

## Curcumin inhibits luteal cell steroidogenesis by suppression of extracellular signal regulated kinase

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### ABSTRACT

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#### BACKGROUND

Curcumin inhibits steroidogenesis in luteal cell cultures by inhibiting progesterone secretion. The site of action of curcumin on steroidogenesis in luteal cell cultures is as yet unknown. The purpose of this study was to determine the influence of curcumin on phosphorylation of extracellular signal regulated kinase (ERK) in steroidogenesis of luteal cell cultures.

#### METHODS

An experimental study with control was conducted to investigate the site of action of curcumin by measurement of ERK phosphorylation in luteal cell cultures (LCC) after administration of luteinizing hormone (LH) and/or prostaglandin F<sub>2</sub> alpha (PGF<sub>2</sub>α). The subjects of this study were luteal cell cultures derived from the corpus luteum of Sprague Dawley rat with superovulation induced by pregnant mare serum gonadotropin. The luteal cell cultures were divided into 16 groups, both with and without the addition of forskolin and each group contained 4 replicates samples. Curcumin was administered immediately following LH and/or PGF<sub>2</sub>α stimulation with or without addition of forskolin. The cell culture was then incubated for 24 h. ERK phosphorylation was measured by immunohistochemistry. Data on ERK phosphorylation was analyzed using one-way Anova, followed by multiple comparison tests.

#### RESULTS

LH significantly increased ERK phosphorylation, whereas PGF<sub>2</sub>α significantly reduced ERK phosphorylation. Forskolin significantly increased ERK phosphorylation to a similar degree as LH. Curcumin inhibited ERK phosphorylation in both LH and forskolin-stimulated luteal cell cultures.

#### CONCLUSION

Curcumin inhibits ERK phosphorylation in luteal cell cultures by suppressing signal transduction upstream of ERK.

**Key words:** Curcumin, signal transduction, forskolin, luteal cells culture

## ***Kurkumin menghambat fosforilasi extracellular regulated kinase pada steroidogenesis kultur sel luteal***

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### **ABSTRAK**

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#### **LATAR BELAKANG**

Senyawa kurkumin dapat menghambat steroidogenesis kultur sel luteal dengan cara menghambat sekresi progesteron. Letak kerja kurkumin pada steroidogenesis kultur sel luteal belum diketahui. Tujuan penelitian ini adalah untuk menentukan pengaruh pemberian kurkumin terhadap sinyal fosforilasi extracellular regulated kinase (ERK) pada steroidogenesis kultur sel luteal

#### **METODE**

Sebuah penelitian eksperimental dengan kontrol digunakan untuk menilai efek kurkumin terhadap fosforilasi ERK setelah diberikan luteinizing hormone (LH) dan/atau prostaglandin F2 alpha (PGF2 $\alpha$ ) pada kultur sel luteal tikus. Subjek penelitian adalah kultur sel luteal yang diperoleh dari tikus Sprague Dawley yang diinduksi dengan pregnant mare serum gonadotropin (PMSG). Kultur sel luteal dibagi menjadi 16 kelompok dengan atau tanpa diberi forskolin, dan setiap kelompok terdiri atas 4 replikasi. Kurkumin diberikan segera setelah distimulasi LH dan atau PGF2 $\alpha$  dengan atau tanpa penambahan forskolin. Kemudian kultur sel luteal diinkubasi selama 24 jam. Fosforilasi ERK diukur dengan metode immunohistochemistry. Data fosforilasi ERK dianalisis menggunakan Anova satu arah dilanjutkan dengan uji perbandingan berganda.

#### **HASIL**

LH meningkatkan fosforilasi ERK secara bermakna, sedangkan PGF2 $\alpha$  mengurangi fosforilasi ERK secara bermakna. Forskolin meningkatkan fosforilasi ERK secara bermakna dan hampir sama dengan yang terstimulasi LH. Kurkumin menghambat fosforilasi ERK pada kultur sel luteal yang terstimulasi LH maupun forskolin.

#### **KESIMPULAN**

Kurkumin menghambat fosforilasi ERK pada kultur sel luteal dengan cara menekan sinyal transduksi di up stream ERK.

