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*P*450scc REGULATION IN PIG GRANULOSA CELLS: INVESTIGATION INTO THE MECHANISM OF INDUCTION

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Summary—*P*450scc catalyses the first and rate-limiting reaction in steroidogenesis and is hormonally regulated. By Northern analysis, using a bovine cDNA probe, we have studied the regulation of *P*450scc mRNA in pig granulosa cells cultivated *in vitro*. Using transcription and translation inhibitors, we show that the gonadotropin-induced accumulation of *P*450scc mRNA mainly results from increased transcription, and that this stimulation, at least in part, is protein synthesis-dependent. Although transcriptional regulation of *P*450scc gene expression is found in other steroidogenic cells, cycloheximide-sensitivity of this regulation is not widespread. Pig granulosa cells thus would constitute a useful model to study this mechanism of regulation.

INTRODUCTION

In pig granulosa cells, as in other steroidogenic tissues, the first step of steroid synthesis, the cholesterol side chain cleavage, is rate-limiting and hormonally regulated. Recent results demonstrate that, in pig granulosa cells, gonadotropins and agonists of the cAMP pathway increase the mRNA level of this side chain cleavage enzyme, *P*450scc [1, 2]. The question then arises as to whether the gonadotropin-induced accumulation of *P*450scc mRNA results from an increased transcription, and as to what may be the underlying mechanisms.

Using inhibitors of transcription and translation, we show that (i) the gonadotropin-induced accumulation of *P*450scc mRNA results from increased transcription, and (ii) that this transcription is protein synthesis-dependent.

Taken together with results already published our data suggest that in cycloheximide-sensitive models, *P*450scc gene transcription could be dependent on *cis*-acting sequence(s) regulated by labile protein(s) or protein complex.

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EXPERIMENTAL

Cell cultures

Ovaries were collected at a local slaughterhouse, and granulosa cells were scraped out from 3 mm diameter healthy follicles, after opening the follicles in the culture medium. Cells were plated at 6 follicle-equivalents per flask in a serum-containing medium in 75 cm^2 Corning culture flasks and grown to confluency (5 days after plating, about 6×10^6 cells). Culture medium was a 1:1 mixture of GC3 [3] and Eagle's minimum essential medium, supplemented with 6% foetal calf serum (Gibco BRL, Cergy Pontoise, France) and 1% serum substitute Ultroser SF (Sepracor IBF, Villeneuve la Garenne, France). At the end of the growing period, medium was replaced by serum-free GC3 medium containing the different agents and incubated as indicated in the figures. Each treatment was repeated at least twice in independent experiments.

Northern analysis

Total RNA was extracted by a LiCl/urea method [4] and 5 μg were size-fractionated through methyl mercury hydroxyde denaturing 1.2% agarose gels [5], blotted by capillary transfer with 0.2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) onto a nylon membrane (Hybond N, Amersham France, Les Ulis) and fixed by u.v. irradiation (0.140 J/cm^2).

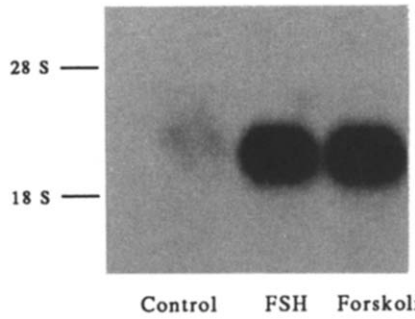


Fig. 1. *P*450scc mRNA induction in pig granulosa cells. Cells were treated for 48 h with 300 ng/ml FSH or 3×10^{-5} M forskolin. Positions of ribosomal RNAs are indicated.

