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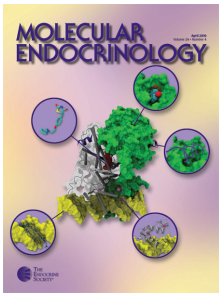

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With the stimulation of many types of cell surface receptors, MAPKs are activated. We have previously demonstrated an effect of extracellular ATP on ERKs and gonadotropin-induced progesterone secretion, implicating the significance of ATP in the regulation of ovarian function. However, little is known about the specific role of ATP in the subsequent MAPK-induced signaling cascade in human granulosa-luteal cells (hGLCs). The present study was designed to examine the effect of ATP on the activation of the MAPK signaling pathway, including nuclear translocation and the expression of the immediate early genes 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 after ATP treatment. To examine the translocation of activated ERKs, fluorescein isothiocyanate-conjugated secondary antibody was used to detect the distribution of total and phosphorylated ERKs. Immunofluorescent staining revealed that phosphorylated ERKs were translocated from cytoplasm into nucleus subsequent to 10 μM ATP treatment. To study the gene(s) induced by exogenous ATP, mRNA was extracted from hGLCs in the presence or absence of 10 μM ATP. 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 and c-raf-1 was increased. To our knowledge, this is the first demonstration of the ATP-induced nuclear translocation of MAPKs in the human ovary. These results suggest that the MAPK signaling pathway plays a role in mediating ATP actions in the human ovary. (*J Clin Endocrinol Metab* 89: 5189–5195, 2004)

AFTER BINDING TO a G protein-coupled P2 purinoceptor, extracellular 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 coreleased 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 coreleased ATP from nerve endings may play a role in regulating ovarian functioning.

MAPKs are a group of serine-threonine kinases involved in converting extracellular stimuli into intracellular signals. ERKs, one of the 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 the 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 human granulosa-luteal cells (hGLCs).

We reported previously the effect of ATP on the activation of ERKs and human chorionic gonadotropin-induced progesterone production in hGLCs (15, 16), 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 the activation of the MAPKs signaling pathway, intracellular translocation, and its action on the expression of members of the mitogenic pathway cascade and immediate early genes in hGLCs.

Materials and Methods

Reagents and materials

ATP was obtained from Sigma Chemical Co. (St. Louis, MO); and PD98059, a MAPK kinase (MEK) inhibitor, was purchased from Cell Signaling Technology (Beverly, MA). DMEM, penicillin-streptomycin, and fetal bovine serum were obtained from GIBCO-BRL (Rockville, MD). PD98059 was dissolved in dimethylsulfoxide, as suggested by the manufacturer. GEArray was purchased from SuperArray Bioscience

Abbreviations: egr-1, Early growth response 1; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G protein-coupled receptor; hGLC, human granulosa-luteal cell; HRP, horseradish peroxidase; MEK, MAPK kinase; PKC, protein kinase C; SDS, sodium dodecyl sulfate.