**Kata kunci:** Kurkumin, sinyal transduksi, forskolin, kultur sel luteal

## **INTRODUCTION**

Regulation of steroidogenesis in luteal cells has an exceedingly complex mechanism, and produces progesterone (P4) as a major component. The regulation of steroidogenesis in luteal cells involves several hormones, such as luteinizing hormone (LH) and prostaglandin F2 alpha (PGF2 $\alpha$ ). LH secretion continuously stimulates the corpus luteum to secrete progesterone,<sup>(1)</sup> by involving signal transduction through the activity of adenylate cyclase within the luteal cell membrane, the formation of cyclic

adenosine 3',5' monophosphate (cAMP) and the activation of protein kinase A (PKA) and steroidogenic enzymes, such as cytochrome P450 side chain cleavage (P450<sub>scc</sub>), and 3 $\beta$ -hydroxysteroid dehydrogenase (HSD).<sup>(2)</sup> The role of PGF2 $\alpha$  as an antigonadotropic is among other things to interfere with the binding of LH to its receptor and decreasing the number of LH receptors, inhibiting the activity of adenylate cyclase and the accumulation of cAMP, reducing the transcription of cytochrome P450<sub>scc</sub> and 3 $\beta$ -HSD, reducing the secretion of progesterone via the protein kinase C (PKC)

pathway,<sup>(3)</sup> and inhibiting the phosphorylation of extracellular signal regulated kinase mitogen activated protein kinase (ERK MAP-Kinase). PKA is involved in the transcriptional regulation of the steroidogenic acute regulatory (StAR) gene and the expression of cytochrome P-450 side chain cleavage (P-450 scc) by increasing the phosphorylation of cAMP response element binding protein (CREB) and extracellular signal regulated kinase (ERK).<sup>(4)</sup> StAR plays a role in cholesterol transport from the cytosol into the mitochondrial outer membrane, while cytochrome P-450sec within the mitochondria converts the cholesterol into pregnenolone. Pregnenolone is then converted into progesterone by 3 $\beta$ -HSD in the smooth endoplasmic reticulum.<sup>(1)</sup>

The compound curcumin (1,7 bis (4-hydroxy-3-methoxyphenyl)-1-6-heptadine-3-5-dion) has been isolated from the extract of the curcuma rhizome and synthesized in the laboratory, and its analogs have been constructed by modification of its terminal aromatic methylenated residues.<sup>(5)</sup> One of the curcumin analogs that has been patented as an anti-inflammatory drug and introduced as National Molecule (*Molekul Nasional*, MOLNAS) is pentagamavunon-0 (PGV-0).

Curcuma extract can cause anovulation and reduce testicular weight, testosterone concentration, and spermatogenesis. In vitro studies on the reproductive system have reported an effect of curcumin on luteal cell cultures following stimulation by human chorionic gonadotropin (hCG).<sup>(6)</sup> The role of curcumin and PGV-0 in the production of progesterone with or without the administration of theophylline, a phosphodiesterase inhibitor, has also been reported. Curcumin has also been shown to be capable of inhibiting progesterone production in cultured granulosa cells.<sup>(7)</sup>

On the other hand, curcumin is known to inhibit COX-2, an enzyme that plays a role in the synthesis of PGF2 $\alpha$  from arachidonic acid. The results of abovementioned studies indicate that even without PGF2 $\alpha$ , curcumin inhibits the

production of progesterone. Thus curcumin inhibits signal transduction in luteal cell steroidogenesis, but its site of action is still not known. The objective of this study was to evaluate the effect of curcumin on steroidogenesis in luteal cells by measurement of ERK phosphorylation.

## METHODS

### Design of the study

An experimental study was conducted from September 2007 to April 2008, to investigate the effect of curcumin on ERK phosphorylation in luteal cell cultures (LCC) after administration of LH and/or PGF2 $\alpha$ . The study involved the following 16 groups, both with and without the addition of forskolin: 1. LCC + methanol 1 % (as control), 2. LCC + curcumin ; 3. LCC + LH ; 4. LCC + PGF2 $\alpha$  ; 5. LCC + LH + PGF2 $\alpha$ ; 6. LCC + LH + curcumin; 7. LCC + PGF2 $\alpha$  + curcumin; 8. LCC + LH + PGF2 $\alpha$  + curcumin. Groups 9-16 are identical to groups 1-8 except for the addition of forskolin. Each group contained 4 replicates samples.