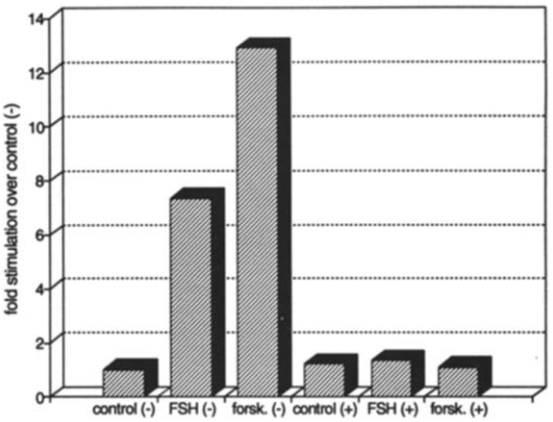


Fig. 3. Effect of actinomycin D treatment on *P*450scc transcription. Cells were treated as in Fig. 2. (actinomycin D: 1 μg/ml, FSH: 100 ng/ml, forskolin: 3×10^{-5} M). Mean values of 3 independent experiments. (−): without, (+): with actinomycin.

The probe was a bovine cDNA [6] ^{32}P-labelled using random hexanucleotide primers and the Klenow fragment of *E. coli* DNA polymerase [7] (Random Primed DNA Labeling Kit, Boehringer Mannheim, France).

Following an overnight prehybridization step at 42°C, the membranes were hybridized with probe (2–4 × 10^6 cpm/ml) for 24–36 h. Hybridization medium was 50% formamide, 5 × SSPE (1 × SSPE = 0.18 M NaCl, 0.01 M sodium phosphate, 0.001 M EDTA pH 7.4), 5 × Denhardt's solution, 1% SDS and 250 μg/ml sonicated denatured salmon sperm DNA. After hybridization, membranes were rinsed at room temperature in 0.1 × SSC, 0.1% SDS, then at 45°C for 10 min in the same solution. Hybridization signals were detected by exposure to Kodak X-OMAT S films at −70°C with intensifying screens for 3 to 7 days. Intensities of autoradiographic bands were measured by densitometric scanning. As a control, membranes were methylene blue stained. The autoradiograms presented are representative of at least two independent experiments, and the size of the *P*450scc transcript is the same in all analysis.

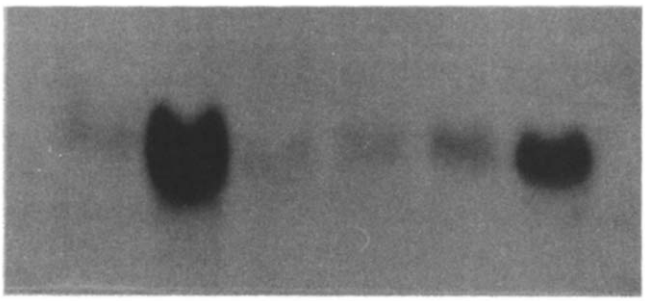
Chemicals

Purified ovine FSH (sp. act. 20–30 × NIH-FSH S1, <3% LH) was given by Dr Y. Combarnous; α-amanitin was from Boehringer Mannheim; [α^{32}P]dCTP was from Du Pont de Nemours NEN (Les Ulis, France) and other biochemicals were from Sigma (L'Isle d'Abeau Chesnes, France).

RESULTS

Gene expression and regulation

Hybridization of total pig granulosa cell RNA with bovine *P*450scc probe reveals a transcript of 2 kb, and treatment of the cells for 48 h with FSH or forskolin results in an increase in



FSH	−	+	−	+	−	+
Actinomycin	−	−	+	+	−	−
Cycloheximide	−	−	−	−	+	+

Fig. 2. Effect of actinomycin D and cycloheximide on FSH-induced accumulation of *P*450scc mRNA. Cells were cultured during 8 h with treatments as indicated (FSH: 100 ng/ml, actinomycin D: 1 μg/ml, cycloheximide: 0.15 μg/ml).

