The effect of ATP on nuclear translocation of mitogen-activated protein kinases, genes expression and cell proliferation in human endometrial stromal cells

Abstract

ATP has been shown to activate the phospholipase C (PLC)/ diacylglycerol/ protein kinase C (PKC) pathway in various systems. However, little is known about the signaling events in human endometrial stromal cells (hESCs). The present study was designed to examine the effect of ATP on the activation of the mitogen-activated protein kinase (MAPK) signaling pathway and its physiological role in hESCs. The present study also examined the expression of P2UR in hESCs by reverse transcription-polymerase chain reaction (RT-PCR). A PCR product corresponding to the expected 599 bp P2UR cDNA was obtained from hESCs. Molecular cloning and sequencing of the PCR product revealed a sequence identical to the reported P2UR cDNA. Western blot analysis, using a monoclonal antibody, which detected the phosphorylated forms of ERK1 and ERK2 ($p42^{mapk}$ and $p44^{mapk}$, respectively), demonstrated that ATP activated MAPK in a dose- and time-dependent manner. Treatment of the cells with suramin (a P2-purinoceptor antagonist), neomycin (a PLC inhibitor), staurosporin (a PKC inhibitor), or PD98059 (a MEK inhibitor) significantly attenuated the ATP-induced activation of MAPK. To study the gene(s) induced by exogenous ATP, mRNA was extracted from hESCs in the presence or absence of 10 μM ATP. The gene array for 23 genes associated with members of the mitogenic pathway cascade and immediate early genes revealed that the expression of early growth response 1 was increased. Besides, it is demonstrated using western blot analysis that ATP induced nuclear translocation of activated MAPK. To our knowledge, this is the first demonstration of the ATP-induced activation of the MAPK signaling pathway in human endometrial cells. These results support the notion that the MAPK signaling pathway is involved in mediating ATP actions in the human reproductive system.

Introduction

Autonomic nerves are known to innervate the female reproductive system (1). Adenosine triphosphate (ATP) is co-released with neurotransmitter granules from autonomic nerves by exocytosis (2). Extracellular ATP binds to a G protein-coupled P2 purinoceptor that activates phospholipase C and phosphatidylinositol hydrolysis, generating diacylglycerol and inositol 1,4,5-triphosphate, which stimulate protein kinase C and cytosolic calcium ($[Ca^{2+}]i$) mobilization, respectively (2,3). Thereafter, ATP may participate in various types of physiological responses, including intracellular signaling transduction, secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function, and muscle contraction (4,5).

The distribution of the autonomic nerves in the female reproductive system leads us to speculate that the co-released ATP from autonomic nerve endings in the uterus may play a role in regulating endometrial functions such as implantation, cell proliferation, or cell differentiation. Our previous data demonstrated that extracellular adenosine triphosphate (ATP), through a transmembrane purinergic receptor (6), plays a crucial role in regulating ovarian functioning by activating the intracellular signaling pathway and modulating human chorionic gonadotropin (hCG) action (7,8). However, the role of ATP in other human reproduction-related cells, such as endometrial stromal cells, remains to be determined. The present study was designed to examine the presence of the purinergic receptor and the effects of exogenous ATP on the intracellular mitogen-activated protein kinases (MAPKs) signaling pathway and genes expression in human endometrial stromal cells.

Mitogen-activated protein kinases (MAPKs) are a group of serine-threonine kinases involved in converting extracellular stimuli into intracellular signals (9). When activated, ERK1 and ERK2 (also known as $p42^{mapk}$ and $p44^{mapk}$, respectively) may be imported into the nucleus and phosphorylate a variety of substrates, including transcription factors, which have been implicated in the control of DNA replication, cell proliferation, differentiation, and genes expression (10-13)**.** The present study was designed to examine the action of ATP- activated MAPKs on the expression of members of the mitogenic pathway cascade and immediate early genes in hESCs.

