Signaling pathways of magnolol-induced adrenal steroidogenesis

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Abstract This study focused on identifying the signalling mediating the effect of magnolol on corticosterone production. Magnolol-induced corticosterone production was completely inhibited by mitogen-activated protein kinase kinase (MEK)-inhibitor PD98059, tyrosine kinase (TK)-inhibitor genistein or Janus tyrosine kinase 2 (JAK2)-inhibitor AG490, suggesting that extracellular signal-regulated kinase (ERK) and JAK2 are both involved in this signaling cascade. Further, magnolol induced the transient phosphorylation of MEK, ERK, cAMP response-element binding protein (CREB) and the expression of 32 and 30 kDa steroidogenic acute regulatory protein (StAR) in a time-dependent manner. Inhibition of TK or JAK2 activities blocked magnolol-induced phosphorylation of MEK and ERK, again supporting the upstream role of JAK2. The activation of JAK2 or MEK apparently mediated the magnolol-induced phosphorylation of CREB and the upregulation of StAR. These findings demonstrate a novel pathway for magnolol to induce the expression of StAR, which regulates the rate-limiting step in sterodiogenesis.

Keywords: Magnolol; JAK2; ERK; CREB; StAR; Steroidogenesis

1. Introduction

Recent studies indicate that signaling pathways other than protein kinase A (PKA) and protein kinase C, including extracellular signal related-regulated kinase (ERK) and tyrosine kinase (TK) pathways regulate steroidogensis in adrenal Y1 and bovine adrenal glomerulosa cells [1–3]. In rat Leydig cells, inhibition of ERK activation suppresses human chorionic gonadotropin-induced steroidogenesis by controlling the synthesis of steroidogenic acute regulatory protein (StAR) protein [4]. In addition, luteinizing hormone and follicle stimulating hormone also induce ERK1/2 phosphorylation and stimulate progesterone production in rat ovarian granulose cells [5]. Genistein inhibits angiotensin II (Ang II)-induced calcium influx and aldosterone production in bovine glomerulosa cells and 3β-hydroxysteroid dehydrogenase activity in H295R cells [6,7]. Moreover, activation of TK by fibroblast growth factor, a receptor TK agonist, rapidly increases the phosphorylation of mitogen-activated protein kinase kinase (MEK) and ERK isoforms in Y1 adrenal tumor cells [8]. Recently, prolactin was found to stimulate porcine adrenal cortisol production via a TK-dependent process [9]. More specifically, Src kinase inhibitor PP2 prevents Ang II-stimulated aldosterone production [7]. Janus tyrosine kinase 2 (JAK2), a member of the family of soluble tyrosine kinases, is activated by auto- or *trans*-phosphorylation of specific tyrosine residues [10]. Inhibition of JAK2 activity by AG490 blocks Ang II-induced StAR promoter activity and steroid production [11]. In 3T3-422A cells, JAK signaling can lead to the recruitment of other SH2 domain-containing proteins to JAK2, such as the Shc adaptor protein, leading to the activation of the Ras-ERK signaling pathway [12].

In a series of magnolol-related studies, we found that magnolol also induces lipolysis in lipid-laden RAW264.7 macrophages [13] and sterol ester-loaded 3T3-L1 preadipocytes, and the signaling pathway of the latter event involves ERK [14]. However, whether the same signaling pathway accounts for magnolol-induced steroidogenesis in adrenal cells has not yet been elucidated. In this study, we examined the involvement of ERK in magnolol-activated signaling pathways in adrenal cells.

2. Materials and Methods

2.1. Cell culture

Adult female Wistar rats (8–12 weeks of age) were purchased from the facility for Research Animal of the National Taiwan University. The maintenance and use of the animals were in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institute of Health (NIH publication No. 85-23, revised 1985). The animals were anesthetized with 7% chloral hydrate (6 ml/kg) by intraperitoneal injection, and adrenocortical cells were prepared by enzymatic dispersion with type II collagenase (Sigma) as described previously [15]. Cells were maintained in 1:1 v/v mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (Gibco, Rockville, MD), supplemented with 25 mM HEPES, 5% horse serum (Gibco), 2.5% fetal bovine serum (Gibco), and 1% penicillin and streptomycin in 24-well plates or 35-mm culture dishes for 3 days at 37 °C in a 95% air and 5% CO_2 humidified atmosphere.