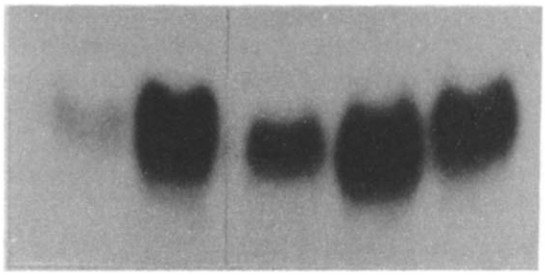


Fig. 4. Effects of actinomycin D and cycloheximide on the stability of induced P450scc mRNA. Pig granulosa cells were incubated for 18 h with or without forskolin (3×10^{-5} M). Forskolin was then withdrawn and cells either harvested or further treated with either actinomycin D (1 μg/ml) or cycloheximide (0.15 μg/ml) for 8 h.

P450scc mRNA level (Fig. 1). As already described [2], this induction is time- and dose-dependent: after 3 h, P450scc mRNA level is slightly increased, and goes up further until 48 h. Maximum response for FSH is obtained with 100 to 300 ng/ml.

Transcription inhibition

Transcription inhibition by actinomycin D, at 1 μg/ml for 8 h, nearly abolishes FSH-induced mRNA levels with a slight effect, if any, on basal level (Fig. 2). Densitometric analysis indicates that actinomycin treatment reduces the stimulation from 7.3-fold over control to 1.3, without effect on control values (Fig. 3).

The same inhibition of P450scc gene expression by actinomycin is observed when cells are stimulated by forskolin, the reduction being from 12.9- to 1-fold over control (Fig. 3).

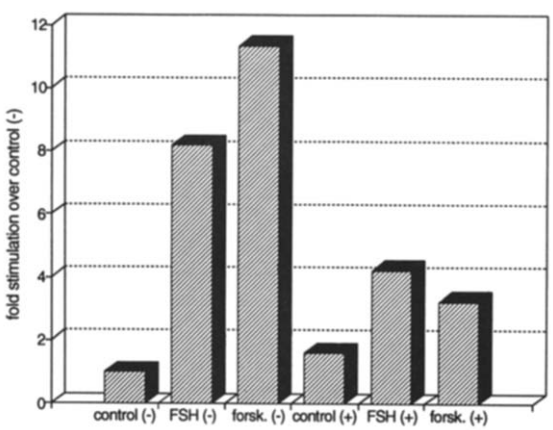


Fig. 5. Effect of cycloheximide treatment on P450scc transcription. Cells were treated as in Fig. 2. (cycloheximide: 0.15 μg/ml, FSH: 100 ng/ml, forskolin: 3×10^{-5} M). Mean values of 3 independent experiments. (–): without, (+): with cycloheximide.

The use of another transcription inhibitor, α-amanitin, also results in an inhibition of the FSH-induced accumulation of P450scc mRNA (not shown).

In order to check that actinomycin D does not reduce P450scc mRNA stability, cells were induced with forskolin for 18 h, then forskolin was withdrawn, and cells treated or not with actinomycin D for 8 h. During this 8 h period, in the absence of treatment, there is a decrease in mRNA level. During this period, actinomycin does not contribute to mRNA degradation, but rather stabilizes P450scc mRNA (Fig. 4). The same effect is observed after FSH induction (not shown).

Protein synthesis inhibition

Protein synthesis inhibition by cycloheximide at 0.15 μg/ml for 8 h clearly results in an important reduction of FSH-induced mRNA level, with a slight stimulatory effect on basal level (Fig. 2). In the presence of cycloheximide, the stimulating effect of FSH is reduced to 2.6 as compared to 8.2 in the absence of inhibitors, and the stimulating effect of forskolin is reduced from 11.3 to 1.6 (Fig. 5).

If the same cells are analysed for the expression of another inducible gene, insulin growth factor I (IGFI), there is only a slight effect of cycloheximide on both basal and FSH- or forskolin-induced IGFI mRNA (not shown), indicating that the effect of cycloheximide of P450scc mRNA level does not result from a general interference of cycloheximide with transcription.

