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Original article

Extracellular signal-regulated kinases (ERK) 1, 2 are required for luteinizing hormone (LH)-induced steroidogenesis in primary Leydig cells and control steroidogenic acute regulatory (StAR) expression

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Abstract – The luteinizing hormone (LH) plays a critical role in steroidogenesis, by stimulating cAMP-dependent protein kinase A (PKA) and phospholipase A_2 activity, and by mobilizing calcium and chloride ions. In contrast, whether the ERK 1, 2 mitogen-activated protein (MAP) kinases are involved in LH-induced steroidogenesis is less obvious. Here, we sought to clarify this point in rat primary Leydig cells, naturally bearing the LH receptor (LH-R) in male, and in the mouse tumoral Leydig cell line (MLTC 1). Pre-incubation of both cell types with the mitogen-activated protein kinase kinase (MEK) inhibitors U0126 and PD98059 reduced LH-induced steroidogenesis, and tonically enhanced the expression of the StAR protein. Furthermore, ERK1, 2 were inducibly phosphorylated following LH exposure of MLTC 1 cells. Altogether, our results indicate that in primary as well as in tumoral Leydig cells, inhibiting MEK dampened LH-induced steroidogenesis but enhanced basal as well as LH-induced StAR expression, suggesting that ERK1,2 could be involved in these responses.

LH / rat Leydig cells / steroidogenesis / MAP kinases / StAR

1. INTRODUCTION

Steroid hormones have proven to be crucial for fertility throughout the animal kingdom. In Mammals, steroidogenesis is primarily controlled by luteinizing hormone (LH), which targets testosterone production in Leydig cells of the testis, and progesterone in granulosa cells of the ovary. The LH-R is a seven-pass transmembrane receptor which mainly couples to Gαs, increasing the canonical second messenger cAMP levels [1], thereby activating PKA which ultimately targets CREB family-dependent transcriptional events. Steroid biosynthesis requires the rapid uptake of intra-cellular stores of cholesterol to the outer mitochondrial membrane and delivery of cholesterol to the inner mitochondrial membrane, where it is cleaved by cholesterol side-chain cleavage cytochrome P450. Importantly, the cholesterol transport is a rate-limiting step in steroidogenesis which depends on de novo synthesis of the StAR protein [2]. Recently,

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transcription of StAR has been shown to be regulated by CREM, a CREB-related transcription factor, in the MA10 Leydig cell line [3].

Besides, following the coupling of LH-R to Gαi or Gαq, other alternative LH-mediated signaling pathways potentially regulating steroidogenesis have been described in rat Leydig cells, including calcium [4, 5] and chloride ion mobilization[6], as well as activation of phospholipase A_2 [7]. In L cells, stably expressing the LH-R, phosphoinositide mobilization has also been evoked [8]. In contrast, the implication of ERK1,2 MAP kinases to transduce LH-induced steroidogenesis has led to more conflicting views. For instance, in MA10 cells, cAMP-induced steroid synthesis depends upon phosphorylation of ERK1,2 MAP kinases, and on a subsequent raise in the StAR gene transcription [9]. Likewise, in primary granulosa cells, progesterone production necessitates ERK1,2 activation [10]. In contrast, in a granulosa cell line overexpressing the LH-R, both StAR transcription and steroidogenesis are hampered by ERK MAP kinases [10, 11]. As mentioned above, these data originate from studies of Leydig or granulosa immortalized cell lines.

To gain a better insight into the control of LH-mediated steroidogenesis in the LH natural target cells, we investigated the dependency of steroidogenesis on the ERK1,2 MAP kinases in MLTC 1 cells, a Leydig tumoral cell line, and in freshly isolated mature rat Leydig cells.

2. MATERIALS AND METHODS

2.1. Pharmacological reagents

Porcine LH (pLH CY 1354; 2.0 × NIH LH S1) was purified by Dr Yves Combarnous. Collagenase, leupeptin, pepstatin A, aprotinin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. Park-Davis (PD) 98059 and U0126 were purchased from New England Biolabs Inc.

2.2. Cells

Leydig cells were isolated from the testes of mature 52-day-old Wistar rats as described [12]. Briefly, the testes were dispersed by collagenase digestion (0.25 $mg{\cdot}mL^{-1}$) in L15 medium (Serva), the tubular tissue was settled in a glass cylinder, the supernatant was recovered, centrifuged for 5 min at 80 *g*, and the cell pellet was placed onto a Percoll discontinuous gradient (17, 42, 70%). After centrifugation at 80 *g* for 30 min, Leydig cells were recovered at the 42–70% interface and washed twice in L15 medium prior to use. The purity of Leydig cells reached 85%, as determined by histochemical staining of 3β– hydroxy-steroid deshydrogenase (3β-HSD) [13]. Macrophages were the other cell type identified in the preparation, as shown by immunochemistry with an anti-ED2 antibody (Serotec). Cell viability reached 90%, as determined by Trypan blue exclusion.