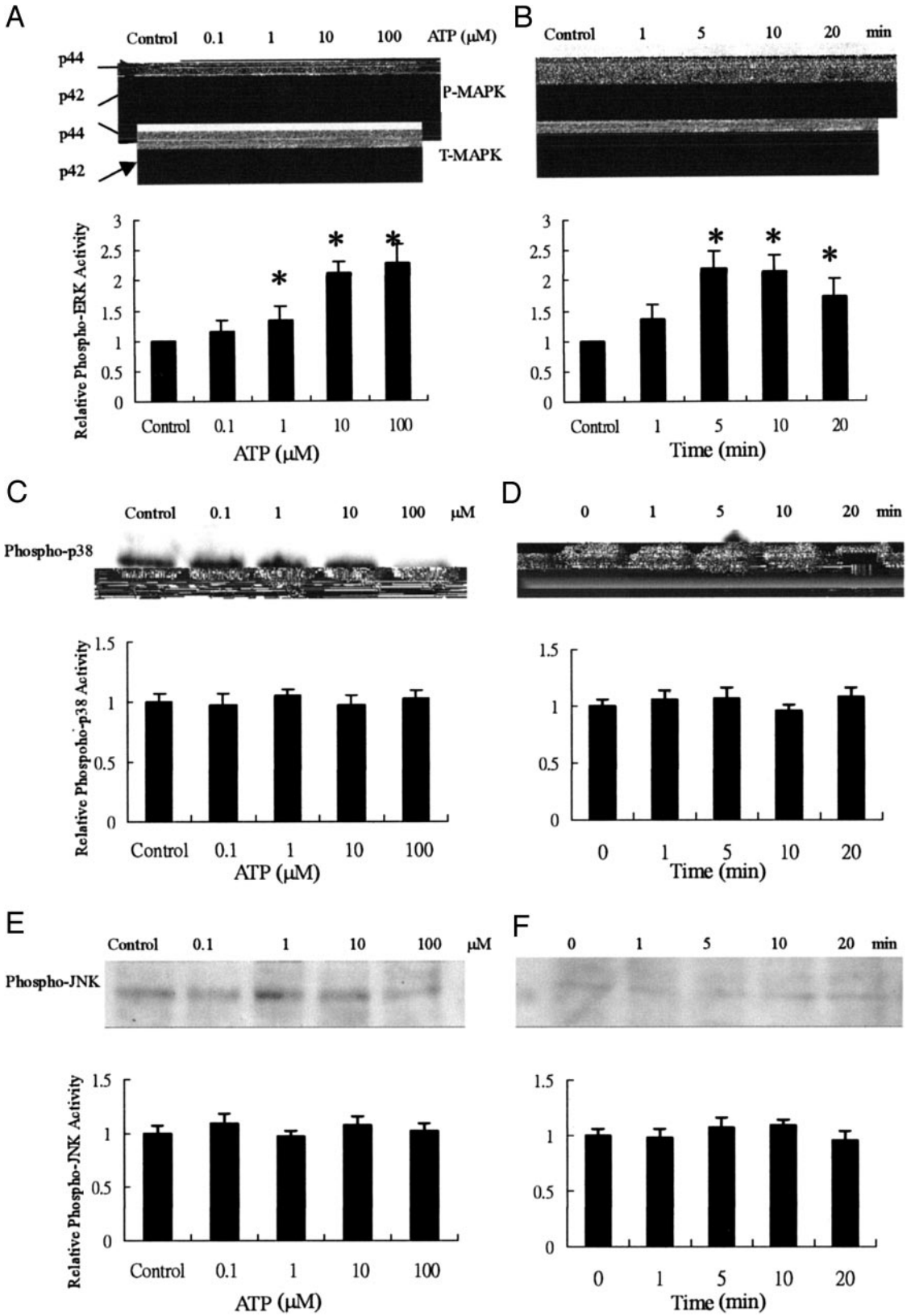

FIG. 1. The effect of ATP on MAPK activation in hGLCs using Western blot analysis. A, The dose-response of ATP on ERK1/2 (p44/p42) activation. hGLCs were treated with increasing concentrations of ATP (0, 100 nM, 1 μM, 10 μM, or 100 μM) for 5 min, as described in *Materials and Methods*. B, The time effect of ATP on ERK1/2 (p44/p42) activation. hGLCs were treated with 10 μM ATP for 0, 1, 5, 10, or 20 min, as described in *Materials and Methods*. C, The dose-response of ATP on p38 activation in hGLCs. D, The time effect of ATP on p38 activation in hGLCs.

Corp. (Bethesda, MD). Phospho-p44/42 MAPK (Thr202/Tyr204) E10 monoclonal antibody (catalog no. 9106), p44/42 MAPK polyclonal antibody (catalog no. 9102), phospho-p38 MAPK polyclonal antibody (catalog no. 9211), and phospho-stress-activated protein kinases/JNK (Thr183/Tyr185) G9 monoclonal antibody (catalog no. 9255) were purchased from Cell Signaling Technology. Goat antimouse IgG horseradish peroxidase (HRP) (catalog no. sc-2005) and donkey antirabbit IgG HRP (catalog no. sc-2313) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

hGLC cultures

The hGLCs were collected from patients undergoing *in vitro* fertilization treatment. The use of hGLCs was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects, in the Department of Obstetrics/Gynecology 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% fetal bovine serum, as described before (16). 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 d.