2.2. Drug treatment

Magnolol, isolated from *Magnolia officialis* with a purity of over 99%, was purchased from the Pharmaceutical Industry Technology and Development Center (Taiwan). The MEK inhibitor, PD98059, and selective JAK2 inhibitor, AG490, were purchased from Calbiochem (La Jolla, CA). TK inhibitor genistein was purchased from Sigma Chemical Co. (St. Louis, MO). Proteaosome inhibitor MG 132 was obtained from Tocris Cookson Ltd. (Avonmounth, UK). The inhibitors were added 30 min prior to the addition of magnolol.

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Abbreviations: Ang II, Angiotensin II; CREB, cAMP response-element binding protein; ERK, extracellular signal related-regulated kinase; HSL, hormone-sensitive lipase; JAK2, Janus tyrosine kinase 2; MEK, mitogen-activated protein kinase kinase; PBS, phosphate-buffered saline; PKA, protein kinase A; SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein; TK, tyrosine kinase

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2.3. Staining for necrotic and apoptotic cells

Cells were treated with DMSO (vehicle control) or magnolol at different concentrations for 6 h, washed with phosphate-buffered saline (PBS) and incubated with propidium iodide (50 μg/ml in serum-free medium) for 1 h in a CO_2 incubator. After a brief wash with PBS, live cells were fixed in 10% formalin for 5 min, washed with PBS and mounted in fluorescence mounting medium. For nuclear staining, cells were fixed in 5% formalin and 0.5% Triton X-100 for 5 min at room temperature, washed with PBS and stained with DAPI (4′,6-diamidino-2-phenylindole, Sigma) solution (1 μg/ml DAPI in 0.9% NaCl) for 15 min. The apoptotic cells were identified by the presence of chromatin condensation or apoptotic body formation. All experiments were performed in triplicate and more than one hundred cells were examined per dish.

2.4. Corticosterone radioimmunoassay

After drug treatments, 5 μl of the culture medium was diluted (1:20) with the assay buffer (0.05 M Tris–HCl, pH 8.0, containing 0.1 M NaCl, 0.1% NaN_3, and 0.1% of bovine serum albumin) and incubated with 500 μl of 1:10 diluted rabbit anti-corticosterone antibody (Sigma). After 20 min at 37 °C, 100 μl of ^{3}H-corticosterone (10 000 cpm in assay buffer) (Amersham Bioscience, UK) was added to the mixture and incubated for 1 h at 37 °C and then 1 h at 4 °C. The free hormones were adsorbed on 200 μl of dextran-coated charcoal (0.5% dextran and 1.25% charcoal) in assay buffer for 10 min and the bound hormones separated by centrifugation at 13 000 rpm for 10 min. The supernatant (about 0.7 ml) was transferred to a counting vial containing 3 ml of counting solution (Ecoscient H) before counting in a β-counter (LS600IC, Beckman, Fullerton, CA). A standard curve was established using corticosterone standard (Sigma).

2.5. Western blot analysis

After various treatments, cells in 35 mm dishes were washed with PBS, collected in 50 μl lysis buffer (0.15% Triton-X 100, 2 mM $MgCl_2$, 25 mM HEPES, 60 mM PIPES and 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotronin, 1 μg/ml pepstatin A and 1 μg/ml leupeptin, pH 6.9) and sonicated for 20 cycles of 10-s pulses. Protein concentrations were determined using a protein assay kit (BioRad Life Sciences, Hercules, CA), and samples stored at −20 °C until further analysis. Cell lysates (50 μg of protein/lane) were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel and the proteins were transferred to nitrocellulose paper as described by Fritz et al. [16]. Membrane strips were blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris Base, pH 8.2) containing 0.1% Tween 20, then incubated overnight at 4 °C with one of the following antibodies including mouse anti-phosphorylated ERK (Santa Crutz Biotechnology, Santa Crutz, CA), rabbit anti-phosphorylated MEK1/2, rabbit anti- phosphorylated CREB (Cell Signaling Technology, Beverly, MA), rabbit anti-StAR antibodies (a kind gift from Dr. Strauss, J. F., III) or mouse anti-β-actin antibodies (Sigma). Immunoblot analyses were performed using alkaline phosphatase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:7500 dilution, Promega Corp., Madison, WI) and bound antibody visualized using a substrate solution (3.3 mg/ml nitro blue tetrazolium and 1.65 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris base, pH 9.5). Some membrane strips were treated with the stripping buffer (1% sodium dodecyl sulfate in 25 mM glycine, pH 2) for 30 min and reprobed for β-actin by reacting with mouse monoclonal anti-β-actin, horseradish peroxidase-conjugated sheep anti-mouse IgG (1:7500 dilution, Santa Crutz) and Western Blotting Luminol Reagent (Santa Crutz). The density of the bands on the nitrocellulose membrane was quantified by densitometry using Gel pro 3.1 (Media Cybernetics, Silver Spring, MD). The density of the band in the control sample was defined as 100% and the densities of the band in the test sample expressed as a percentage of this value.