Materials and Methods

Reagents and materials

ATP was obtained from Sigma Chemical Co. (St. Louis, MO). PD98059, a MEK inhibitor, was purchased from New England Biolabs Inc., Berverly, MA. Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin, and fetal bovine serum (FBS) were obtained from GIBCO-BRL. PD98059 was dissolved in dimethyl sulfoxide (DMSO), as suggested by the manufacturer. GEArray was purchased from SuperArray Bioscience Corporation (Bethesda, MD, USA) (www.superarray.com). (GEArray^{TM} Original Series Human MAP Kinase Signaling Pathways Gene Array).

Tissue collection and human endometrial stromal cell cultures (hESCs)

Samples of endometrium were obtained from women undergoing hysterectomy who

had no history of malignancy. The Clinical Screening Committee for Research and Other Studies Involving Human Subjects, in the Department of OB/GYN of Taipei Medical University Hospital, approved the use of human endometrium.

Isolation of human endometrial stromal cells was performed following the protocol reported by Shiokawa et al (14). Briefly, the endometrium was minced and subjected to 0.1% collagenase (type IV, Sigma Chemical Co.) and 0.1% hyaluronidase (type I-S, sigma Chemical Co.), and was digested in a shaking water bath at 37C for one hour. The cells were pelleted by centrifugation at 800 × *g* for 10 min at room temperature. The cell pellet was resuspended in DMEM. The endometrial cells were collected in a 50 ml tube after being passed through a nylon sieve (100μm) to remove the large cell mass, and collected cells were passed through a smaller size nylon sieve (40μm) to separate stromal cells from epithelial cells. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and incubated at 37 C under a water-saturated atmosphere of 5% CO_2 in air. The purity of the stromal cell cultures was determined by immunostaining for vimentin and cytokeratin.

Treatments

Human ESCs were incubated in a serum-free medium for 4 h prior to treatment. To examine the dose-response relationship, hESCs were treated with increasing concentrations of ATP or UTP (100 nM, 1 M, 10 M, or 100 M) for 5 min. For time-course experiments, hESCs were treated with 10 M ATP or UTP for 1, 5, 10, or 20 min.

To determine the intracellular signaling pathway, hESCs were treated with suramin (300 M, an inhibitor of the P2 purinergic receptor), neomycin (10 mM, a PLC inhibitor), staurosporin (1 M, a PKC inhibitor), or PD98059 (50 M, a MEK inhibitor) in the presence or absence of 10 M ATP. Human ESCs were pretreated with suramin for 30 min, neomycin for 15 min, staurosporin for 15 min, and PD98059 for 30 min prior to ATP treatment. The cells were collected 10 min after ATP exposure.

To study the action on the expression of members of the mitogenic pathway cascade and immediate early genes induced by ATP, hESCs were treated with 10 M ATP for 30 min, and the mRNA was extracted.

Total RNA isolation

Total RNA was isolated using an RNeasy Mini Kit (Qiagen). Briefly, cells were disrupted in a buffer containing guanidine isothiocyanate and homogenized following the manufacturer's protocol. Ethanol was then added to the lysate, creating conditions that promote the selective binding of RNA to the RNeasy silica-gel membrane. The sample was then applied to the RNeasy mini column. Total RNA was bound to the membrane, contaminants were efficiently washed away, and high-quality RNA was eluted in RNase-free water. The RNA concentration was determined based on absorbance at 260 nm.

RT-PCR

One microgram of total RNA obtained from hESCs was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Morgan, Canada). One set of oligonucleotide primers (5- CCTGGAATGCGTCCACCACATAT-3 and 5-GACGTGGAATGGCAGGAAG CAGA -3), based on the published human P2U receptor sequence (15), was designed for polymerase chain reaction (PCR) to amplify the P2UR from hESCs. PCR reactions were performed in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 400 µM dNTPs, 0.25 U Taq DNA polymerase, 2 M primers, and 1 l cDNA template per 25 l reaction. Amplification was carried out for 33 cycles under a condition of denaturation at 94 C for 60 seconds, annealing at 64 C for 35 seconds and extension at 72 C for 90 seconds, and a final extension at 72 C for 15 minutes.

