Curcumin down regulates 8-br-cAMP-induced steroidogenesis in mouse Leydig cells by suppressing the expression of Cyp11a1 and StAR independently of the PKA-CREB pathway

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Abstract. Although curcumin was widely applied as a functional food for different diseases, it was found to reduce serum testosterone level and fertility in male animals by unknown molecular mechanisms. Here in our study, we investigated the possible mechanisms of curcumin-suppressed testosterone production in Leydig cells. Our enzyme immunoassay results showed that curcumin cell-autonomously suppressed ovine luteinizing hormone-stimulated testosterone production in primary Leydig cells and 8-bromo-cyclic adenosine monophosphate (8-br-cAMP)-induced progesterone production in MA-10 cells. Furthermore, our real-time PCR, Western blot, and 22R-OHC/pregnenolone supplementing experiment data demonstrated that curcumin suppressed 8-br-cAMP-induced steroidogenesis in Leydig cells by inhibiting the expression of StAR and Cyp11a1. Interestingly, our Western blot data showed that although curcumin suppressed PKA activity, it did not alter the 8-br-cAMP-induced phosphorylation of CREB. On the contrary, the real-time PCR results showed that curcumin suppressed 8-br-cAMP-induced expression of Nr5a1 and Fos, which are crucial for cAMP-stimulated StAR and Cyp11a1 expression in Leydig cells. Collectively, our data demonstrated that curcumin may suppress cAMP-induced steroidogenesis in mouse Leydig cells by down-regulating Nr5a1/Fos-controlled StAR and Cyp11a1 expression independently of the PKA-CREB signaling pathway.

Key words: Curcumin, Steroidogenesis, StAR, CYP11A1, Protein kinase A

CURCUMIN (C₂₁H₂₀O₆) is an active component of turmeric (Curcuma longa), which is commonly used as a food additive. Recent studies have shown that curcumin possesses numerous beneficial properties such as anti-cancer [1-3], antioxidant [4], anti-inflammatory [5], and anti-arthritis [6]. Beyond these beneficial effects, however, studies also revealed the antifertility effects of curcumin. It was reported that a 60-day treatment of curcumin reduced the fertility of rats [7]. In addition, long-term administration of an aqueous rhizome extract of C. longa was also found to reduce the weight of the testis, epididymis, and seminal vesicle in mice. The same study also showed increased percentage of morphologically abnormal spermatozoa and reduced sperm count, as well as the serum testosterone level in mice [8]. Of note, an in vitro study had demonstrated that curcumin also cell-autonomously inhibited the LH-stimulated testosterone production in rat Leydig cells [9].

Although the molecular mechanisms of curcumin-suppressed steroidogenesis in Leydig cells are still unclear, a previous study has demonstrated that curcumin inhibits the adrenocorticotropic hormone- (ACTH) or 8-(4-chlorophenylthio)-cAMP-stimulated cortisol synthesis in bovine adrenal zona fasciculata cells by suppressing the mRNA expression of STAR and Cyp11a1 [10]. Because the expression of STAR and Cyp11a1, which are two rate-limiting enzymes in steroidogenesis, is known to be tightly controlled by the cAMP-PKA signaling path-
way in steroidogenic cells, we hypothesized that curcumin may also inhibit cAMP-stimulated steroidogenesis in Leydig cells by regulating PKA-mediated induction of StAR and Cyp11a1 expression.

Materials and Methods

Reagents and chemicals
Culture medium DMEM/F12, Medium 199 (M199), trypsin-EDTA 0.25%, antibiotics (penicillin G and streptomycin sulfate), fetal bovine serum, Hank’s balanced salt solution, and trypan blue were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Type 1 collagenase was bought from Worthington Biochemical Corporation (Lakewood, NJ, USA). BSA and 22R-hydroxycholesterol (22R-OHC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 8-bromo-cyclic adenosine monophosphate (8-br-cAMP) was ordered from Tocris Bioscience (Ellisville, MO, USA). Pregnenolone was bought from Steraloids (Newport, RI, USA).

Animals
Male C57BL/6 mice (5 weeks old) were obtained from the National Taiwan University Experimental Animal Center. All animals were fed ad libitum with a 12-hour light (0900–2100)/12-hour dark (2100–0900) cycle until they were 10–12 weeks old. All experimental protocols were approved by the National Taiwan University Institutional Animal Care and Use Committee (NTU-101-EL-120). All procedures were complied with the National Institutes of Health Guide for the care and use of laboratory animals.