2.6. Statistical analysis

All experiments were performed at least three times, and the values are expressed as mean ± S.D. Statistical differences between treatments and the control were determined by Student's *t* test procedure. $P < 0.05$ was considered a statistically significant difference.

3. Results

3.1. Involvement of ERK in magnolol-induced steroidogenesis

Magnolol (10–30 μM) significantly induced the corticosterone secretion at 3 h of treatment, and this effect was maintained for up to 24 h (Fig. 1). Only few propidium iodide-positive necrotic and apoptotic cells were detected in DMSO groups, and the percentages of necrotic and apoptotic cells in 30 μM-treated groups showed no significant difference when compared with the DMSO groups (data not shown). Higher concentrations of magnolol ($\geqslant 40$ μM) increased the percentages of both necrotic and apoptotic cells. To obtain evidence for the activation of ERK by magnolol, we assessed the phosphorylation levels of MEK and ERK. Treatment with magnolol resulted in a transient phosphorylation of MEK1/2 and ERK1/2 in a time-dependent manner. The protein levels of the phosphorylated MEK, ERK1 and ERK2 increased to 2.7-, 1.7- and 1.6-fold, respectively, at 5 min after magnolol treatment (Fig. 2A).We further tested the effect of MEK inhibitor on magnolol-induced steroidogenesis and ERK phosphorylation. Pretreatment with 30 μM of PD98059 resulted in a dose-dependent inhibition of magnolol-induced corticosterone production (Fig. 3A) and also significantly blocked magnolol-induced phosphorylation of ERK 1 and ERK2 (Fig. 2B). Another MEK inhibitor U0126 similarly inhibited magnolol-induced steroidogenesis (data not shown). These data suggest that the MEK–ERK signaling pathway is required for magnolol-induced steroidogenesis.

3.2. Involvement of JAK2 in magnolol-induced steroidogenesis and biochemical evidence for the link between JAK2 and ERK pathway

To assess the potential role of TK in the stimulatory effect of magnolol on steroidogenesis, the action of the TK inhibitor

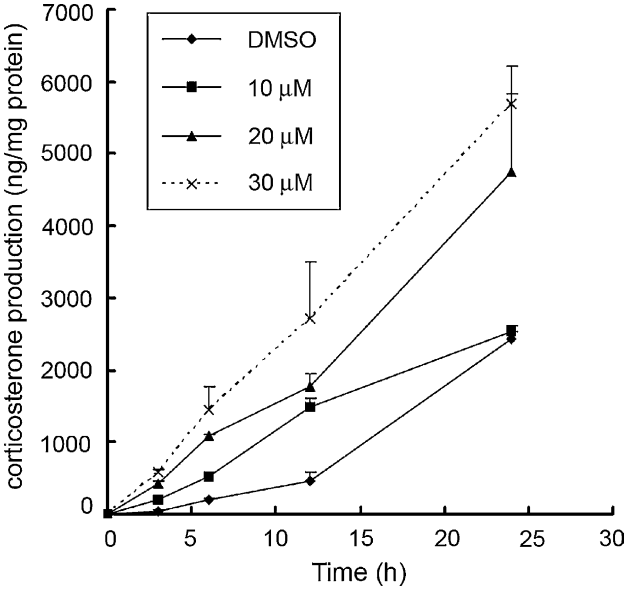

Fig. 1. Time- and dose-dependent response of magnolol on adrenal steroidogenesis. Adrenal cells were treated with 0.1% DMSO (vehicle control) or different concentrations of magnolol (10, 20, 30 μM) for 3, 6, 12 and 24 h. The culture supernatants were assayed for corticosterone. Results given are from a representative of three separate experiments performed in triplicate.