Cloning and sequencing of RT-PCR product

Ten l of PCR products of P2UR were fractionated in a 1% agarose gel stained with ethidium bromide. The expected PCR products (599 bp) were isolated from gel, cloned using the TA cloning kit (Invitrogen), and sequenced by the dideoxy chain termination method using a T7 DNA polymerase sequencing kit (Pharmacia Biotech, Morgan, Canada). The sequence of the cDNA was sent to the GenBank at the National Center for Biotechnology Information NCBI (NCBI) through the internet (www.ncbi.nlm.nih.gov), in order to compare the identity with published human P2UR.

Western blot analysis

The hESCs were washed with ice-cold PBS and lysed with 100 L of cell lysis buffer (RIPA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 % Nonidet P-40, 0.5 % deoxycholate, 0.1% SDS, 1.0 mM PMSF, 10 g/mL leupeptin, and 100 g/mL aprotinin) at 4 C for 30 min. The cell lysate was centrifuged at 10,000 x g for 5 min, and the supernatant was collected for Western blot analysis. The amount of protein was quantified using a Bio-Rad protein assay kit (Bio-Rad Laboratories), following the manufacturer's protocol. Aliquots (30 g) were subjected to 10% SDS-polyacrylamide gel electrophoresis under a reducing condition, as previously described (16). The proteins were electrophoretically transferred from the gels onto nitrocellulose membranes (Amersham Pharmacia Biotech), following the procedures of Towbin et al. (17). These nitrocellulose membranes were probed with a mouse monoclonal antibody directed against the phosphorylated forms of ERK1 and ERK2 (P-MAPK, $p42^{mapk}$ and $p44^{mapk}$, respectively) at 4C for 16 h. Alternatively, membranes were probed with a rabbit polyclonal antibody for p42/p44 MAPK, which detects total MAPK (T-MAPK) levels (New England Biolabs Inc.). After washing, the membranes were incubated with HRP-conjugated goat anti- mouse secondary antibody, and the signal was visualized using a ECL system (Amersham Pharmacia Biotech) followed by exposure to X-ray film. The autographs were scanned and quantified with Image Pro Plus software (Media Cybernetics, Inc., USA).

MAP Kinase Assay

To measure MAP kinase activity, a nonradioactive method was used (p44/42 MAP Kinase Assay Kit, New England Biolabs Inc.). Briefly, active MAP kinase of cell lysate

(200 g) from hESCs treated with 10 M ATP for 10 min was selectively immunoprecipitated with an immobilized monoclonal antibody to phospho-p44/42 MAP kinase. For a positive control, active MAP kinase (provided by the manufacturer) was added to the control cell extract. The resulting precipitate was incubated with an Elk-1 fusion protein in the presence of ATP which allowed immunoprecipitated active MAPK to phosphorylate Elk-1. Phosphorylated Elk-1 was detected by Western blot using a phospho-Elk-1 antibody.