### Materials

The subjects of this study were luteal cells derived from the 4-day old corpus luteum of a premature female Sprague Dawley rat (*Rattus norvegicus L*) with superovulation induced by pregnant mare serum gonadotropin/PMSG (Gestyl, Organon). The luteal cells were mechanically and enzymatically separated from the corpus luteum. Reagents used for the luteal cell cultures were minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin (PenStrep), gentamycin, fungizone, phosphate buffered saline (PBS), collagenase, trypan blue (Gibco, BRL, Life Technologies, Rockville, MD). Reagents for assessment of ERK phosphorylation were rabbit phosphorylated ERK1/ERK2 pAb (Promega, UK), streptavidine-biotin, decaminobenzidine tetrahydrochloride (DAB), theophylline and

forskolin (both from Sigma Aldrich Inc, St. Louis, MO). Chemicals required for treatment and tests were synthesized curcumin and pentagamavunon-0 (Lab. Molnas, Fakultas Farmasi UGM), LH, PGF2 $\alpha$ , and forskolin (Sigma Aldrich Inc, St. Louis, MO).

### Preparation of MEM culture medium

MEM powder from the sachets was dissolved in an Erlenmeyer flask containing one liter of sterile twice-distilled water using a magnetic stirrer. To the MEM solution was added 2.2 gram sodium bicarbonate, imparting a red color to the solution. The pH of the medium was then measured by means of a electric pH meter. The pH was adjusted to 7.4 by the addition of 0.1 M sodium hydroxide or 0.1 M hydrochloric acid as required. The MEM solution was filtered using a peristaltic pump in a sterile room. From the Erlenmeyer flask the medium was passed into the pump through a plastic pipe, at the end of which a sterile 0.22  $\mu$ m Millipore filter was attached. The filtered solution was collected in a sterile medium flask and stored in the refrigerator at 2-8 $^{\circ}$ C. To prepare a dispersion medium, to the MEM solution was added Pen-Strep 5%, Fungizone 1% and collagenase 1mg/mL. For a washing medium, to the MEM solution was added serum 10%, while for a growth medium to the MEM solution was added PenStrep 5%, Fungizone 0.7% and FBS 10%.

### Preparation of LCC

The corpus luteum was surgically removed from the ovary in a cool steril laminar-airflow room. After dressing, the corpus luteum was enzymatically dispersed in a medium containing collagenase/dispase 1 mg/ml and 5% Penstrep. The preparation was subsequently incubated in CO $_2$  5% at 37 $^{\circ}$ C for 30 minutes, then dispersed mechanically by vortexing and shaking for 3 minutes. This procedure of incubation and subsequent dispersion was repeated three times. The cell suspension was then filtered through

sterile gauze, and cleansed in washing MEM containing fetal bovine serum [FBS] 10% (Gibco), then it was centrifuged for 10 minutes 4 $^{\circ}$ C in a Sorvall super T21 centrifuge at a speed of 720 g (2.000 rpm). The washing was repeated three times. At the third washing, the supernatant was discarded and growth MEM was added, containing FBS 10%, penstrep 5%, gentamycin 5% and fungizone 0.7%.

Before seeding, viable cells were counted in a hemocytometer after the addition of Trypan blue. The suspension was diluted with growth medium to obtain a luteal cell concentration of 10 $^5$ -10 $^6$  cells/ml. Luteal cells were then seeded in 24-well culture plates at a volume of 0.5 ml per well or on 96-well culture plates at a volume of 0.1 mL per well, and incubated in CO $_2$  5% at 37 $^{\circ}$ C for 72 hours. The culture medium was renewed once in 24 hours and checked by observation of the cell cultures under an inverted microscope. Viable cells adhered to the bottom of the wells, while dead cells floated in the medium and were removed on renewal of medium. The cells were then treated with a single dose of curcumin at a dosage of 100  $\mu$ M/L in accordance with the study design. Before treatment the cells in one of the sample wells were also counted.

### Determination of ERK phosphorylation

After treatment, coverslips were placed in the wells and the luteal cell cultures incubated for 24 hours. The luteal cells adhering to the coverslips were immunohistochemically stained in the Pathology Laboratory of Dr. Sardjito Central Hospital, Yogyakarta. The procedure used to measure ERK phosphorylation by immunohistochemistry (IHC) in this study was a modification of the method of Cicero & Herrup,<sup>(8)</sup> as follows: The treated cell culture on the coverslip was incubated in 5% CO $_2$  at 37 $^{\circ}$ C for 24 hours, after which the coverslip was placed on a poly-l-lysine slide, and fixed in acetone or methanol for 10 minutes at -20 $^{\circ}$ C. The slide was then washed three times in PBS,