Treatments

The hGLCs were incubated in a serum-free medium for 4 h before 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 treated with 10 μM ATP for 5 min and fixed in 3.7% formaldehyde in Dulbecco's PBS. To study the 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, then the mRNA was extracted. To examine the direct effect of MAPK in gene expression, hGLCs were pretreated with PD98059 for 30 min before 10 μM ATP exposure, and the mRNA was extracted.

Western blot analysis

The hGLCs were washed with ice-cold PBS and lysed with 100 μl cell lysis buffer, RIPA [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1.0 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, and 100 μg/ml aprotinin] at 4 C for 30 min. The cell lysate was centrifuged at 10,000 $\times$ *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, Hercules, CA), following the manufacturer's protocol. Aliquots (30 μg) were subjected to 10% SDS-PAGE under a reducing condition, as previously described (17). The proteins were then electrophoretically transferred from the gels onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ), following the procedures of Towbin *et al.* (18). 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 (Cell Signaling Technology). After washing, the membranes were incubated with HRP-conjugated goat antimouse or donkey antirabbit secondary antibody, and the signal was visualized using an ECL system (Amersham Pharmacia Biotech) followed by exposure to x-ray film. The autographs were then scanned and quantified with Scion Image-Released β 3b (Scion Corp., Bethesda, MD).

Immunofluorescence microscopy

hGLCs were seeded onto glass cover slips (5000/slip) and incubated for 3 d at 37 C in humidified air with 5% CO_2 before immunofluorescence microscopy experiments. Cells were treated with 10 μM ATP for 5 min in the absence or presence of PD98059 (pretreated for 30 min before ATP exposure), fixed in 3.7% formaldehyde in Dulbecco's PBS for 10 min, rinsed in PBS, and permeabilized for 10 min in PBS containing 1% Nonidet P-40 before 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 fluorescein isothiocyanate (FITC)-conjugated goat antimouse or FITC-conjugated goat antirabbit IgG for 60 min at room temperature. After the antibody incubations, the coverslips were washed in PBS, and nuclei were stained with Hoechst 33342 reagent (Molecular Probes, Eugene, OR). 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 (Media Cybernetics, Inc., Silver Spring, MD).

Total RNA isolation

The hGLCs were treated with 10 μM ATP for 30 min before RNA extraction. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, cells were disrupted in a buffer containing guanidine isothiocyanate, and then 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 minicolumn. With total RNA bound to the membrane, contaminants were efficiently washed away, and high-quality RNA was eluted in ribonuclease-free water. The RNA concentration was determined based on absorbance at 260 nm.

Gene array analysis

Biotinylated cDNA probes were synthesized from 5 μg 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 Corporation). Briefly, total RNA was used as a template for the synthesis of cDNA probes with deoxynucleotide triphosphate mix containing biotin-16-deoxy-UTP. Annealing of RNA with primers was performed in a preheated heat block at 70 C for 2 min. Samples were cooled to 42 C and kept at 42 C for 2 min before labeling with biotin-16-deoxy-UTP. The cDNA probe was denatured by heating at 94 C for 5 min, and quickly chilled on ice. The GEArray membrane spotted with 23 gene-specific cDNA fragments was wet with deionized H_2O and prehybridized the membrane with GEAhyb hybridizational solution containing heat-denatured sheared salmon sperm DNA at 68 C for 1–2 h. The membrane was then incubated with the denatured cDNA probe overnight with continuous agitation at 68 C, then washed twice with prewarmed 2$\times$ SSC containing 1% SDS for 20 min at 68 C, and twice with prewarmed 0.1$\times$ SSC containing 0.5% SDS for 20 min at 68 C. 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, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The relative abundance of a particular transcript can be estimated by comparing its signal intensity to the signal derived from β-actin and GAPDH. The intensity of the array of spots was converted into numerical data using the Image Pro Plus software.