Gene array analysis

Human ESCs were treated with 10 M ATP for 30 min prior to RNA extraction. Biotinylated cDNA probes were synthesized from 5 g total RNA of ATP-treated or control samples using SuperArray's proprietary GEAprimer mix as reverse transcriptase primers and hybridized to the GEArray membrane spotted with 23 gene-specific cDNA fragments, following the manufacturer's instructions (SuperArray Bioscience). Briefly, total RNA was used as a template for the synthesis of cDNA probes with dNTP mix containing biotin-16-dUTP. Annealing of RNA with primers was performed in a preheated heat block at 70C for 3 min. Samples were cooled to 42 C, and kept at 42 C for 2 min before labeling with biotin-16-dUTP. The cDNA probe was denatured by heating at 94 C for 5 min, and chilling quickly on ice. The GEArray membrane spotted with 23 gene-specific cDNA fragments was wet with deionized H2O, and was prehybridized with GEAhyb hybridizational solution containing heat-denatured sheared salmon sperm DNA at 68 C for 1 to 2 hours. The membrane was incubated with the denatured cDNA probe overnight with continuous agitation at 68 C, then washed twice with pre-warmed 2XSSC containing 1% SDS for 20 min at 68 C, and twice with pre-warmed 0.1XSSC containing 0.5% SDS for 20 min at 68C. After blocking with GEAblocking solution, the membrane was incubated with alkaline phosphatase-conjugated streptavidin, washed with washing buffer, incubated with CDP-*Star*, a chemiluminescent substrate, and exposed to X-ray film. Each GEArray membrane was spotted with a negative control of pUC18 DNA as well as two positive control genes, -actin and GAPDH. The relative abundance of a particular transcript was estimated by comparing its signal intensity to the signal derived from -actin and GAPDH. The intensity of the array of spots was converted into numerical data using the Image Pro Express 4.5 software.

Cell proliferation assay

Cell viability was assessed by MTT assay, following manufacturer's protocol (ATCC 30-1010K). Briefly, human ESCs were plated in a 96-well culture dish. Cells were incubated in the absence or presence of increasing concentrations of ATP for 24 hours. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) was reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The hESCs were seeded into 96-well plates at a cell density of 1×10^4 cells per well and incubated in 100μl (DMEM＋10％FBS). The cells were then treated with increasing concentration of ATP (0, 0.1, 1, 10, 100μM) for 24 hours. Ten microliter MTT Reagent were added into each well and incubated for 2 to 4 hours until intracellular purple precipitate was visible under an inverted microscope. One hundred microliter

Detergent Reagent were added into each well and left at room temperature in the dark for 3 hours. The precipitated intracellular purple formazan was solubilized and absorbance was recorded and quantified at 550 nm by scanning with an ELISA reader (Molecular Devices, Sunnyvale, CA).

Subcellular Fractionation

Cells were treatment in the absence or presence of 10μM ATP for10 min. To prepare the nuclear extracts of cells, the human ESCs pellets were suspended in a hypotonic buffer (20mM HEPES (pH 7.4), 1mM $MgCl_2$, 10mM KCl, 0.5% Nonidet P-40, 0.5mM dithiothreitol (DTT), and protease and phosphatase inhibitors) at 4 °C for 30 min. After centrifugation at 4,000 x *g* at 4 °C for 10 min, the pellets of nuclei were resuspended in a high salt buffer (20mM HEPES (pH 7.4), 0.4 M NaCl, 1mM $MgCl_2$, 10mM KCl, 0.5mM dithiothreitol, 1mM henylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10μg/ml leupeptin, and 100mM sodium fluoride) and then incubated on ice for 30 min. The supernatants recovered after centrifugation were treated as nuclear extracts.

Statistical Analysis

MAPKs activity were expressed as a relative ratio of basal levels. Independent replicates of experiments in this study were performed with cells from different patients. Data were represented as means ± standard errors (SE). Statistical analysis was performed by one-way analysis of variance followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$.

Results

Expression of the P2U receptor mRNA in human endometrial stromal cells (hESCs)

The expression of the P2U receptor mRNA in hESCs was examined by RT-PCR using one set of primers designed on the basis of the published human P2UR expressed in airway epithelium. An expected 599-bp DNA fragment was observed in ethidium bromide-stained gel from hESCs isolated from 3 different patients (Fig. 1). No product was obtained from the negative control (without a first strain cDNA template in the PCR reaction). The PCR products from hESCs were subcloned and sequenced. Sequence analysis revealed that the cloned cDNA was identical to nucleotide position 436-1034 of the published human P2U receptor (15).

