (25). Another study has indicated that PI3-K is activated downstream of FAK (47). The relationship between FAK and PI3-K activation by extracellular nucleotides in EC requires further evaluation.

Interactions between the P2Y receptors and $\alpha_v\beta_3$ integrin and their respective signaling pathways also require additional investigation. It has been shown that $\alpha_v\beta_3$ integrin promotes cell migration and provides key signals for the regulation of cell proliferation and

differentiation. The expression of $\alpha_v\beta_3$ integrin is upregulated in new capillaries and its inhibition with specific antibodies blocks capillary tube formation induced in Matrigel (48). We have observed an increase in the expression of α_v integrin at the protein level following stimulation of HUVEC with UTP (Fig. 7) or ATP (data not shown). Similarly, rapid upregulation of this integrin subunit has been previously shown for microvascular EC stimulated with a metabolite of arachidonic acid (49). Detailed mechanisms for upregulation of α_v integrin by extracellular nucleotides are not clear at this time. It is possible that nitric oxide (NO) may be implicated in this process, since extracellular nucleotides can stimulate NO secretion (50) with consequent upregulation of $\alpha_v\beta_3$ integrin (48).

In conclusion, our data indicate that extracellular nucleotides, ATP and UTP, induce endothelial cell responses such as cell spreading and PI-3 kinase-dependent migration. These responses are likely due to activation of P2Y2 and P2Y4 receptors. We further demonstrate that changes in $[Ca^{2+}]_i$ are crucial for both FAK phosphorylation and cell migration. Our results suggest that endothelial cell P2Y receptors should be regarded as a new group of receptors that modulate vascular inflammation, wound healing, angiogenesis and presumably atherosclerosis via pathways involving regulation of the expression and transactivation of integrins.

Acknowledgments

This project was supported by grants: HL66167 (EK), KBN3P05A08723 (KK), AG09525 (JKB), NEI EY06000 (VTR), AG18357 (GAW), and HL57307/HL63972 (SCR).

Abbreviations used

HUVEC	human umbilical vein endothelial cells
IP3-K	phosphatidylinositol 3-kinase
BAPTA-AM	1,2-bis-(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester
$[Ca^{2+}]_i$	intracellular free calcium ion concentration

REFERENCES

1. Filippini A, Taffs RE, Sitkovsky MV. Extracellular ATP in T-lymphocyte activation: possible role in effector functions. *Proc Natl Acad Sci USA* 1990;87:8267–71. [PubMed: 2146684]
2. Gordon JL. Extracellular ATP: effects, sources and fates. *Biochem J* 1986;233:309–19. [PubMed: 3006665]
3. Dubyak GR, el Moatassim C. Signal transduction via P2-purinergic receptors for extracellular ATP and other nucleotides. *Am J Physiol* 1993;265:c577–606. [PubMed: 8214015]
4. Vischer UM, Wollheim CB. Purine nucleotides induce regulated secretion of von Willebrand factor: involvement of cytosolic Ca^{2+} and cyclic adenosine monophosphate-dependent signaling in endothelial exocytosis. *Blood* 1998;91:118–27. [PubMed: 9414275]
5. Seye CI, Yu N, Jain R, et al. The P2Y2 nucleotide receptor mediates UTP-induced vascular cell adhesion molecule-1 expression in coronary artery endothelial cells. *J Biol Chem* 2003;278:24960–5. [PubMed: 12714597]
6. Di Virgilio F, Solini A. P2 receptors: new potential players in atherosclerosis. *Br J Pharmacol* 2002;135:831–42. [PubMed: 11861311]
7. Burnstock G. Purinergic signaling and vascular cell proliferation and death. *Arterioscler Thromb Vasc Biol* 2002;22:364–73. [PubMed: 11884276]
8. Luttikhuisen DT, Harmsen MC, de Leij LF, et al. Expression of P2 receptors at sites of chronic inflammation. *Cell Tissue Res* 2004;317:289–98. [PubMed: 15290227]