E, The dose-response of ATP on JNK activation in hGLCs. F, The time effect of ATP on JNK activation in hGLCs. The amount of loading was normalized by total ERK. The data are shown as relative ratio to basal levels. Values are presented as the mean $\pm$ SE of three individual experiments. Statistical analysis was performed by one-way ANOVA followed by the Tukey test. *, Differences were considered significant at $P < 0.05$.

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 ± SE of two individual experiments with triplicate samples. Statistical analysis was performed by one-way ANOVA 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. 1A, ATP activated ERK1/2 in hGLCs in a dose-dependent manner. A significant effect was observed at 1 μM ATP, with a maximum effect noted at 10 μM, and there was no statistical difference between cells treated with 10 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 lasted for at least 15 min (Fig. 1B). In contrast, p38 and JNK were not activated by ATP in this study (Fig. 1, C–F).

Subcellular ERK localization

As shown in Fig. 2A, the use of a polyclonal antibody against total ERKs demonstrated that ERKs (nonphosphorylated and phosphorylated) were distributed in both the cytoplasm and the nucleus. To observe the base line level of phosphorylated ERKs, cells were fixed in the absence of ATP, and the faint intracellular fluorescence revealed that small amounts of phosphorylated ERK1/2 were located in the nucleus (Fig. 2C). To examine the distribution of ATP-activated ERKs, hGLCs were treated with 10 μM ATP for 5 min. Once activated, phosphorylated ERKs were translocated into the nuclei, which were detected by a monoclonal antibody against phosphorylated ERK1/2 (Fig. 2E). Figure 2G demonstrates that, in the presence of PD98059, the effect of ATP on ERK1/2 translocation was completely blocked. A faint fluorescent staining revealed the distribution of phosphorylated ERK1/2 in both the cytoplasm and nucleus. The nuclear translocation of phosphorylated ERK1/2 was not significant when compared with the cells in Fig. 2C. The nuclei of hGLCs in the present study were stained with Hoechst and emitted blue fluorescence (Fig. 2, B, D, F, and H).

Gene array analysis

Total RNA, extracted from hGLCs incubated in the absence or presence of 10 μM 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 and 2F) and c-raf-1 (spots 8A and 8B) were increased (Fig. 3, A and B). The relative abundance of egr-1 in the ATP-treated group was 4.3-fold greater than the control group, when comparing their signal intensities to the signals derived from GAPDH. The expression of c-raf-1 was increased by 2.7-fold in the presence of ATP. To examine the direct effect of MAPK in gene expression, hGLCs were pre-