In order to detect a negative effect of cycloheximide on P450scc mRNA stability, we used

the same experimental design as for actinomycin D, that is stimulation of mRNA accumulation by forskolin, then treatment by the inhibitor. In these conditions, the presence of cycloheximide does not result in a reduction, but rather in a stabilization of *P*450scc mRNA (Fig. 4). The same effect is observed after FSH induction (not shown).

DISCUSSION

Our data suggest that the gonadotropin induction of *P*450scc mRNA is of transcriptional nature, and that this regulation is partially protein synthesis-dependent. They also confirm the results of Mulheron *et al.* [1] and Urban *et al.* [2] who have shown that gonadotropins and cAMP agonists stimulate *P*450scc mRNA accumulation in pig granulosa cells.

Transcriptional regulation

mRNA accumulation could result from an increased transcription, an increased stability, or both.

We used actinomycin D to detect an effect of FSH or forskolin on transcription. To avoid as much as possible toxic or side effects associated with actinomycin, we used it in short term experiments (8 h). Control experiments indicated that, in pig granulosa cells, more than 90% inhibition of uridine incorporation is obtained with 1 μg/ml for 8 h.

In these conditions, we show that the gonadotropins-induced accumulation of *P*450scc mRNA results from increased transcription. Moreover, the experiment of Fig. 4 shows that, after the 8 h period, *P*450scc mRNA levels are higher in actinomycin-treated cells than in untreated cells indicating that not only does actinomycin not reduce *P*450scc mRNA stability but rather it slows down *P*450scc mRNA decay. However, a stabilization of the mRNA by gonadotropins should be directly appreciated by measuring *P*450scc mRNA half-life after labelling of the cells.

Using actinomycin D, a transcriptional stimulation has been reported for aromatase mRNA in the same pig granulosa cell model [8], and for *P*450scc mRNA in forskolin-stimulated rat granulosa cells [9]. An increased transcription of the *P*450scc gene in response to cAMP has also been demonstrated in other models, either by nuclear run-on transcription as in the murine Leydig cell line MA-10 [10] or again by using actinomycin D as in the human choriocarcinoma cell line JEG-3 [11].

In bovine adrenocortical cells, transcriptional stimulation is likewise involved in the regulation of the expression of *P*450scc [12] and other genes of the steroidogenic pathway, *P*450c17, *P*450c11, *P*450c21 and adrenodoxin [13], but, in addition, there is an increase in *P*450scc mRNA stability [14].

Thus, even if not exclusive, increased transcription seems to be a general response to hormonal stimulation in steroidogenic tissues.

Protein synthesis inhibition

Our data demonstrate that, in pig granulosa cells, the inhibition of protein synthesis reduces the hormonally-induced accumulation of *P*450scc mRNA. This effect does not seem to result from mRNA half-life reduction or from a side effect of cycloheximide, as this inhibitor seems to stabilize preinduced *P*450scc mRNA (Fig. 4). An analogous situation is found in bovine adrenocortical cells, where cycloheximide inhibits the ACTH-induced *P*450scc mRNA synthesis [12, 13], and is interpreted by the intervention of short-lived, cAMP-induced proteins which activate the transcription of the *P*450scc gene. These hypothetical proteins, termed SHIP (steroid hydroxylase inducing proteins) [13], would confer cycloheximide sensitivity to *P*450scc mRNA induction. However, sequences conferring cAMP responsiveness have since been found in the 5′ region of the bovine *P*450scc gene [15] indicating that cAMP can directly activate transcription without the intervention of an inducible protein.

If the SHIP hypothesis can be ruled out, the cycloheximide inhibitory effect on *P*450scc mRNA accumulation remains to be explained: it is also found in a human cytotrophoblastic cell line JEG-3 [11] and in a mouse tumor Leydig line MA-10 [16]. By contrast, *P*450scc gene expression is not inhibited by cycloheximide in human granulosa cells [17], rat follicles *in vitro* [18], and also, according to Mellon and Vaisse [10], the same MA-10 cells.