9. Jin J, Dasari VR, Sistare FD, et al. Distribution of P2Y receptor subtypes on haematopoietic cells. *Br J Pharmacol* 1998;123:789–94. [PubMed: 9535005]
10. Burnstock G, Kennedy C. Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* 1985;16:433–40. [PubMed: 2996968]
11. Wang L, Karlsson L, Moses S, et al. P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 2002;40:841–53. [PubMed: 12451317]
12. Communi D, Raspe E, Pirotton S, et al. Coexpression of P2Y and P2U receptors on aortic endothelial cells. Comparison of cell localization and signaling pathways. *Circulation Research* 1995;76:191–8. [PubMed: 7834829]
13. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 1998;392:933–6. [PubMed: 9582075]
14. BurrIDGE K, Turner CE, Romer LH. Tyrosine phosphorylation of paxillin and pp125FAK accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J Cell Biol* 1992;119:893–903. [PubMed: 1385444]
15. Cary LA, Han DC, Polte TR, et al. Identification of p130Cas as a mediator of focal adhesion kinase-promoted cell migration. *J Cell Biol* 1998;140:211–21. [PubMed: 9425168]
16. Penta K, Varner JA, Liaw L, et al. Del1 induces integrin signaling and angiogenesis by ligation of alphaVbeta3. *J Biol Chem* 1999;274:11101–9. [PubMed: 10196194]
17. Shattil SJ, Haimovich B, Cunningham M, et al. Tyrosine phosphorylation of pp125FAK in platelets requires coordinated signaling through integrin and agonist receptors. *J Biol Chem* 1994;269:14738–45. [PubMed: 7514181]
18. Nebe B, Bohn W, Sanftleben H, et al. Induction of a physical linkage between integrins and the cytoskeleton depends on intracellular calcium in an epithelial cell line. *Exp Cell Res* 1996;229:100–10. [PubMed: 8940254]
19. Erb L, Liu J, Ockerhausen J, et al. An RGD sequence in the P2Y(2) receptor interacts with alpha(V) beta(3) integrins and is required for G(o)-mediated signal transduction. *J Cell Biol* 2001;153:491–501. [PubMed: 11331301]
20. Lowry OH, Rosebrough NJ, Farr AL, et al. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;193:265–75. [PubMed: 14907713]
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5. [PubMed: 5432063]
22. Palazzo AF, Eng CH, Schlaepfer DD, et al. Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling. *Science* 2004;303:836–9. [PubMed: 14764879]
23. Anfosso F, Bardin N, Vivier E, et al. Outside-in signaling pathway linked to CD146 engagement in human endothelial cells. *J Biol Chem* 2001;276:1564–9. [PubMed: 11036077]
24. Cary LA, Guan JL. Focal adhesion kinase in integrin-mediated signaling. *Front Biosci* 1999;4:D102–13. [PubMed: 9889179]
25. Reiske HR, Kao SC, Cary LA, et al. Requirement of phosphatidylinositol 3-kinase in focal adhesion kinase- promoted cell migration. *J Biol Chem* 1999;274:12361–6. [PubMed: 10212207]
26. Giannone G, Ronde P, Gaire M, et al. Calcium oscillations trigger focal adhesion disassembly in human U87 astrocytoma cells. *J Biol Chem* 2002;277:26364–71. [PubMed: 12011063]
27. Berthet V, Rigot V, Nejjar M, et al. The endoproteolytic processing of alphavbeta5 integrin is involved in cytoskeleton remodelling and cell migration. *FEBS Lett* 2004;557:159–63. [PubMed: 14741360]
28. Ikari Y, Yee KO, Schwartz SM. Role of alpha5beta1 and alphavbeta3 integrins on smooth muscle cell spreading and migration in fibrin gels. *Thromb Haemost* 2000;84:701–5. [PubMed: 11057873]
29. von Albertini M, Palmethofer A, Kaczmarek E, et al. Extracellular ATP and ADP activate transcription factor NF-kappa B and induce endothelial cell apoptosis. *Biochem Biophys Res Commun* 1998;248:822–9. [PubMed: 9704012]
30. Goepfert C, Imai M, Brouard S, et al. CD39 modulates endothelial cell activation and apoptosis. *Molecular Medicine* 2000;6:591–603. [PubMed: 10997340]
31. Greenberg S, Di VF, Steinberg TH, et al. Extracellular nucleotides mediate Ca²⁺ fluxes in J774 macrophages by two distinct mechanisms. *J Biol Chem* 1988;263:10337–43. [PubMed: 3392016]