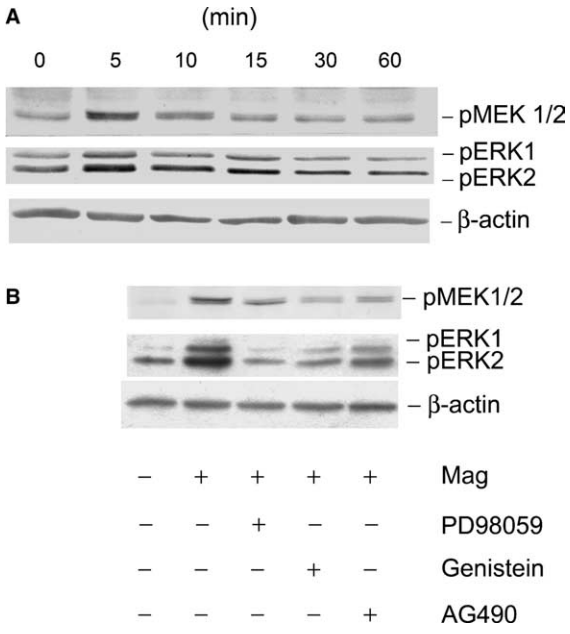

Fig. 2. Involvement of TK and JAK2 in magnolol-induced phosphorylation of MEK and ERK. (A) Adrenal cells were incubated with 30 μM magnolol for different intervals. Cell homogenates were analyzed for phosphorylated MEK1/2, phosphorylated ERK1/2 or β-actin (loading control) by immunoblotting. (B) Adrenal cells were treated for 30 min with different inhibitors (30 μM PD98059, 30 μM genistein, 50 μM AG490) prior to the incubation with 0.1% DMSO or 30 μM magnolol (Mag) for 5 min. The cell homogenates were immunoblotted for phosphorylated MEK1/2 or phosphorylated ERK1/2. β-Actin served as loading control. Results are from a representative experiment.

genistein on this pathway was examined. Genistein (30 μM) was found to significantly abolish magnolol-induced steroidogenesis (Fig. 3B). Moreover, genistein inhibited magnolol-induced MEK and ERK phosphorylation (Fig. 2B). These data indicate that TK plays a role in magnolol-induced MEK and ERK activation and in the subsequent stimulation of steroidogenesis. In order to clarify the involvement of Src kinase, we investigated the effect of the Src kinase inhibitor PP1 on magnolol action. Magnolol transiently increased the protein levels of phosphorylated Src kinase, however, cotreatment with PP1 did not prevent magnolol-induced steroidogenesis (data not shown). Thus, activation of the Src kinase by magnolol is unlikely to contribute to the increased steroid production. We then examined the role of another possible candidate of nonreceptor TK, JAK2. The JAK2 inhibitor AG490 significantly inhibited magnolol-induced corticosterone production (Fig. 3C). The presence of the link between JAK2 and ERK pathway was supported by the observation that AG490 inhibited magnolol-induced phosphorylation of MEK and ERK (Fig. 2B). These data provided evidence that JAK2 is involved in the magnolol-induced steroidogensis and acts as the upstream regulator of the MEK–ERK pathway.