MLTC 1 cells [14]**,** generously provided by Dr E.M. McNamara (Université de Liège, Belgium), were grown in RPMI 1640 with L-Glutamine and 25 mM Hepes, supplemented with 0.1% gentamycin, 10 $U{\cdot}mL^{-1}$ penicillin, 10 $\mu g{\cdot}mL^{-1}$ streptomycin and with 10% fetal calf serum, at 37 °C with 5% CO_2.

2.3. Biological responses and pharmacological treatments

Freshly isolated Leydig cells (8×10^5 cells per aliquot in L15 medium) were stimulated for 3 h at 34 °C under continuous shaking. Then the cells were centrifuged at 80 *g* for 10 min, and secreted testosterone was quantified in the media by a radioimmunoassay (RIA), as previously described [12]. MLTC 1 cells were seeded at 1.5×10^5 cells per well in 24-well plates. After overnight plating, the cells were serum-starved for one hour before stimulation for 3 h at 37 °C, then the media were harvested, boiled for 10 min, centrifuged and the supernatants were assayed for progesterone content [15, 16].

The concentrations of PD98059 (100 µM) and U0126 (10 µM) were similar to those commonly used with transformed cell lines. Controls incubated with similar doses of DMSO, the MEK inhibitor solvent, were performed. Pharmacological treatments were generally more detrimental to Leydig cell viability when compared to LH-R-overexpressing cell lines (our unpublished observations). Therefore, our experimental procedures were standardized in Leydig cells and similar doses were assayed in MLTC 1 cells. In addition, intracellular cAMP production was quantified in any experimental condition, to ensure primary Leydig cell viability and responsiveness to LH stimulation. For example, in the control experiments, the basal cAMP level was 18.7 ± 1.2 nM and LH-induced cAMP level was 41.75 ± 1.2 nM. Likewise, in the presence of UO126, the basal cAMP level was 18.25 ± 2.5 nM and LH-induced cAMP level was 44.75 ± 0.01 nM.

2.4. Immunoblot analysis and antibodies

After hormone stimulation, the cells were disrupted on ice for 30 min in a lysis buffer containing 20 mM Tris (pH 7.8), 50 mM NaCl, 5 mM EGTA, 1 mM PMSF, 4mM sodium orthovanadate, 5 $\mu g \cdot mL^{-1}$ leupeptin, 5 $\mu g \cdot mL^{-1}$ pepstatin A, 5 $\mu g \cdot mL^{-1}$ aprotinin and 1% v/v Triton X100. After centrifugation at 10 000 *g*, cell supernatants were recovered and protein content was estimated by a Bradford reaction measured at 595 nm. A hundred micrograms of total cell lysates were resolved by SDS-PAGE (10% acrylamide 37.5:1, Biorad), electrophoretically transferred to PVDF membrane (NEN Life Science Products) for 2 h at 100 V, and probed with the respective antibodies. The rabbit anti-StAR antibody, generously provided by Dr Hales [17], was diluted 1:5000, and the anti-phospho-$p44^{ERK1}/p42^{ERK2}$ monoclonal antibody (New England Biolabs Inc.) was diluted 1:2000. Horseradish peroxydase-coupled anti-mouse or anti-rabbit antibodies (Sanofi/Pasteur) diluted 1:5000 were used to detect antigen-antibody interactions by enhanced chemiluminescence (NEN Life Science Products). The ImageScan Software (Amersham-Pharmacia) was used for scanning and quantification. The blotting membranes were stained with Coomassie blue, scanned and used to normalize the immunodetections.

2.5. Statistical analysis

Each experiment was reproduced more than 3 times, with each sample in quadruplicate for steroid quantifications. To compare the significance of the results obtained from drug-treated and drug-untreated samples, statistical analysis was performed with the Fisher *F* test.