Primary mouse Leydig cell culture
To obtain primary Leydig cells, all mice were sacrificed by cervical dislocation. The theca of the testes was removed, and the remaining seminiferous tubules were washed with M199 and collected. Seminiferous tubules were then incubated with disassociation buffer (Hank’s balanced salt solution with 10 mg/mL BSA and 235 U/mL type 1 collagenase) at room temperature. After the seminiferous tubules and interstitial cells were filtered with a 250-mesh filter and the seminiferous tubules were removed, the interstitial cells were suspended in M199 without serum supplementation. The live cells were cultured (10^6 cells/tube, supplie with 1 mL medium) and treated with ovine LH (oLH) or curcumin and incubated at 37°C with 5% CO₂. The medium was collected 4 hours after incubation and stored at −20°C until assay.

Cell culture
The MA-10 cell line was a kind gift from Dr. Ing-Cherng Guo (School of Veterinary Medicine, National Taiwan University). The MA-10 cells were cultured in T-75 cell culture flasks (Thermo Fisher Scientific) with DMEM/F-12 (containing 10% fetal bovine serum, 2.2 mg/mL sodium bicarbonate, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) and incubated at 37°C with 5% CO₂. For progesterone assay and Western blot analysis, cells were seeded in 24-well culture plates (Thermo Fisher Scientific) at the density of 1 × 10^5 cells/well overnight for cell attachment. For progesterone assay, cells were treated with 8-br-cAMP and different dosages of curcumin, prepared in DMEM/F12 medium without serum. The applied medium was collected after a 4-hour treatment and stored at −20°C until assay. For 22R-OHC and pregnenolone supplementing tests, cells were seeded in 48-well culture plates at the density of 5 × 10^4 cells/well.

Enzyme immunoassay of testosterone and progesterone
The antibodies for enzyme immunoassay of testosterone and progesterone were developed in our laboratory as described before [11, 12]. Briefly, all samples and standards were co-incubated with horseradish peroxidase-conjugated progesterone or testosterone in antibody-coated 96-well plates. The signal was developed by adding 3.7 mM o-phenylenediamine as the substrate of horseradish peroxidase. The developing reaction was stopped by 8 N H₂SO₄. The optical density at 490 and 630 nm (as background reference) was measured with a μQuant spectrophotometer (Biotek). By comparing the optical density values of a standard curve, the steroid hormone level of each sample was calculated.

Reverse transcription and real-time PCR
Total RNA was extracted from MA-10 cells using TRizol reagent (Thermo Fisher Scientific) following the manufacturer’s protocol. For reverse transcription, 1 μg of total RNA with 20 units of SuperScript III reverse transcriptase were used (Thermo Fisher Scientific).

For Real-time PCR, the following protocol was performed: initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and annealing extension at 60°C for 30 seconds. The melt-curve assay was performed immediately after the 40-cycle amplification. The PCR reactions were executed using the StepOne Real-Time PCR System (Applied Bio-
systems, Foster City, CA, USA) with SYBR Green PCR master mix (Applied Biosystems). The primer pairs used in this study are listed in Table 1.

### Protein extraction and Western blot

After a 4-hour treatment of 8-br-cAMP and curcumin, MA-10 cells were rinsed twice with cold phosphate-buffered saline and harvested in 50 μL of Laemmli dye containing 10 mM dithiothreitol. 8 μL sample was loaded into each well of 10% or 12% SDS-polyacrylamide gels and separated by electrophoresis. The separated proteins were transferred to a polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA) using a semi-dry blotter (Bio-Rad) at 25 V/0.4 A for 30 minutes. The membrane was blocked in TBST containing 5% skim milk for 1 hour at room temperature, and followed by an incubation with primary antibodies specific for StAR [12, 13], CYP11A1 [12, 14], phosphorylated-PKA substrate (Cell Signaling Technology, Boston, MA, USA), and ACTB (Millipore, Menlo Park, CA, USA), prepared in 1% BSA with TBST overnight at 4°C. After a three-time TBST wash, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit or antimouse IgG (Santa Cruz Biotechnology, Dallas, Texas, USA) in 5% skim milk with TBST for another 1 hour. Finally, the membranes were washed with TBST and visualized by adding the enhanced chemiluminescence substrates (GE, Aurora, OH, USA, for CYP11A1 protein detection; Millipore, Menlo Park, CA, USA, for other proteins) in a Bio-Rad Chemidoc Touch Imaging System.

### Data analysis

Statistical analyses were carried out using SigmaPlot 12.5 (Aspire Software International, Ashburn, VA, USA). One-way analysis of variance (ANOVA) was conducted followed with Duncan’s multiple comparison as the post hoc test. Differences were considered significant when $p < 0.05$. All results were presented as the means ± standard error of the mean (SEM).

### Results

To confirm the inhibitory effect of curcumin on steroidogenesis in Leydig cells, we treated primary mouse Leydig cells with different doses of curcumin (5, 10, 20, and 40 μM) in the presence or absence of oLH for 4 hours, and measured the testosterone production. As shown in Fig. 1A and B, curcumin significantly suppressed both the basal and oLH-stimulated testosterone production in primary mouse Leydig cells. Although curcumin had no effects on the basal steroidogenesis in MA-10 cells (Fig. 1C), it dose-dependently decreased 8-br-cAMP-induced progesterone production in MA-10 cells (Fig. 1D).