3.3. Involvement of CREB and StAR in magnolol-induced steroidogenesis

A transient increase on the phosphorylation of CREB with time was noted after magnolol treatment (Fig. 4A). We next examined the roles of TK, JAK2 and ERK in magnolol-induced CREB phosphorylation. Pretreatment with PD98059,

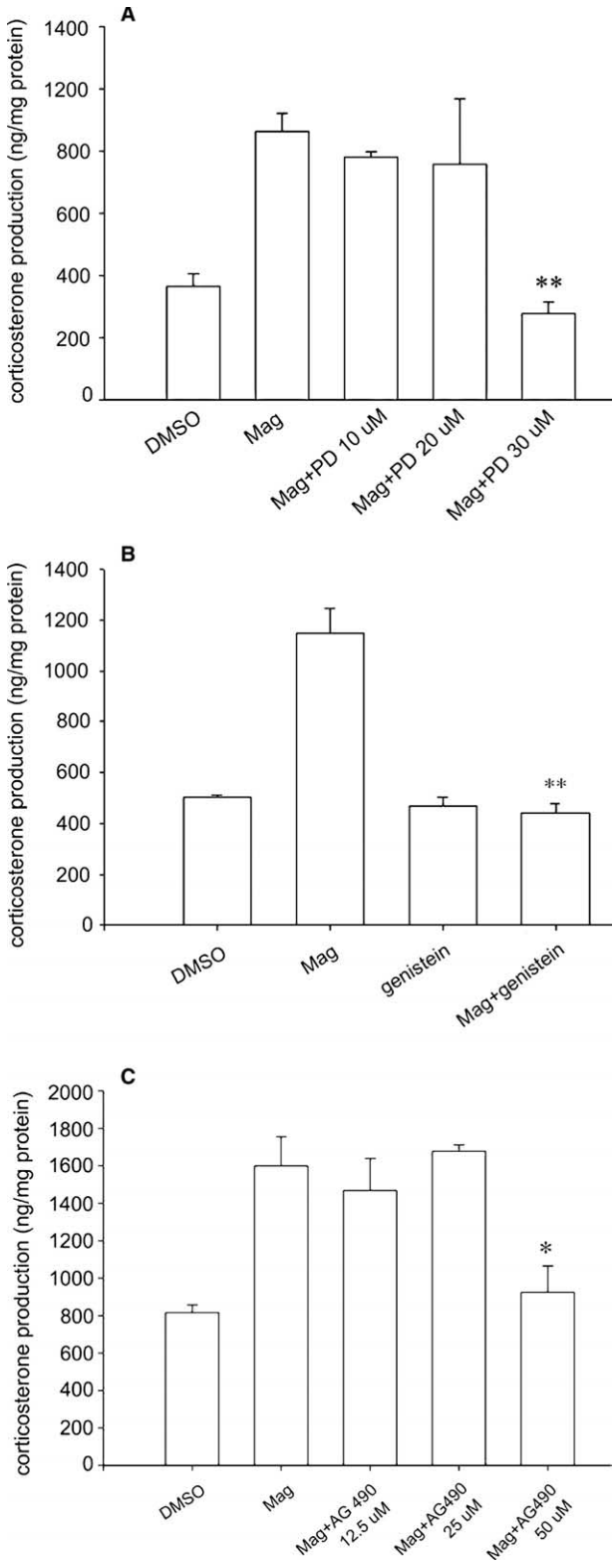

Fig. 3. Effects of MEK- , TK- and JAK2-inhibitors on magnolol-induced steroidogenesis. (A) Adrenal cells were treated with 0.1% DMSO, 30 μM magnolol (Mag), or magnolol plus different concentrations of PD98059 for 6 h. (B) Cells were treated with DMSO, genistein (30 μM) or magnolol (30 μM) plus genesitein for 6 h. (C) Cells were treated with DMSO, magnolol (30 μM) or magnolol plus different concentrations of AG490 for 6 h. The culture supernatants were assayed for corticosterone. $^{*}P < 0.05$; $^{**}P < 0.01$ compared to the Mag group.

