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ATP　對人類卵巢顆粒細胞中 Mitogen-activated Protein kinases 的影響

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ATP Activates Nuclear Translocation of Mitogen-activated Protein Kinases in Human Granulosa-luteal Cells

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Running Title: ATP activates nuclear translocation of MAPKs in hGLCs

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Abstract

Upon stimulation of many types of cell surface receptors, mitogen-activated protein kinases (MAPK) are activated by the signaling cascade of Ras, Raf and MEK. However, little is known about the role of extracellular adenosine triphosphate (ATP) in mitogen-activated protein kinase signaling events in human granulosa-luteal cells (hGLCs). The present study was designed to examine the effect of ATP on activation of MAPKs signaling pathway, translocation and its action on expression of members of the mitogenic pathway cascade and immediate early genes induced by mitogenic factors in hGLCs. Western blot analysis, using a monoclonal antibody, which detected the total and phosphorylated forms of ERK1 and ERK2 ($p42^{mapk}$ and $p44^{mapk}$, respectively), demonstrated that exogenous ATP evoked ERKs in a dose- and time-dependent manner. In contrast, p38 and JNK were not significantly activated following ATP treatment. To examine the translocation of activated ERKs, FITC-conjugated secondary antibody was used to detect the distribution of total and phosphorylated ERKs. Immunofluorescent staining revealed that phosphorylated ERK was translocated from cytoplasm into nucleus subsequent to 10μM ATP treatment. To our knowledge, this is the first demonstration of ATP-induced nuclear translocation of MAPKs in the human ovary. These results support the notion that the MAPKs signaling pathway plays a role in mediating ATP actions in the human ovary.

Introduction

After binding to a G protein-coupled P2 purinoceptor, extracellular adenosine triphosphate (ATP) may participate in various types of physiological responses, including secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function and muscle contraction (1,2). ATP is co-released with neurotransmitter granules from nerve endings by exocytosis (3). Considering that the ovary is a well-innervated organ, it is tempting to speculate that the co-released ATP from nerve endings may play a role in regulating ovarian function.

Mitogen-activated protein kinases (MAPKs) are a group of serine-threonine kinases involved in converting extracellular stimulus into intracellular signals. Extracellular signal regulated kinases (ERKs), one of MAPKs subfamilies, have been shown to be activated by extracellular agonists such as cytokines, growth factors and neurotransmitters (4,5). It is believed that two classes of cell surface receptors, G-protein-coupled receptor (GPCR) and receptor tyrosine kinases are associated with the activation of MAPKs (6-8). The nucleus has been shown to be a critical site for phosphorylated p42/p44 MAPKs localization. When activated, ERK1 and ERK2 (also known as $p42^{mapk}$ and $p44^{mapk}$, respectively) may be imported into nucleus and phosphorylate a variety of substrates, including transcription factors, which have been implicated in the control of DNA replication, cell proliferation and differentiation (9-14). However, the translocation of MAPKs is still unknown in hGLCs.

We reported previously the effect of ATP on activation the ERKs and hCG-induced progesterone production in hGLCs（15）, highlighting the significance of ATP in regulating

ovarian function, but little is known about the signaling events and gene responses related to activated MAPKs in the human ovary. The present study was designed to examine the effect of ATP on activation of MAPKs signaling pathway and translocation in hGLCs.

Materials and Methods

Reagents and Materials

ATP was obtained from Sigma Chemical Co. 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 were dissolved in dimethyl sulfoxide (DMSO) as suggested by manufacturers.

Human granulosa-luteal cells culture

Human GLCs were collected from patients undergoing in vitro fertilization treatment. The use of human GLCs was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects, in the Department of OB/GYN of Taipei Medical University Hospital. Granulosa cells were separated from red blood cells in follicular aspirates by centrifugation through Ficoll Paque, washed and suspended in DMEM containing 100 U penicillin G / ml, 100 μg streptomycin/ml and 10% FBS as described before (5). The cells were plated at a density of approximately 150,000 cells in 35-mm culture dishes. Cells were incubated at 37 C under a water-saturated atmosphere of 5% CO_2 in air for 3 days.

Treatments

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

To determine the translocation of MAPKs, hGLCs were fixed 5 min after 10 μM ATP exposure. To study the action on expression of members of the mitogenic pathway cascade and immediate early genes induced by ATP, hGLCs were treated with 10 μM ATP for 30 min and mRNA was extracted.

Western blot analysis

The hGLCs were washed with ice-cold PBS and lysed with 100 μL 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 μg/mL leupeptin and 100 μg/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 Bio-Rad protein assay kit (Bio-Rad Laboratories) following manufacturer's protocol. Aliquots (30 μg) were subjected to 10 % SDS-polyacrylamide gel electrophoresis under reducing condition, as previously described (16). The proteins were then electrophoretically transferred from the gels onto nitrocellulose membranes (Amersham Pharmacia Biotech) according to 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), phospho JNK or phospho p38 at 4 C for 16 h. Alternatively, the membranes were

probed with a rabbit polyclonal antibody for p42/p44 MAPK, which detected 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 ECL system (Amersham Pharmacia Biotech) followed by autoradiogrphy. The radioautograms were then scanned and quantified with Scion Image-Released Beta 3b (Scion Corp., Bethesda, MD).