To further investigate the possible mechanisms involved in curcumin-suppressed steroidogenesis in Leydig cells, we first analyzed the mRNA expression of four important steroidogenic genes including Tspo, Hsd3b1, Star, and Cyp11a1 in MA-10 cells treated with or without 8-br-cAMP (100 μM) and curcumin (20 μM) for 4 hours. As shown in Fig. 2A and B, both curcumin and 8-br-cAMP did not alter the Tspo and Hsd3b1 mRNA expression in MA-10 cells. However, our results showed that curcumin significantly suppressed the 8-br-cAMP-induced mRNA expression of Star and Cyp11a1 (Fig. 2C and D), which are well-known downstream targets of the PKA signaling.

In order to confirm the involvement of post-PKA signaling, STAR, and Cyp11a1 in curcumin-suppressed steroidogenesis in MA-10 cells, we used Western blot to analyze the protein levels of StAR, Cyp11a1, Phospho-

### Table 1

Primer pairs used in this study.

| Gene Name | Accession Number | Sense (5’ to 3’) | Antisense (5’ to 3’) | Product Size (bp) |
|-----------|------------------|-----------------|---------------------|------------------|
| Tspo [31] | NM_009775.4      | GCTGGCTTTTGCCACCCGTGC | TGGCTGGCACGGCTGATTCC | 119              |
| Star [31] | NM_011485.4      | CGTGAGCGTGCCGTGACCA | TGACACCACCTGCTCGGCA  | 95               |
| Cyp11a1 [31] | NM_019779.3 | ACCGAGATGCTGGCAGGGAGG | CGGGCAGCCAGGACTTCAGC | 119              |
| Hsd3b1 [31] | NM_008293.3 | CAGCCAGGGGCCTGGGAGA | GCTGGGCTTAGGCGGAGAC  | 143              |
| Nr5a1    | NM_139051.3      | GTCAGGGGAGGGGGCAATA | CAACCTGCCCCCGCCCAATC | 154              |
| Fos      | NM_010234.2      | GCGTACCTCCCGCTGCA   | CAGCCACTGCAAGCTGCA   | 197              |
| Actb     | NM_007393.3      | GTGCGTGACATCAAGAG   | CAAGAGGAAGGGCTGGA    | 177              |
PKA substrates, and phospho-CREB. Similar to the mRNA expression patterns, our results showed that curcumin dramatically blocked the 8-br-cAMP-induced StAR and Cyp11a1 protein expression in MA-10 cells (Fig. 3A and B). Interestingly, the Western blot data showed that the phosphorylation of CREB in MA-10 cells was not altered by curcumin, although curcumin treatment had strong inhibition on PKA activity (Fig. 3A and B). Instead, we found that the 8-br-cAMP-induced mRNA expression of Nr5a1 and Fos, two other transcription factors of Star and Cyp11a1, were suppressed by curcumin (Fig. 3C). Collectively, these results suggested that curcumin may block cAMP-induced expression of StAR and Cyp11a1 by down-regulating Nr5a1 and Fos in a PKA-CREB independent manner.

To further confirm the involvement of StAR and Cyp11a1 in curcumin-suppressed steroidogenesis, we co-treated MA-10 cells with 22R-OHC and pregnenolone in the presence of 8-br-cAMP and curcumin. 22R-OHC and pregnenolone are two cell-permeable steroidogenesis intermediates which can bypass the StAR and CYP11A1 respectively. Our results showed that 22R-OHC supplementation only partially restored curcumin-suppressed progesterone production (Fig. 4A), while the adding of pregnenolone fully recovered the curcumin-impaired steroidogenesis (Fig. 4B). Collectively, these results confirmed the involvement of both StAR and Cyp11a1 in curcumin-suppressed Leydig cell steroidogenesis.

**Discussion**

To test whether curcumin can cell autonomously affect steroidogenesis in Leydig cell, the mouse Leydig cell line MA-10 was used in the present work. Because MA-10 cells express low level and activity of Cyp17a1, the enzyme to convert progesterone into 17-OH progesterone for testosterone synthesis, MA-10 cells produce progesterone as the major product during stimulated-steroidogenesis [15]. By comparing the results obtained from primary mouse Leydig cells and MA-10 as the
cells, we demonstrated that curcumin not only reduces serum testosterone level \textit{in vivo} [8], but also cell-autonomously suppressed the steroidogenesis in Leydig cells (Fig. 1).