3. RESULTS

3.1. ERK1,2 MAP kinases are involved in steroidogenesis

In Leydig cells, pre-treatment with PD98059 (Fig. 1A) strongly impeded LH-induced testosterone biosynthesis (up to 80%), and also the basal response (50%), suggesting the involvement of MEK in driving the steroidogenic response. To minimize the risk of studying non-specific effects, another widely used MEK inhibitor, U0126, was assayed. This set of experiments led to similar results, i.e. 70% inhibition of the LH-stimulated response, and 40% inhibition of the basal response (Fig. 1B). MEK are dual-specificity Thr- and Tyr kinases which specifically activate ERK1,2 MAP kinases. Hence, we sought to investigate by immunoblotting whether ERK1,2 were dually phosphorylated in basal and LH-induced conditions. Surprisingly, in response to LH stimulation, we failed to visualize any modulation of ERK1,2 phosphorylation level, whatever the dose of LH (Fig. 1C), or the hormone stimulation time (Fig. 1D). However, ERK1,2 were basally phosphorylated, which may further prevent LH-induced phosphorylation, and this basal phosphorylation was completely abrogated by U0126.

In MLTC 1 cells, pre-treatment with U0126 caused a decrease in the basal (insert) as

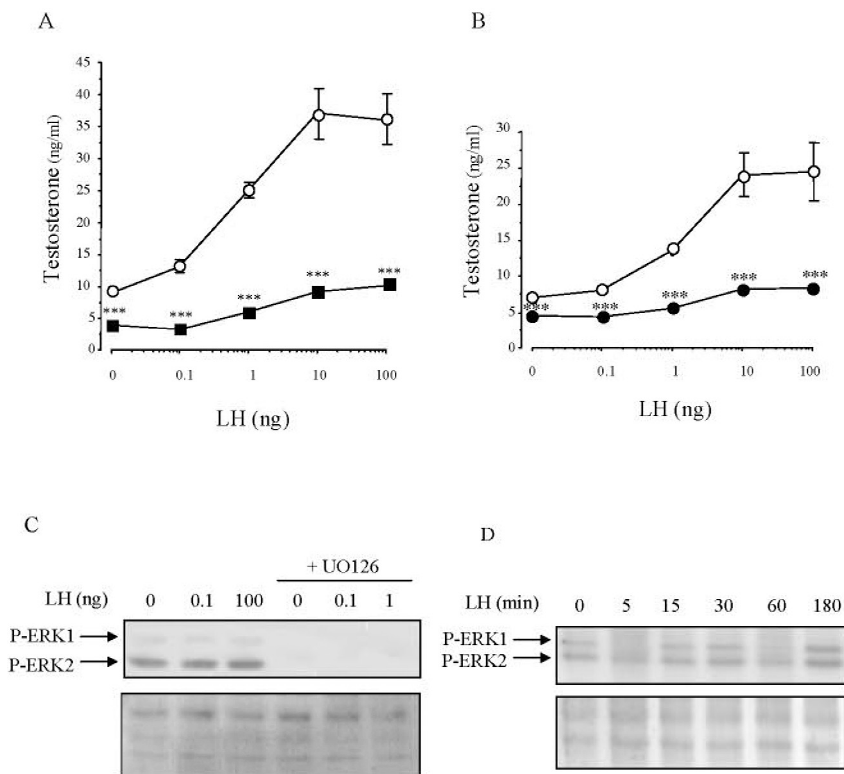

Figure 1. MAPK-dependent steroidogenesis in Leydig cells. Leydig cells (8×10^5 cells) were pre-incubated for 30 min with (A) 100 mM PD98059 (black squares) or (B) with 10 µM U0126 (black dots), or with 0.5% DMSO (open dots), prior to stimulation with increasing doses of LH for 3 h. Then, testosterone synthesis was quantified. The results are expressed as mean values of quadruplicates ± S.D. The statistical analysis was performed with the Fisher F test, with *** for $P < 0.001$. In the inhibitor-treated series, the error bars are too tiny to be visible. (C) Immunodetection of ERK1,2 MAP kinase phosphorylation level, with or without U0126 as indicated, following 2 h of LH stimulation. (D) Kinetics of ERK1,2 MAP kinase phosphorylation level, following LH stimulation ($100\ ng \cdot mL^{-1}$). Beneath is shown a Coomassie blue staining of the membranes. Data shown are representative of three independent experiments.

well as in the LH-induced progesterone response (Fig. 2A). The same results were obtained with PD98059 (data not shown). As opposed to Leydig cells, in MLTC 1 cells, phosphorylation of ERK1,2 was enhanced by increasing amounts of LH (Fig. 2B). Consistently, pre-treatment with U0126 completely abolished ERK1,2 basal and LH-induced phosphorylation.