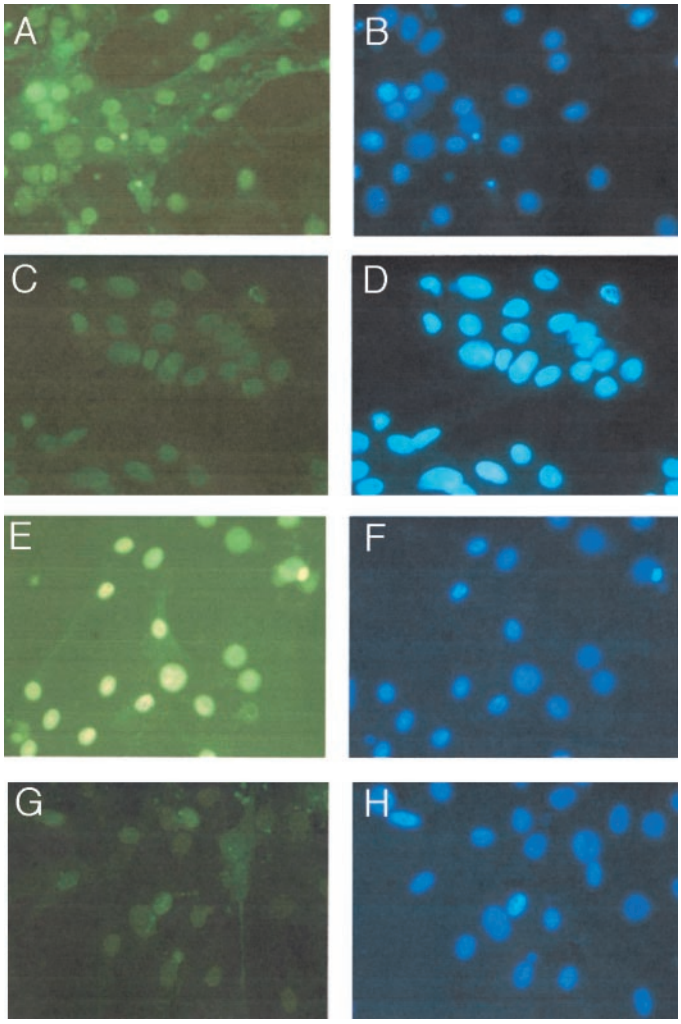

FIG. 2. The distribution of p42/p44 and the effect of ATP on phospho-p42/p44 translocation in hGLCs. A, The distribution of p42/p44 in hGLCs. Formaldehyde-fixed cells were incubated with the primary polyclonal anti-p42/p44 MAPKs antibody, and the primary antibody staining was detected with the FITC-conjugated antirabbit IgG. B, Nuclei of cells in A were stained with Hoechst 33342. C, The localization of the phospho-p42/p44 in hGLCs. Formaldehyde-fixed hGLCs, in the absence of ATP, were incubated with the primary monoclonal anti-phospho-p42/p44 antibody, and primary antibody staining was detected with the FITC-conjugated antimouth IgG. D, Nuclei of cells in C were stained with Hoechst 33342. E, The localization of the activated p42/p44 (phospho-p42/p44) in hGLCs. hGLCs were treated with 10 μM ATP for 5 min. Formaldehyde-fixed cells were incubated with the primary monoclonal anti-phospho-p42/p44 antibody, and the primary antibody staining was detected with the FITC-conjugated antimouth IgG. F, Nuclei of cells in E were stained with Hoechst 33342. G, The effect of PD98059 on ATP induced-translocation of the activated p42/p44 in hGLCs. hGLCs were treated with 10 μM ATP in the presence of PD98059 for 5 min. Formaldehyde-fixed cells were incubated with the primary monoclonal anti-phospho-p42/p44 antibody, and the primary antibody staining was detected with the FITC-conjugated antimouse IgG. H, Nuclei of cells in G were stained with Hoechst 33342.

treated with PD98059 for 30 min before 10 μM ATP exposure, and the mRNA was extracted. As shown in Fig. 3C, the effects of ATP on the expression of egr-1 and Raf were significantly down-regulated in the presence of PD98059.