32. Viana F, de Smedt H, Droogmans G, et al. Calcium signalling through nucleotide receptor P2Y2 in cultured human vascular endothelium. *Cell Calcium* 1998;24:117–27. [PubMed: 9803312]
33. Bird GS, Aziz O, Lievreumont JP, et al. Mechanisms of phospholipase C-regulated calcium entry. *Curr Mol Med* 2004;4:291–301. [PubMed: 15101686]
34. Erb L, Lustig KD, Sullivan DM, et al. Functional expression and photoaffinity labeling of a cloned P2U purinergic receptor. *Proc Natl Acad Sci U S A* 1993;90:10449–53. [PubMed: 8248130]
35. Charlton SJ, Brown CA, Weisman GA, et al. Cloned and transfected P2Y4 receptors: characterization of a suramin and PPADS-insensitive response to UTP. *Br J Pharmacol* 1996;119:1301–3. [PubMed: 8968535]
36. Avraham HK, Lee TH, Koh Y, et al. Vascular endothelial growth factor regulates focal adhesion assembly in human brain microvascular endothelial cells through activation of the focal adhesion kinase and related adhesion focal tyrosine kinase. *J Biol Chem* 2003;278:36661–8. [PubMed: 12844492]
37. Soltoff SP. Related adhesion focal tyrosine kinase and the epidermal growth factor receptor mediate the stimulation of mitogen-activated protein kinase by the G-protein-coupled P2Y2 receptor. Phorbol ester or $[Ca^{2+}]_i$ elevation can substitute for receptor activation. *J Biol Chem* 1998;273:23110–7. [PubMed: 9722539]
38. Seye CI, Yu N, Gonzalez FA, et al. The P2Y2 nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). *J Biol Chem* 2004;279:35679–86. [PubMed: 15175347]
39. Eliceiri BP, Puente XS, Hood JD, et al. Src-mediated coupling of focal adhesion kinase to integrin $\alpha(v)\beta(5)$ in vascular endothelial growth factor signaling. *J Cell Biol* 2002;157:149–60. [PubMed: 11927607]
40. Vanhauwe JF, Thomas TO, Minshall RD, et al. Thrombin receptors activate G(o) proteins in endothelial cells to regulate intracellular calcium and cell shape changes. *J Biol Chem* 2002;277:34143–9. [PubMed: 12039967]
41. Sauzeau V, Le Jeune H, Cario-Toumaniantz C, et al. P2Y(1), P2Y(2), P2Y(4), and P2Y(6) receptors are coupled to Rho and Rho kinase activation in vascular myocytes. *Am J Physiol Heart Circ Physiol* 2000;278:H1751–61. [PubMed: 10843869]
42. Richardson A, Parsons T. A mechanism for regulation of the adhesion-associated protein tyrosine kinase pp125FAK. *Nature* 1996;380:538–40. [PubMed: 8606775]
43. Ilic D, Furuta Y, Kanazawa S, et al. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature* 1995;377:539–44. [PubMed: 7566154]
44. Sieg DJ, Hauck CR, Schlaepfer DD. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci* 1999;112:2677–91. [PubMed: 10413676]
45. Klepeis VE, Weinger I, Kaczmarek E, et al. P2Y receptors play a critical role in epithelial cell communication and migration. *J Cell Biochem* 2004;93:1115–33. [PubMed: 15449317]
46. Kaczmarek E, Koziak K, Sevigny J, et al. Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J Biol Chem* 1996;271:33116–22. [PubMed: 8955160]
47. Qi JH, Claesson-Welsh L. VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase. *Exp Cell Res* 2001;263:173–82. [PubMed: 11161716]
48. Lee PC, Kibbe MR, Schuchert MJ, et al. Nitric oxide induces angiogenesis and upregulates $\alpha(v)\beta(3)$ integrin expression on endothelial cells. *Microvasc Res* 2000;60:269–80. [PubMed: 11078643]
49. Tang DG, Diglio CA, Bazaz R, et al. Transcriptional activation of endothelial cell integrin αv by protein kinase C activator 12(S)-HETE. *J Cell Sci* 1995;108:2629–44. [PubMed: 7593304]
50. Buvinic S, Briones R, Huidobro-Toro JP. P2Y(1) and P2Y(2) receptors are coupled to the NO/cGMP pathway to vasodilate the rat arterial mesenteric bed. *Br J Pharmacol* 2002;136:847–56. [PubMed: 12110609]