Interestingly, in JEG-3 cells, cycloheximide inhibits the cAMP-induced accumulation of *P*450scc mRNA, but stimulates adrenodoxin mRNA accumulation. All these data indicate that the mechanisms of regulation of the endogenous expression of *P*450scc vary according to the cell model under study.

To identify the 5′ region required for cAMP induction, transient expression of chimeric

plasmids containing the promoter/regulatory region of P450scc gene in various cell lines has been analysed: the human gene expressed in the transformed murine adrenal cell line Y1 was found insensitive to cycloheximide [19], as well as the bovine gene expressed in either Y1 or bovine adrenocortical cells [15]. This last result is surprising as, in this model, endogenous stimulation of P450scc gene expression is cycloheximide-sensitive [12, 13]., Similarly, in the same cells, P450c17 constructs are cycloheximide-insensitive, although the endogenous gene is inhibited by cycloheximide [20].

So, cycloheximide results are rather controversial, and the link between cycloheximide and cAMP stimulation of P450scc gene expression remains to be determined. Multiple cAMP responsive regions or sequences are present in the 5′ region of the human P450scc gene [19, 21] and possibly in the bovine P450scc gene [15]. If such multiple cAMP responsive regions are found in the 5′ region of the pig P450scc gene, some (but not all) proteic *trans*-acting factors which interact with these regions could be short-lived and thus confer a partial cycloheximide-sensitivity to the model.

Thus, as far as P450scc is concerned, pig granulosa cells provide a useful model to study this particular mechanism of regulation. Nuclear run-on should be used to confirm the transcriptional regulation and the protein-synthesis dependence of the cAMP regulation, and when P450scc genomic clones will be available, transient expression of chimeric constructs will be used to analyse this mechanism.