Effect of ATP on MAPK activation

To demonstrate the ability of ATP in activating MAPK, hESCs were treated with increasing concentrations (100 nM-100 M) of ATP for 5 min. For time-course analysis, the cells were treated with 10 M ATP for varying time intervals (1-20 min). As shown in Fig. 2A, ATP activated MAPK in hESCs in a dose-dependent manner. A significant effect was observed at 10 M with a maximum effect noted at 100 M. ATP was capable of rapidly inducing MAPK activity. A significant effect was seen within 5 min after treatment, and the activation of MAPK was sustained for at least 15 min. As shown in the study, the effect of ATP reached maximum at 10 min. (Fig.2B).

Effect of UTP on MAPK activation

To demonstrate the ability of UTP in activating MAPK, hESCs were treated with increasing concentrations (100 nM-100 M) of UTP for 5 min. For time-course analysis, the cells were treated with 10 M UTP for varying time intervals (1-20 min). As shown in Fig. 3A, UTP activated MAPK in hESCs in a dose-dependent manner. A significant effect was observed at 1 M, with a maximum effect noted at 100 M; there was a statistical difference between the cells treated with 10 M and those treated with 100 M of UTP. In time-course experiments, UTP was capable of inducing MAPK activity promptly. A significant effect was seen within 5 min after treatment, and the activation of MAPK was sustained for at least 15 min. As shown in Fig. 3B, the effect of UTP reached maximum at 10 min, 5 times greater than the basal level.

P2-purinergic receptor and ATP-induced MAPK activation

As shown in Fig.1, the P2U purinergic receptor was expressed in hESCs. To investigate the involvement of the P2 purinoceptor in ATP-induced MAPK activation, hESCs were pretreated with 300 M suramin, a P2 purinoceptor antagonist (18), for 30 min prior to the administration of ATP. As demonstrated in Fig.4, ATP activated MAPK to about 550% of the basal (control) level. The co-treatment with suramin and ATPcompletely reduced MAPK activity, when compared to ATPtreatment alone.

PLC and ATP-induced MAPK activation

Neomycin, an aminoglycoside antibiotic, has been demonstrated to inhibit PLC (19). In this study, hESCs were pretreated with 10 mM neomycin for 15 min prior to the stimulation of ATP. As shown in Fig.5, treatment of hESCs with neomycin significantly inhibited the ATP-induced activation of MAPK. The combined treatment with neomycin and ATPsignificantly attenuated MAPK activity by 80**%**, when compared with ATPtreatment alone.

PKC and ATP-induced MAPK activation

Staurosporin, a potent inhibitor of protein kinase C (20), significantly attenuated the ATP-induced activation of MAPK (Fig.6). Concomitant treatment with the PKC inhibitor and ATPattenuated MAPK activation by 60**%**, when compared to the level stimulated by ATPalone.

MEK and ATP-induced MAPK activation

In the MAPK activation cascade, MEK is the immediate activator of MAPK. MEK is also known as MAPK Kinase (21). The MEK inhibitor, PD98059, significantly decreased the ATP-induced activation of MAPK in hESCs. Simultaneous treatment with PD98059 and ATPreduced MAPK activity to about 80% of the level stimulated by ATPalone (Fig. 7).

MAP kinase activity

In vitro MAP kinase activity was detected using a p44/42 MAP kinase assay kit. As shown in Fig. 8, ATP significantly increased MAPK activity by 250% of the control level.

Gene array analysis

Total RNA extracted from hESCs incubated in the absence or presence of 10 M ATP for 30 min was converted to cDNA. Superarray analysis for 23 genes related to members of the mitogenic pathway cascade and immediate early genes revealed that the expression of early growth response 1 (egr-1:spots 2E & 2F) was increased (Figs. 9-(I) & 9-(II)). The relative abundance of egr-1 in the ATP-treated group was 5.6-fold of the control group, when comparing their signal intensities to the signals derived from GAPDH. To examine the direct effect of MAPK in gene expression, hESCs were pretreated with PD98059 for 30 min prior to 10 M ATP exposure, and the mRNA was extracted. As shown in Fig. 10-(II), the effects of ATP on the expression of Early Growth Response 1 (Fig. 10-(I)) was significantly downregulated in the presence of PD98059.

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