Immunofluorescence microscopy

Human GLCs were seeded onto glass cover slips (5,000 /slip) and incubated for 3 days at 37 °C in humidified air with 5% CO_2 prior to **i**mmunofluorescence microscopy **e**xperiments. Cells were treated with 10 μM ATP for 5 min in the presence or absence of PD98059, fixed in 3.7% formaldehyde in Dulbecco's phosphate buffered saline (D-PBS) for 10 minutes, rinsed in PBS, and permeabilized for 10 minutes in PBS containing 1% NP-40 prior to staining. Nonspecific staining was blocked with 5% Goat serum/PBS. Cells were incubated with the antibody against phosphorylated forms of ERKs or total ERKs overnight at 4°C. Coverslips were rinsed extensively in PBS and then incubated with either FITC-conjugated goat anti-mouse or FITC-conjugated goat anti-rabbit IgG for 60 minutes at room temperature. Following the antibody incubations, the coverslips were washed in PBS, and nuclei were stained with Hoechst 33342 reagent (Molecular Probes). Coverslips were mounted onto slides with Fluoromount-G and viewed on a Nikon microscope equipped with E600 epi-fluorescence set and CoolSNAP-Pro Digital Kits.

Statistical Analysis

MAPKs 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

Effect of ATP on MAPK activation

For the dose effect of ATP on activating MAPK, hGLCs were treated with increasing concentrations (100 nM-100 μM) of ATP for 5 min. For time-course analysis, the cells were treated with 10 μM ATP for varying time intervals (1-20 min). As shown in Fig. 1, ATP activated ERK1/2 in hGLCs in a dose-dependent manner. A significant effect was observed at micro levels with a maximum effect noted at 10 μM, and there is no statistic difference between cells treated with 10 μM and 100 μM ATP. ATP was capable of rapidly inducing ERK1/2 activity. A significant effect was seen within 5 min after treatment, and the activation of ERK1/2 was lasting for at least 15 min (Fig.2). In contrast, p38 and JNK were not activated by ATP in this study（Fig. 3）.

Subcellular ERK localization

As shown in Fig. 4A, antibody against total ERKs demonstrated that ERKs (nonphosphorylated and phosphorylated) were distributed in both cytoplasm and nucleus. To examined the distrubution of ATP-activated ERKs, hGLCs were treated with 10 μM ATP for 5 min. Once activated, phosphorylated ERKs were translocated into the nucleus, which were detected by monoclonal antibody against phosphorylated ERK1/2（Fig. 5A）. In the presence of PD98059, the effect of ATP was blocked and no nuclear translocation was noted（Fig. 6A）. Nucleus of hGLCs were counterstained with Hoechst and emitted with blue fluorescence （Fig. 4B, 5B and 6B）.

Discussion

The present study demonstrated that ATP was able to activate the ERK1/2, induce the nuclear translocation of phosphorylated ERKs and increase the expression of MMK3 in hGLCs. MAP Kinases have been identified in several steroidogenic cells (8, 19). Recently, Kang et al. reported that MAPKs mediate the inhibitory effect of gonadotropin-releasing hormone in progesterone production in hGLCs (20), indicating the role of MAPKs in steroidogenesis. Previously, we demonstrated that ATP is capable of activating ERK1/2 in hGLCs through the signaling cascade of P2-purinoceptor, G-protein, PLC, PKC and MEK,and furthermore, MAPKs mediated the anti-gonadotropic action of ATP in steroidogenesis in hGLCs（15）.

The MAP kinases have been implicated in the regulation of cell growth and differentiation

(21). MAP kinases are classified into three subfamilies: (I) ERKs (extracellular signal-regulated kinases), including ERK1 and ERK2, (II) SAPKs (stress-activated protein kinase), also called c-jun N-terminus kinases (JNKs), and (III) p38 kinase (7). The first MAPKs to be cloned are MAPK/ERK 1 and 2, which are phosphorylated and activated by MEKs (22, 23). Since the ERKs are only one class of MAP kinase, we extended studies to include both JNK and p38 MAP kinases. The present study revealed that ATP activated the ERK1/2 but not JNKs or p38. The concentration of ATP in adrenergic granules of sympathetic nerves and in acetylcholine-containing granules of parasympathetic nerves can be as high as 150 mM (24). Our results demonstrated that 10 μM ATP was able to activate ERKs in a dose- and time-dependent manner, and the functional role of activated ERKs has partially been revealed in our previous study as an anti-gonadotropic effect（15）.

GPCRs use to control the activity of MAP kinases vary between receptor and cell type but fall broadly into one of three categories: （1）signals initiated by classical G protein effectors, （2）signals initiated by cross-talk between GPCRs and classical receptor tyrosine kinases, and （3）signals initiated by direct interaction between b-arrestins and components of the MAP kinase cascade（25）. Various functions were observed in each of these pathways. ERKs activation occurring via the GPCRs/PKC pathway and EGF receptor transactivation leads to nuclear translocation of the kinases and stimulates cell proliferation, while MAP kinases activation via b-arrestin scaffolds primarily boost cytosolic kinase activity. （12, 25）MAPKs nuclear translocation has been shown to be essential for growth factor-induced DNA replication and cell transformation（12-14）. When activated, ERKs phosphorylate a variety of substrates in nucleus, including transcription factors, which have been implicated in the control of cell

proliferation and differentiation (9-11). The import of ERK2 reached the maximum in several min, and then the imported ERK2 was exported from the nucleus. MEK mainly resided in the cytoplasm （26）. In addition, sustained calcium increase was required for the optimal translocation of ERK2 into the nucleus（26）. It is believed that the nucleus is also a significant site for mitogenic signal termination by nuclear sequestration of p42/p44 MAPKs away from MEK, their cytoplasmic activator, and dephosphorylation by certain nuclear phosphatases（15）. Interestingly, transient and sustained ERK phosphorylation varies in effect on cell growth. The phosphatase inhibitor may cause growth inhibition as a consequence of prolonged ERK phosphorylation（27）.

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