each time for 5 minutes, and H<sub>2</sub>O<sub>2</sub> 0.3 % was added dropwise for 20 minutes. Normal mouse serum (1:50) was also added dropwise for 15 minutes. The liquid covering the slide was discarded but the slide was not rinsed. This was followed by the addition of the primary rabbit anti-phosphorylated ERK antibody ERK1/ERK2 pAb, at a dilution of 1:200 for 60 minutes. The slide was washed three times in PBS, each washing lasting 5 minutes, then it was incubated with biotinylated secondary antibody for 5-10 minutes, and again washed three times 5 minutes in PBS. This was followed by incubation with streptavidin-peroxidase for 5-10 minutes, washing in PBS 3 x 5 minutes, incubating with the chromogen deaminobenzidine tetrahydrochloride for 5-10 minutes, at a chromogen-substrate ratio of 1:20, and finally washing in distilled water. The preparation was then immersed in hematoxylin for 3-5 minutes as counterstain, rinsed in distilled water, dehydrated with ethanol 95%, and immersed in xylene for 10 minutes. To the preparation was added mounting medium (Canada balsam), then it was covered with a coverslip. ERK phosphorylation was observed by light microscopy. Phosphorylation-positive cells had a dark brown color, whereas phosphorylation-negative cells were violet. A total of 200 luteal cells were counted and the results were expressed in percentages.

### Data analysis

The percentages of ERK phosphorylation were analyzed by one-way Anova, followed by Least Significant Difference multiple comparison tests.

### Ethical clearance

This study on rat luteal cell cultures obtained ethical clearance from the Commission for Research Ethics for the Medical and Health Sciences, Faculty of Medicine, Gadjah Mada University, Yogyakarta.

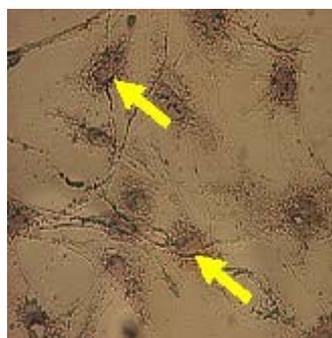
### RESULTS

ERK1/ERK2 phosphorylation in luteal cell cultures was detected by immunohistochemistry (IHC). The calculation was based on the percentage of phosphorylation-positive cells obtained by IHC. The phosphorylation value considered positive was the ERK1/ERK2 detected by IHC analysis, ERK1/ERK2 cells having a brown-colored nucleus and cytoplasm after staining with DAB chromogen (Figure 1).

The results of calculation of ERK1/ERK2 phosphorylation (percentage of phosphorylation-positive cells) in all groups after administration of curcumin are presented in Table 1. There was a significant differences between treatment groups.



Control (solvent)



Solvent + curcumin

Figure 1. IHC ERK phosphorylation results in control and treatment groups. Magnification 400x brown color (arrows) in nucleus and cytoplasm indicates positive ERK phosphorylation

Table 1. ERK phosphorylation in each group after curcumin administration with and without forskolin

| Treatment group                                 | ERK % (Mean ± SD) |
|---|-------------------|
| Solvent (control) without forskolin             | 37.25 ± 0.50      |
| Solvent + curcumin without forskolin            | 32.00 ± 0.82      |
| LH without forskolin                            | 41.25 ± 0.50      |
| LH + curcumin without forskolin                 | 32.50 ± 0.50      |
| PGF2 $\alpha$ without forskolin                 | 34.50 ± 0.58      |
| PGF2 $\alpha$ + curcumin without forskolin      | 30.00 ± 0.82      |
| LH + PGF2 $\alpha$ without forskolin            | 40.75 ± 0.50      |
| LH + PGF2 $\alpha$ + curcumin without forskolin | 31.75 ± 0.50      |
| Solvent (control) with forskolin                | 40.75 ± 0.50      |
| Solvent + curcumin with forskolin               | 36.75 ± 0.50      |
| LH with forskolin                               | 42.50 ± 0.58      |
| LH + curcumin with forskolin                    | 34.75 ± 0.82      |
| PGF2 $\alpha$ with forskolin                    | 36.00 ± 0.82      |
| PGF2 $\alpha$ + curcumin with forskolin         | 32.25 ± 0.50      |
| LH + PGF2 $\alpha$ with forskolin               | 42.00 ± 0.82      |
| LH + PGF2 $\alpha$ + curcumin with forskolin    | 32.75 ± 0.50      |
| One way Anova                                   | p < 0.05          |

Values are mean  $\pm$  standard deviation

ERK phosphorylation after curcumin administration in the solvent LCC group with or without forskolin and in the LCC groups stimulated by LH, PGF2 $\alpha$  and LH + PGF2 $\alpha$  showed significantly lower values ( $p < 0.05$ ) compared with the groups without curcumin. Administration of forskolin to the solvent LCC group significantly increased ERK1/ERK2 phosphorylation ( $p < 0.05$ ) compared with the solvent-only group.