Albeit modulation of ERK1,2 MAP kinase phosphorylation upon LH stimulation cannot be detected in Leydig cells, it appears that their inhibition has more pronounced outcomes on steroidogenesis than in MLTC 1 cells.

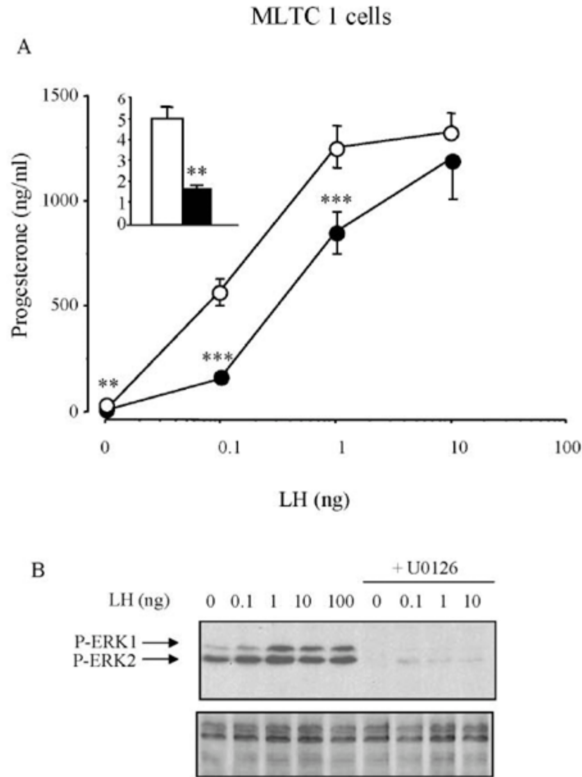

Figure 2. MAPK-dependent steroidogenesis in MLTC 1 cells. (A) MLTC 1 cells (1.5×10^5 cells) were pre-incubated with 10 μM U0126 (black dots) or not (open dots) for 30 min, prior to stimulation with increasing doses of LH for 3 h, and progesterone synthesis was quantified. The inserted graph is a magnification of the basal levels, in both conditions. The results are expressed as above, with ** for $P < 0.01$. (B) Immunodetection of ERK1,2 MAP kinase phosphorylation level after one hour of LH stimulation. Beneath is shown a Coomassie blue staining of the membranes. Data shown are representative of three independent experiments.

3.2. LH regulates StAR protein expression differently in Leydig cells and in MLTC 1 cells

We next investigated whether the LH-dependent signaling events that modulate steroidogenesis could converge on expression of the StAR protein as an intermediate. The 30 kDa mature form of StAR was up-regulated by LH stimulation in Leydig cells (Fig. 3A), as previously reported [18]. However and quite strikingly, pre-treatment of cells with U0126 enhanced the basal StAR expression level so much, that the inducibility upon LH stimulation was greatly reduced.

In MLTC 1 cells, stimulation with LH (Fig. 3B, lane 2) for 3 h increased the expression of the StAR protein in the range of 2-fold for both the 37 kDa precursor and the 30 kDa protein [19]. Pre-treatment with U0126 led to a 1.5-fold increase in the StAR basal level (lanes 1 and 3), hence reducing StAR inducibility (lanes 1 and 2 vs. 3 and 4). Therefore, these results suggest that in Leydig cells, ERK1,2 would dampen LH-induced

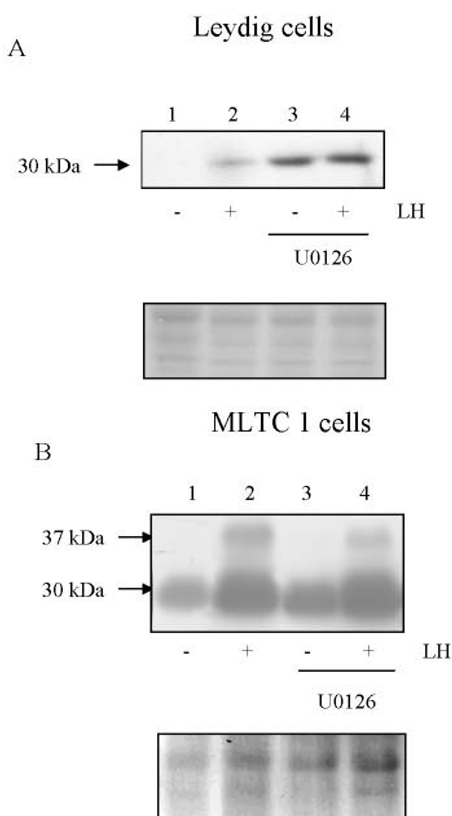

Figure 3. StAR expression level in Leydig cells (A) and in MLTC 1 cells (B) following stimulation with 10 ng·mL^{-1} LH (+), for 2 (A) or 3 h (B), as visualized by immunoblotting. Cells were pre-incubated with U0126 as above. Beneath is shown a Coomassie blue staining of the membranes. Data shown are representative of three independent experiments.