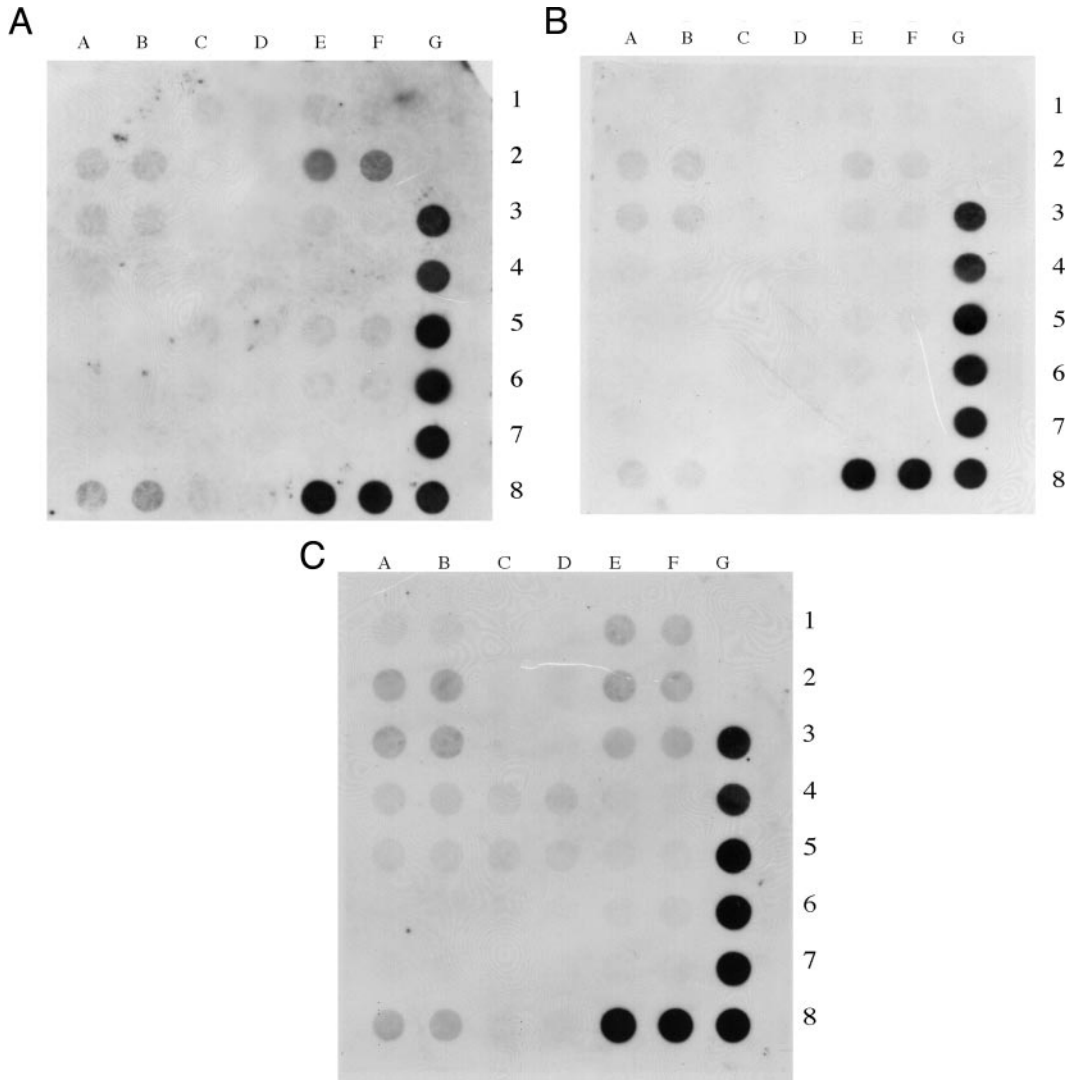

FIG. 3. Biotinylated cDNA probes were synthesized from 5 μg total RNA of hGLCs in the absence (A) or presence (B) of 10 μM ATP and hybridized to the GEArray membrane spotted with 23 gene-specific cDNA fragments. After the hybridization, the membrane was incubated with alkaline phosphatase-conjugated streptavidin, and the signal was visualized with CDP*Star*, a chemiluminescent substrate, and exposed to x-ray film. C, Biotinylated cDNA probes were synthesized from 5 μg total RNA of hGLCs pretreated with MEK inhibitor (PD98059) before 10 μM ATP exposure (2E and 2F, egr-1; 8A and 8B, c-raf-1; 8E and 8F, GAPDH).

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 egr-1 and c-raf-1 in hGLCs. MAPKs have been identified and play important roles in several steroidogenic cells (8, 19). Recently, Kang *et al.* (20) reported that MAPKs mediate the inhibitory effect of GnRH in progesterone production in hGLCs, 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-purinoceptors, G protein, phospholipase C, protein kinase C (PKC), and MEK and, furthermore, that MAPKs mediated the antigonadotropic action of ATP in steroidogenesis in hGLCs (15).