Figure 1. BAPTA-AM and thapsigargin attenuate extracellular nucleotide-induced increases in $[Ca^{2+}]_i$ in HUVEC

HUVEC were loaded with fluo-3 and preincubated for 20 min with 10 μ M BAPTA-AM or 1 μ M thapsigargin. These experiments were performed in the presence of extracellular Ca^{2+} (3 mM). In a parallel set of experiments, the reaction buffer did not contain Ca^{2+} , and was supplemented with 3 mM EGTA. Increases in $[Ca^{2+}]_i$ in response to UTP or ATP were determined by a confocal microscopy. Data are representative of results from three independent experiments.

Figure 2. Phosphorylation of FAK, p130^{cas} and paxillin in response to ATP

Western blot analysis of HUVEC stimulated with ATP (100 μ M) for the indicated time. Cell lysates were immunoprecipitated with anti-phosphoTyr antibody and immunoblotted with antibodies to human-FAK (A), p130^{cas} (B) or paxillin (Pax) (C). In another set of experiments, cell lysates were immunoprecipitated with anti-paxillin and immunoblotted with anti-phosphoTyr (P-Tyr) (D) or anti-paxillin (E) antibodies. The data are representative of results from at least three experiments.

Figure 3. Effect of UTP on phosphorylation of FAK, p130^{cas} and paxillin in HUVEC
Western blot analysis of HUVEC stimulated with UTP (100 μ M) for the indicated time. Cell lysates were immunoprecipitated with anti-phosphoTyr antibody and immunoblotted with antibodies to human FAK (A), p130^{cas} (B) and paxillin (Pax) (C). The data are representative of results from three experiments.

Figure 4. Dependence of UTP-induced FAK phosphorylation on increases in $[Ca^{2+}]_i$
 Western blot analysis of HUVEC stimulated with UTP (100 μ M) for the indicated time, with or without BAPTA-AM. (A) Cell lysates were immunoblotted with anti-phospho-Tyr₃₉₇-FAK antibody or anti-FAK antibody. (B) Cell lysates (200 μ g) were immunoprecipitated with anti-phospho-Tyr antibody and immunoblotted with anti-FAK antibody.

Figure 5. Extracellular nucleotides induce cytoskeletal changes in HUVEC

Fluorescence microscopy of (A) control, (B) ATP- or (C) UTP-treated HUVEC stained with Alexa Fluor 488 Phalloidin (magnification $\times 100$). Stress fibers (D) formed in response to ATP are shown at higher magnification ($\times 200$). Data are representative of results from three independent experiments.

Figure 6. Extracellular ATP or UTP enhances migration of HUVEC

HUVEC seeded in the upper chamber of Transwells were incubated with (A) 0, 1, 10 or 100 μ M ATP, (B) UTP or (C) ADP added to the lower chamber, and cell migration was determined after 5 h. Migration of HUVEC induced by UTP (100 μ M) also was determined in the presence or absence of (D) wortmannin (W; 200 nM) or (E) LY294002 (10 μ M), inhibitors of PI3-K. (F) HUVEC were treated for 20 min with BAPTA-AM (10 μ M) and UTP-induced cell migration was determined. Data are representative of results from at least three independent experiments and are expressed as means $\pm$ SD. The data (D, E and F) were analyzed by two-way analysis of variance followed by application of the Tukey test. * $p < 0.01$ was considered to be statistically significant.

Figure 7. Upregulation of α_v integrin expression in HUVEC treated with UTP
HUVEC were incubated with 100 μ M UTP from 0 to 3 h, and Western blot analysis was performed with (A) anti-human α_v and or (B) anti-human α_5 antibody. (C) Bands representing α_v and α_5 integrins were scanned, and data shown are means $\pm$ S.E. of results from three experiments.