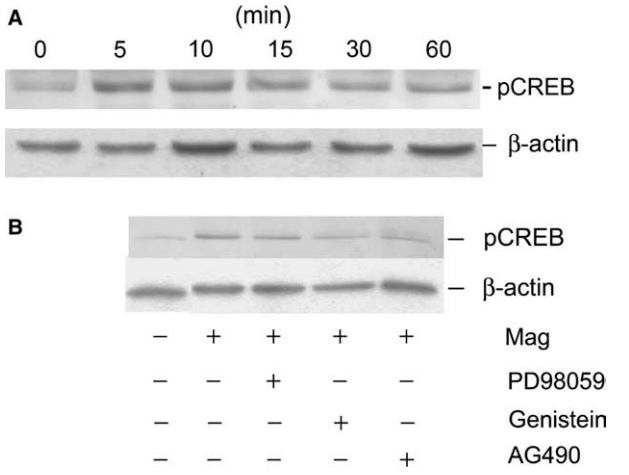

Fig. 4. Effects of MEK-, TK- and JAK2-inhibitors on magnolol-induced CREB phosphorylation. (A) Adrenal cells were treated with DMSO or 30 μM magnolol for different intervals, followed by analyses of cell homogenates for phosphorylated CREB or β-actin. (B) PD98059 (30 μM), genistein (30 μM) or AG490 (50 μM) were added 30 min prior to the addition of magnolol (30 μM, 10 min). Results are from a representative experiment performed in triplicate with β-actin as loading control.

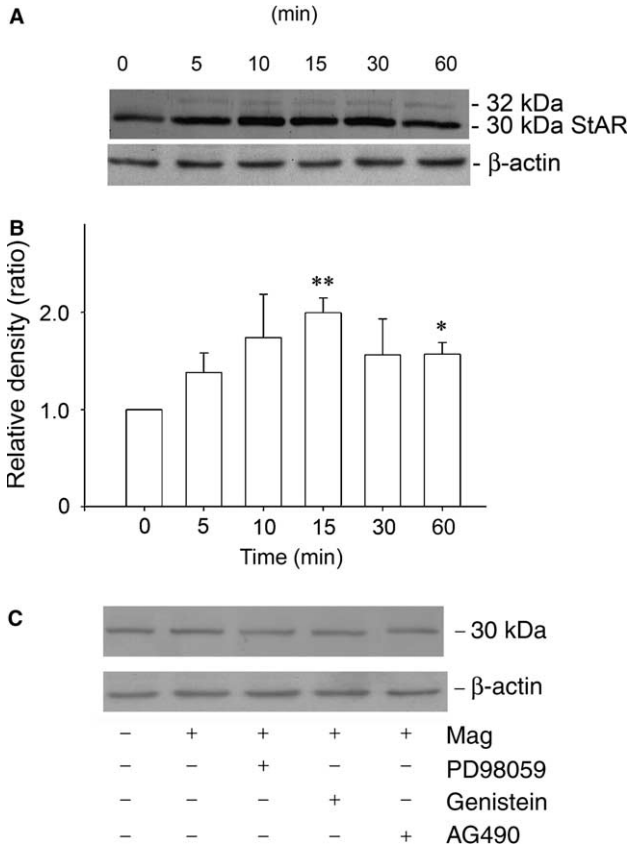

Fig. 5. A time-course study of magnolol-induced expression of 32 and 30 kDa StAR proteins. (A) Adrenal cells were treated with DMSO or 30 μM magnolol for different intervals, followed by analyses of cell homogenates for StAR protein. (B) Densitometric scans of triplicate blots for 30 kDa StAR protein levels from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the 0-min group. (C) PD98059 (30 μM), genistein (30 μM) or AG490 (50 μM) were added 30 min prior to the addition of magnolol (30 μM, 60 min). Cell homogenates were analyzed with rabbit anti-StAR antibodies or mouse anti-β-actin antibody by immunoblotting. Results are from a representative experiment.

genistein or AG490 prevented the phosphorylation of CREB induced by magnolol (Fig. 4B). Thus, magnolol increases CREB phosphorylation via the JAK2–MEK–ERK pathway in adrenal cells. Efforts were then made to identify the possible downstream targets of the CREB phosphorylation. The rabbit anti-StAR antibody used in this study detects a major 30 kDa band and a minor 32 kDa band only in overloaded samples from DMSO-treated cells (Fig. 5A). Magnolol increased the protein levels of both 32 and 30 kDa StAR proteins in a time-dependent manner (Fig. 5B). The increase of the 30 kDa StAR was detected as early as 15 min after magnolol stimulation and was sustained for a period of at least 6 h (data not shown). In order to examine whether proteasomes were involved in the initial increase of 30 kDa StAR, the effect of a nonclassical proteasome inhibitor MG132 was examined. Upon pretreatment with MG132, no significant effects on the protein levels of 32 and 30 kDa were observed, as compared with those in the magnolol-treated group (data not shown). To examine whether the activation of TK, JAK2 and ERK regulate the expression of StAR proteins, the effects of specific inhibitors were investigated. Magnolol-induced 30 kDa StAR protein expression was slightly decreased by the pretreatment with PD98059, genistein or AG490 after short-term treatment (15 min) (not shown) and more pronounced after long-term treatment (60 min) (Fig. 5C). In conclusion, these results demonstrate that the magnolol-induced increase in 30 kDa StAR protein expression is mediated by the JAK2–MEK–ERK–CREB pathway.