The MAPKs have been implicated in the regulation of cell growth and differentiation (21). MAPKs are classified into three subfamilies: 1) ERKs, including ERK1 and ERK2; 2) stress-activated protein kinases, also called c-jun N terminus kinases (JNKs); and 3) p38 kinase (7). The first MAPKs to be cloned are MAPK/ERK 1 and 2, which are phosphorylated and activated by MEKs (22, 23). Because the ERKs are only one class of MAPK, we extended our studies to include both JNK and p38 MAPKs. The present study revealed that ATP activated the ERK1/2 but not JNKs or p38 (Fig. 1). The concentration of ATP in the adrenergic granules of sympathetic nerves and in the acetylcholine-containing granules of parasympathetic nerves can be as high as 150 mM (24). Our results demonstrated that ATP was able to activate ERKs in a dose- and time-dependent manner; the functional role of activated ERKs was partially revealed in our previous study as an antigonadotropic effect (15). The current study further examined the intracellular performance of activated ERKs in human ovarian cells.

Extracellular ATP binds to purinergic receptors, which belong to one of the GPCRs. The GPCRs used to control the activity of MAPKs vary between receptor and cell types 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 MAPK 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 the nuclear translocation of the kinases and stimulates cell proliferation, whereas MAPKs activation via b-arrestin scaffolds primarily boosts 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 the nucleus, including transcription factors, which have been implicated in the control of cell proliferation and differentiation (9–11). In the present study, we demonstrated the ATP-induced nuclear translocation of activated ERK1/2 (Fig. 2E). We reported previously that PD98059 (a MEK inhibitor) significantly attenuated the ATP-induced activation of MAPK (15). We hereby showed that, in the presence of PD98059, the effect of ATP on ERK1/2 translocation was blocked (Fig. 2G).

The import of ERK into the nucleus reaches a maximal level in several min, after which the imported ERK is exported from the nucleus (26). It is believed that the nucleus is also a significant site for mitogenic signal termination by the 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).

In the nucleus, activated ERK1/2 continuously phosphorylates elk-1, leading to the nuclear accumulation of transcription factors, such as c-*fos*, which is responsible for DNA synthesis (28, 29). In the human ovarian cells, GnRH agonist stimulates a significant increase in c-*fos* mRNA expression, and the maximal effect is observed within 30 min (30). In the present study, we examined the effect of ATP on 23 genes of members of the mitogenic pathway cascade and immediate early genes. These include: ATF-2 (creb-2), c-fos, c-jun, c-myc, CREB, egr-1, elk-1, elk-3, ERK1 protein kinase, ERK2 (MAPK1), JNK1, JNK2, Max, MEK1, MEK2, MEKK1, MEKK3, MKK3, MKK4 (JNKK1), MKK6, p38 MAPK, Raf (c-raf-1), and SRF (serum response factor). Among these genes, the expression of egr-1 and c-raf-1 were elevated significantly (Fig. 3). It has been reported that insulin rapidly increased the transcription of egr-1 through ERK1/2 activation (31); egr-1 is an immediate early gene, which is rapidly activated in quiescent cells by mitogens and has been involved in diverse biological functions such as cell growth and differentiation. It was also demonstrated that the enforced expression of the egr-1 gene induces apoptosis (32). Extracellular ATP elevates the expression level of egr-1 protein in a human osteoblastic cell line via a PKC-dependent pathway (33). In addition, it has been reported that the activation of ERKs 1 and 2 is related to an increased expression of c-fos, egr-1, and junB (34). We reported previously that extracellular ATP induced the activation and translocation of PKCα in hGLCs. Taken together, our results indicate that extracellular ATP plays an important role in inducing the expression of immediate early response genes via the PKC/ERK signaling pathway.

c-raf-1 is an upstream activator in the Ras/Raf/MEK/ERK signaling cascade (35, 36). ATP has been reported to induce cell proliferation via activation of the Ras/Raf/MEK/MAPK pathway (37). In the present study, the increased expression of c-raf-1 after ATP treatment further supports the existence of an autoregulation system in the ATP-evoked MAPK pathway.

To our knowledge, this is the first demonstration of the ATP-induced nuclear translocation of phosphorylated ERKs and the induction of egr-1 and c-raf-1 expression 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.

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