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REFERENCES

1. Mulheron G. W., Wise T. H., Stone R. T. and Ford J. J.: Follicle-stimulating hormone regulation of cytochrome P-450 side-chain cleavage messenger ribonucleic acid accumulation by porcine granulosa cells isolated from small and medium follicles. *Biol. Reprod.* **43** (1990) 828–834.
2. Urban R. J., Garmey J. C., Shupnik M. A. and Veldhuis J. D.: Follicle-stimulating hormone increases concentrations of messenger ribonucleic acid encoding cytochrome P450 cholesterol side-chain cleavage enzyme in primary cultures of porcine granulosa cells. *Endocrinology* **128** (1991) 2000–2007.
3. Gasser F., Mulsant P. and Gillois M.: Long term multiplication of the chinese hamster ovary (CHO) cell line in a serum-free medium. *In Vitro Cell. Dev. Biol.* **21** (1985) 588–592.
4. LeMeur M., Glanville N., Mandel J. L., Gerlinger P., Palmiter R. and Chambon P.: The ovalbumin gene family: Hormonal control of X and Y gene transcription and mRNA accumulation. *Cell* **23** (1981) 561–571.
5. Bailey J. M. and Davidson N.: Methylmercury as a reversible denaturing agent for agarose gel electrophoresis. *Analyt. Biochem.* **70** (1976) 75–85.
6. John M. E., John M. C., Ashley P., MacDonald R. J., Simpson E. R. and Waterman M. R.: Identification and characterization of cDNA clones specific for cholesterol side-chain cleavage cytochrome P-450. *Proc. Natn. Acad. Sci. U.S.A.* **81** (1984) 5628–5632.
7. Feinberg A. P. and Vogelstein B.: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analyt. Biochem.* **132** (1983) 6–13.
8. Chan W. K. and Tan C. H.: Induction of aromatase activity in porcine granulosa cells by FSH and cyclic AMP. *Endocrine Res.* **13** (1987) 285–299.
9. Kurten R. C. and Richards J. S.: An adenosine 3,5′-monophosphate-responsive deoxyribonucleic acid element confers forskolin sensitivity on gene expression by primary rat granulosa cells. *Endocrinology* **125** (1989) 1345–1357.
10. Mellon S. H. and Vaisse C.: cAMP regulates P450scc gene expression by a cycloheximide-insensitive mechanism in cultured mouse Leydig MA-10 cells. *Proc. Natn. Acad. Sci. U.S.A.* **86** (1989) 7775–7779.
11. Picado-Leonard J., Voutilainen R., Kao L. C., Chung B. C., Strauss J. F. and Miller W. L.: Human adrenodoxin: cloning of three cDNAs and cycloheximide enhancement in JEG-3 cells. *J. Biol. Chem.* **263** (1988) 3240–3244.
12. John M. E., Simpson E. R., Waterman M. R. and Mason J. I.: Regulation of cholesterol side-chain cleavage cytochrome P450 gene expression in adrenal cells in monolayer culture. *Molec. Cell. Endocr.* **45** (1986) 197–204.
13. John M. E., John M. C., Boggaram V., Simpson E. R. and Waterman M. R.: Transcriptional regulation of steroid hydroxylase genes by corticotropin. *Proc. Natn. Acad. Sci. U.S.A.* **83** (1986) 4715–4719.
14. Boggaram V., John M. E., Simpson E. R. and Waterman M. R.: Effect of ACTH on the stability of mRNAs encoding bovine adrenocortical P-450scc, P-45011β, P-45017α, P-450C21, and adrenodoxin. *Biochem. Biophys. Res. Commun.* **160** (1989) 1227–1232.
15. Ahlgren R., Simpson E. R., Waterman M. R. and Lund J.: Characterization of the promoter/regulatory region of the bovine CYP11A (P-450scc) gene. Basal and cAMP-dependent expression. *J. Biol. Chem.* **265** (1990) 3313–3319.
16. Hales D. B., Sha L. and Payne A. H.: Glucocorticoid and cyclic adenosine 3′5′-monophosphate-mediated induction of cholesterol side-chain cleavage cytochrome P450 (P450scc) in MA-10 tumor Leydig cells. Increases in mRNA are cycloheximide sensitive. *Endocrinology* **126** (1990) 2800–2808.
17. Golos T. G., Miller W. L. and Strauss J. F.: Human chorionic gonadotropin and 8-bromo cyclic adenosine monophosphate promote an acute increase in cytochrome P450scc and adrenodoxin messenger RNAs in cultured human granulosa cells by a cycloheximide-insensitive mechanism. *J. Clin. Invest.* **80** (1987) 896–899.
18. Oonk R. B., Krasnow J. S., Beattie W. G. and Richards J. S.: Cyclic AMP-dependent and -independent regulation of cholesterol side chain cleavage cytochrome P-450 (P-450scc) in rat ovarian granulosa cells and corpora lutea. cDNA and deduced amino

acid sequence of rat *P*-450scc. *J. Biol. Chem.* **264** (1989) 21934–21942.
19. Moore C. C. D., Brentano S. T. and Miller W. L.: Human *P*450scc gene transcription is induced by cyclic AMP and repressed by 12-*O*-tetradecanoylphorbol-13-acetate and A23187 through independent cis elements. *Molec. Cell. Biol.* **10** (1990) 6013–6023.
20. Lund J., Ahlgren R., Wu D. H., Kagimoto M., Simpson E. R. and Waterman M. R.: Transcriptional regulation of the bovine CYP17 (*P*-450 17α) gene. Identification of two cAMP regulatory regions lacking the consensus cAMP-responsive element (CRE). *J. Biol. Chem.* **265** (1990) 3304–3312.
21. Inoue H., Watanabe N., Higashi Y. and Fujii-kuriyama Y.: Structures of regulatory regions in the human cytochrome-*P*-450scc (desmolase) gene. *Eur. J. Biochem.* **195** (1991) 563–569.