4. Discussion

Recent studies have shown that nonreceptor TK, including JAK2 and Src kinase, are important for steroidogensis [7,11,17,18]. In this study, pretreatment with a Src kinase inhibitor PP1 had no effect on magnolol-induced steroidogenesis, thus excluding the involvement of Src kinase in this event. We further demonstrated that inhibition of TK with genistein or selective inhibition of JAK2 with AG490 blocked magnolol-induced corticosterone production. Moreover, AG490 pretreatment decreased magnolol-induced phosphorylation of MEK and ERK and blocked magnolol-induced StAR protein levels. Thus, JAK2 is a major upstream activator of MEK–ERK pathway.

Several lines of evidence support the role of ERK1/2 in adrenal steroidogenesis [19]. In other cell types, MEK–ERK acts as an integrator of mitogen signals originating from receptor TK and G-protein coupled receptors. Previously, ERK1/2 activation has been implicated in both positive and negative regulation of steroidogenesis, depending on the agonists used or the cell type studied [3,5,20–23]. In our study, magnolol rapidly stimulated the phosphorylation of MEK and ERK1/2. Moreover, PD98059 completely inhibited magnolol-stimulated steroidogenesis and ERK1/2 phosphorylation, suggesting that ERK1/2 activation is positively correlated with the magnolol-induced steroidogenesis.

The downstream targets of activated ERK may include hormone sensitive lipase (HSL), steroidogenic factor 1 (SF-1), CREB and StAR proteins [3,4,24,25]. Several investigations have shown that the phosphorylation of CREB is critical for steroidogenesis and StAR protein expression [26–28]. In the classical cAMP-PKA signaling pathway, activated PKA phosphorylates CREB on Ser133, which subsequently increases the association with the CREB binding protein co-activator and results in histone modification and increased transcription [29]. In this study, we observed the transient phosphorylation of CREB by magnolol. We then investigated the possibility for magnolol-induced activated JAK and ERK to phosphorylate CREB. The data suggested that JAK and ERK were responsible for magnolol-induced CREB phosphorylation, since inhibition of TK, JAK2 or MEK blocked CREB phosphorylation stimulated by magnolol, and the time course of ERK phosphorylation matched temporally with that for CREB phosphorylation. Thus, it is possible that magnolol-induced CREB phosphorylation may contribute to steroid synthesis by stimulation of StAR pre-protein synthesis via a direct binding of pCREB to the StAR promoter as observed in Leydig cells [28]. In the neuronal system, Burton's tyrosine kinase, a member of Tec family of TK, has been shown to phosphorylate CREB protein in immortalized hippocampal progenitor cells [30]. Although our data demonstrated that activation of JAK2–MEK–ERK cascade increased CREB phosphorylation, the possibility that JAK2 might directly phosphorylate CREB should be considered.

It has been indicated that phosphorylated CREB can synergistically interact with SF-1 in upregulating the expression of StAR pre-protein in MA-10 mouse Leydig tumor cells and Y1 adrenal tumor cells [26,31]. SF-1 is shown to be essential to both basal and hormone-induced regulation of the StAR gene [32,33]. Gyles et al. [3] have demonstrated that the ERK-dependent phosphorylation of SF-1 enhances its binding to the StAR promoter. Therefore, further studies are needed to determine whether SF-1 is phosphorylated by ERK activation induced by magnolol.