StAR inducibility. In MLTC 1 cells, StAR inducibility would depend on ERK1,2 to a minor extent. And importantly, inhibiting ERK1,2 MAP kinases elevates the StAR steady-state level in primary Leydig cells as well as in MLTC 1 cells.

4. DISCUSSION

The present study reports the involvement of ERK1,2 MAP kinases in LH-controlled testosterone biosynthesis in primary cultures of adult rat Leydig cells, and highlights their unexpected impact on StAR expression. In these cells, we show that LH stimulates testosterone production via ERK1,2 activation, as demonstrated by pre-treatment with the U0126 and PD98059 MEK-1 inhibitors. Furthermore, albeit both phosphorylated ERK1,2 (as presented herein) and up-regulation of StAR [18] are required for LH-induced testosterone production, we found that ERK1,2 inhibition did not lead to StAR down-regulation. This suggests that MAP kinase-mediated steroidogenesis stimulation might not primarily impact on StAR. Instead, ERK1,2 appear to inhibit basal StAR expression, which is similar to data obtained from immortalized granulosa cells over-expressing the LH-R [11]; but these results contrast with our data, in that the inhibition of ERK1,2 activation also enhanced progesterone production, hence directly linking ERK1,2, StAR and progesterone biosynthesis. This discrepancy may be explained by the time-course of LH stimulation and by the duration of the pharmacological inhibitor treatments. In our study, incubation times with LH and with pharmacological inhibitors did not overpass 3.5 h, in contrast to 24 to 48 h in the immortalized granulosa cells [11]. This leads us to the hypothesis that ERK1,2 MAP kinases might serve to level off StAR to restrain the steady-state testosterone biosynthesis in Leydig cells. Interestingly, a hitherto uncharacterized steroid-dependent feedback inhibition of StAR expression has recently been shown [20, 21]. Whether this step could be accomplished by ERK1,2 is addressed by our work. An important outcome of this finding is that StAR up-regulation, although necessary, may not be sufficient to trigger steroidogenesis.

Our claim that in Leydig cells testosterone production is activated by LH through a MAP kinase-dependent pathway is based on the use of two structurally distinct MEK inhibitors, namely U0126 and PD98059. MEK inhibitors can also serve as MEK-5 inhibitors [22]; nevertheless, despite extensive attempts, ERK5, although expressed in

Leydig cells, was never found phosphorylated in our hands (our unpublished observations). Furthermore, in Leydig cells, we were not able to directly visualize an increase in ERK1,2 phosphorylation upon LH exposure, and the following reasons can be inferred : first, phosphorylated ERK1,2 may be differentially dispatched between the cytoplasm, where steroidogenesis occurs, and the nucleus where ERK1,2 exert their transcription-devoted functions. Such a compartmentalization would not be detected when assaying whole-cell extracts, as we did herein. Second, a very weak and undetectable LH-induced ERK phosphorylation may be sufficient to sustain steroidogenesis. Third, only 6×10^3 LH-R are expressed at the Leydig cell surface [23], which may not be sufficient to induce a detectable ERK activation. In contrast, in MLTC 1 cells, a higher receptor number, i.e. 3×10^4 sites per cell [14], could amplify all the signaling mechanisms. We favor the latter hypothesis because, according to previous studies, ERK1,2 phosphorylation level increases in parallel to the LH-R number [24, 25].

In proliferating Leydig cell precursors, hCG (human choriogonadotropin), although functionally different from LH [26, 27], binds to LH-R and induces ERK phosphorylation [28]. These cells are still devoid of steroidogenic function [29] and ERK1,2 phosphorylation could reflect their level of proliferation. Here, we have shown that ERK1,2 could also play a role in Leydig cell differentiated function, since they exert a stimulatory role in LH-controlled steroidogenesis, and could behave as brakes to regulate StAR expression. By this means, ERK1,2 MAP kinases could contribute to fine-tuning the primary differentiated function of Leydig cells.

NOTE ADDED IN PROOF

While this work was being reviewed, Martinelle et al. published that ERK enhanced the stimulatory effect of hCG on the de novo synthesis of StAR, in plated Leydig cells from immature rats.

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