## DISCUSSION

LH stimulation increased ERK phosphorylation, similar to previous studies.<sup>(9)</sup> In previous studies forskolin was used for detecting ERK phosphorylation, because forskolin is capable of increasing ERK phosphorylation by increasing adenylate cyclase activity.<sup>(10,11)</sup> ERK1/ERK2 phosphorylation in the groups receiving forskolin was almost equal that in the groups with LH stimulation only. Luteinizing hormone can activate ERK directly from G. protein-coupled receptor (GPCR).<sup>(9)</sup> The increase in ERK1/ERK2 phosphorylation by LH may also be the result of increased cAMP concentration through PKA activation. Both LH

and cAMP are capable of activating ERK phosphorylation. Forskolin increases ERK phosphorylation in the same manner as LH, because both substances increase adenylate cyclase activity.<sup>(12)</sup> The administration of PGF2 $\alpha$  is antigonadotropic, as it inhibits ERK phosphorylation, which is consistent with the opinion of previous investigators. PGF2 $\alpha$  in luteal cells can influence ERK phosphorylation through the Ca<sup>2+</sup>/calmodulin pathway by inducing the expression of the Nur 77 gene.<sup>(13)</sup>

Administration of curcumin causes a significantly lower ERK phosphorylation in comparison with the solvent group. Curcumin shows antigonadotropic activity by inhibiting the increase in ERK phosphorylation by LH alone or by LH+PGF2 $\alpha$ . Inhibition of ERK phosphorylation by PGF2 $\alpha$  was stronger after addition of curcumin. Curcumin also causes a significant inhibition of ERK phosphorylation after the addition of forskolin. This indicates that curcumin has antigonadotropic activity against the increase in ERK phosphorylation by forskolin. The increase in ERK phosphorylation by forskolin is almost equal to that by LH.<sup>(12)</sup> Inhibition of ERK phosphorylation by curcumin is the result of

inhibition of cAMP accumulation, because ERK is situated downstream to cAMP/PKA. PKA phosphorylation can activate ERK and the transcription factor CREB.<sup>(14)</sup> There is a possibility that curcumin also inhibits ERK phosphorylation via signal transduction of the GPCR complex. Previous studies reported that P4 concentration in LH-stimulated LCC was slightly higher than P4 concentration due to forskolin.<sup>(15)</sup> This indicates that apart from signal transduction through cAMP/PKA, curcumin also uses the direct signal transduction pathway from GPCR.

Another possibility is that curcumin interferes with  $G\alpha_i$ , one of the  $G\alpha$  members from the GPCR complex that activates signal transduction directly to ERK. There are four  $G\alpha$  members, namely  $G\alpha_{q/11}$  which activates signal transduction from GPCR to PLC/PKC;  $G\alpha_s$ , which activates signal transduction via cAMP/PKA;  $G\alpha_i$  which activates signal transduction directly from GPCR to ERK, and  $G\alpha_{12/13}$ .<sup>(16,17)</sup> ERK phosphorylation can be activated from the GPCR complex via cooperation between  $G\alpha_q$  and  $G\alpha_i$ . Whether curcumin can inhibit the cooperation of both  $G\alpha$  members is unclear. The obtained results indicate that curcumin inhibits ERK phosphorylation both in the control group and the groups stimulated by LH with or without PGF<sub>2</sub>α.

Curcumin exhibits an antigonadotropic effect on steroidogenesis in LCC, especially on ERK phosphorylation. Based on its activity as revealed in this study, curcumin may be developed into an antifertility agent with anti-implantation action by inhibiting P4 production through inhibition of signal transduction in steroidogenesis of luteal cells. Apart from its anti-inflammatory activity, curcumin may be developed as an antiovarulatory agent. Previous studies have demonstrated that curcumin inhibits P4 and E2 production in granulosa cell cultures by stimulating FSH.<sup>(7)</sup>

It is hoped that this investigation may become a valuable contribution to the

community and the pharmaceutical industry as a reference on the use of curcumin as an alternative component for developing medications for fertility control, such as contraceptives. One limitation of the present study is that it did not study the factors playing a role on signal transduction upstream to ERK, such as adenylate cyclase activity, expression of PKA and expression of transcription factors. Further studies are necessary to study the effect of curcumin on these parameters.

## CONCLUSION

Curcumin inhibits signal transduction for steroidogenesis in luteal cells by acting on a site upstream to ERK.

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