In response to hormonal stimulation, StAR pre-protein (37 kDa) is rapidly synthesized in the cytosol, phosphorylated by PKA and imported into the mitochondria while it actively transports cholesterol from the cytoplasmic pool into the mitochondria [34–36]. The 37 kDa pre-protein is subsequently cleaved by mitochondrial matrix proteases and generates 32 and 30 kDa proteins [37,38]. Since the 37 kDa pre-protein and 32 kDa protein have short half-lives of 5.4 ± 1.1 and 4.4 ± 0.8 min, respectively, they are detected in small quantities in the basal state. In contrast, the 30 kDa StAR protein has a longer half-life (4–5 h), and predominates over the other two forms [37]. Thus, the expression of 30 kDa StAR protein directly correlates with the steroidogenic activity in the previous study [37]. Due to the failure of our anti-StAR antibodies to detect the 37 kDa pre-protein, we only observed the time-dependent increases of 32 and 30 kDa StAR proteins by magnolol stimulation. The time-course of magnolol-induced steroid production was consistent with the expression of 32 and 30 kDa StAR proteins which maintained a high level up to at least 6 h after magnolol stimulation. In rat adrenal cells, pretreatment with MG 132 did not prevent the initial rise on the 30 kDa StAR protein induced by magnolol. In this cell type, the transport of the 37 kDa pre-protein may be too rapid to be degraded by the proteasome. Tajima et al. [39] showed that pretreatment with MG132 increased the contents of 37 kDa pre-protein in human granulose cells at 15 min after stimulation with human chorionic gonadotrophin. Moreover, 32 kDa protein could only be detected 1–2 h later, and no change was found in 30 kDa StAR. Although the 30 kDa StAR could be degraded from the pre-existing 37 kDa pre-protein in a proteasome-dependent way in the cytosol [38], our experiment favors the point that the observed increase of the 30 kDa StAR might derive from the cleavage of newly synthesized 37 kDa pre-proteins. This is supported by the observations that both the levels of 32 and 30 kDa proteins increased simultaneously in the initial phase and the failure of MG132 to block the initial increase of 32 and 30 kDa proteins. This hypothesis is supported by the facts that newly synthesized S^{35} labelled 32 and 30 kDa StAR proteins could be detected within 15 min after dibutyryl cAMP stimulation in a pulse-chase time course study in adrenal cells [37] and that 90–97% of S^{35}-labelled 37 kDa pre-protein can be degraded into S^{35}-30 and -32 kDa proteins within 15 min after cAMP stimulation [40]. The increased StAR pre-protein mRNA expression induced by ERK activation is responsible for steroidogensis [3]. In Y1 mouse adrenal cells, both PD98059 and U0126 treatment decreased forskolin-induced progesterone production and StAR pre-protein mRNA levels [3,41]. In primary culture of rat ovarian granulosa cells, MEK inhibitor U0126 also blocked follicle-stimulating hormone-stimulated StAR pre-protein mRNA [23]. The present study demonstrated that pretreatment of PD98059, genistein or AG490 prevented magnolol-induced expression of 30 kDa StAR protein. Our data are consistent with the involvement of ERK1/2 in the regulation of StAR mRNA expression. Previous studies indicate that the 37 kDa pre-protein can only be phosphorylated by PKA [42,43]. The question then arises as to which kinases are responsible for the phosphorylation of 37 kDa pre-protein in the PKA-independent magnolol action.

Another contribution for activated ERK in steroidogenesis may be mediated by manipulating HSL activity. In 3T3-L1 adipocytes, ERK activation phosphorylates HSL and increases the activity of HSL, which in turn stimulates lipolysis [44]. In addition, HSL has recently been proven to be responsible for the majority of the neutral cholesterol ester hydrolase activity in the adrenal cells [45]. Cherradi et al. [24] indicate that activation of ERK stimulates aldosterone production by increasing HSL activity in adrenal glomerulosa cells. Therefore, magnolol-induced ERK activation may directly phosphorylate and activate the HSL, which in turn increases steroid production, although this needs to be examined. Our previous study has shown that the inhibition of protein synthesis by cycloheximide could only prevent half of the steroid production induced by magnolol [46]. This indicates that magnolol-induced expression of 30 kDa StAR was not completely responsible for magnolol-induced steroidogenesis.

In summary, we propose a novel pathway that contributes to magnolol-induced adrenal steroidogenesis. Activation of JAK2–MEK–ERK is required for this process. We further provide evidence that the CREB phosphorylation and the expression of 30 kDa StAR proteins are the events subsequent to ERK activation.

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