By checking the expression of four well-known steroidogenesis genes including \textit{Tspo} [16], \textit{Hsd3b1} [17], \textit{Star} [18], and \textit{Cyp11a1} [19], our data showed the possible involvement of post-PKA signaling in curcumin-suppressed steroidogenesis in Leydig cells. According to our data, the mRNA expression levels of \textit{Tspo} and \textit{Hsd3b1} in MA-10 cells were not altered by both 8-br-cAMP and curcumin (Fig. 2A and B). The results, whereas, showed that the mRNA expression of \textit{Star} and \textit{Cyp11a1} was significantly stimulated and suppressed by 8-br-cAMP and curcumin respectively (Fig. 2C and D). Consistently, Western blot data showed that the protein levels of StAR and Cyp11A1 in MA-10 cells were also regulated similarly (Fig. 3A and B).

Because the cAMP-stimulated StAR and Cyp11A1 expression is known to be largely regulated by PKA signaling [20, 21], we next investigated the involvement of post-PKA signaling in curcumin-suppressed Leydig cell steroidogenesis. By analyzing the level of phospho-PKA substrates in MA-10 cells, we found that curcumin potently inhibited both the basal and 8-br-cAMP-stimulated PKA activity in MA-10 cells (Fig. 3A). Except for a previous study showed the \textit{in vitro} inhibitory effect of curcumin on purified PKA [22], this was the first time to report that curcumin potently suppresses PKA activity in mammalian cells. In steroidogenic cells, the transcription of \textit{StAR} or \textit{Cyp11a1} is known to be largely controlled by the transcription factors including NR5A1 [23], CREB [24], and FOS [24]. Under the stimulation of cAMP, the activated PKA increases the expression of NR5A1 [25] and FOS [26], and the phosphorylation of CREB to activate the downstream gene transcription [27, 28]. Surprisingly, it seemed that the curcumin-suppressed expression of StAR and Cyp11A1

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**Fig. 2** The effect of curcumin on mRNA expression of steroidogenic genes in MA-10 cells.

The mRNA levels of \textit{Tspo} (A), \textit{Hsd3b1} (B), \textit{Star} (C) and \textit{Cyp11a1} (D) in curcumin- (20 μM for 4 hours) and 8-br-cAMP-treated (100 μM for 4 hours) MA-10 cells were quantified by real-time PCR. Results are expressed as means ± SEM ($n = 3$). Different letters above each column indicate significant difference ($p < 0.05$).
were not mediated by the conventional PKA-CREB signaling pathway, although our data showed that curcumin suppressed the 8-br-cAMP-stimulated PKA activity in Leydig cells. As shown in Fig. 3A and B, curcumin had no effect on the phosphorylation of CREB under both the basal and 8-br-cAMP-stimulated conditions. However,
real-time PCR results showed that curcumin potently blocked the 8-br-cAMP-induced Nr5a1 and Fos mRNA expression in MA-10 cells (Fig. 3C), which suggested the involvement of Nr5a1 and Fos in curcumin-regulated StAR and Cyp11A1 expression. Although the activity of CREB is known to be regulated by PKA-mediated phosphorylation at the Ser 133 residue, this phosphorylation site was also reported to be modulated by many other kinases, such as the extracellular signal-regulated kinase, p38 MAP, and MAPKAP kinase-2 [29, 30]. This may explain the reason why curcumin suppressed PKA activity in MA-10 cells without decreasing the phosphorylation of CREB.

To further evaluate the dependency of StAR and Cyp11a1 in curcumin-suppressed Leydig cell steroidogenesis, we repeated the 8-br-cAMP and curcumin experiments in MA-10 cells supplied with 22R-OHC or pregnenolone, which can increase steroidogenesis bypass of StAR or Cyp11a1 respectively. As shown in Fig. 4, supplement of 22R-OHC alone did not completely prevented curcumin-suppressed progesterone production in MA-10 cells, while adding of pregnenolone fully reversed curcumin-inhibited steroidogenesis in MA-10 cells. These results indicated that the down-regulated StAR and CYP11A1 may both contribute to curcumin-suppressed steroidogenesis in Leydig cells.

In conclusion, our study have clearly confirmed the inhibitory effects of curcumin on Leydig cell steroidogenesis. And importantly, our data demonstrated that curcumin may suppress the cAMP-stimulated steroidogenesis in Leydig cell by inhibiting StAR and CYP11A1 expression independently of the conventional PKA-CREB signaling pathway. Although the detail of this signaling pathway still needs more experiments to clarify, our preliminary data suggest the possible involvement of Nr5a1 and Fos.

Acknowledgements

The authors acknowledge the funding support by Ministry of Science and Technology, Taiwan [NSC-102-2313-B-002-025]. We appreciate the expert contribution of Dr. Mu-En Wang for his critical reading of this manuscript.

Disclosure

None of the authors have any potential conflicts of interest in this research.

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