A DNA topoisomerase I inhibitor blocks the differentiation of rat granulosa cells induced by follicle-stimulating hormone

J. Lino BARAÑAO,*† Miguel A. BLEY,* Facundo D. BATISTA† and Gerardo C. GLIKIN†

*Instituto de Biología y Medicina Experimental and †Instituto de Investigaciones en Ingeniería Genética y Biología Molecular (Consejo Nacional de Investigaciones Científicas y Técnicas) and Facultad de Ciencias Exactas y Naturales (Universidad de Buenos Aires), Vuelta de Obligado 2490, 1428 Buenos Aires, Argentina

Follicle-stimulating hormone (FSH), acting through a cyclic AMP-mediated mechanism, promotes differentiation of rat granulosa cells cultured in a defined medium. Camptothecin, a DNA topoisomerase I blocker, inhibited the increase in progesterone and oestradiol production stimulated by FSH. This effect was not due to non-specific inhibition of protein synthesis, as shown by measurement of [³⁵S]methionine incorporation. A transient increase in DNA topoisomerase I activity was observed after 24 h of culture in the presence of FSH or dibutyryl cyclic AMP. Our results are consistent with a key role for DNA topoisomerase I in the modulation of gene expression by FSH in rat granulosa cells.

INTRODUCTION

During the development of the ovarian follicle, the granulosa cell proliferates and undergoes cytodifferentiation into a mature, steroidogenically active cell that provides an adequate environment for oocyte maturation. Follicle-stimulating hormone (FSH), the main physiological stimulus for granulosa cell differentiation, has been shown to induce steroidogenic enzymes and increase the luteinizing hormone (LH) and prolactin receptor content in rat granulosa cells cultured in serum-free medium [1]. This process is mediated by cyclic AMP and seems to involve an increase in gene expression.

Cyclic AMP-responsive elements have been identified in a variety of genes [2]. These cis-regulatory elements in the promoter region interact with trans-acting factors that mediate the actions of cyclic AMP. We have recently shown that regulation of fibronectin gene expression by cyclic AMP in bovine granulosa cells is mediated by a mechanism that suppresses transcriptional activation of the cyclic AMP-responsive element [3]. However, the mechanisms by which the cyclic nucleotide modulates these factors and the relationship between its binding to the regulatory elements and the increased transcription are still poorly understood.

Recent evidence from several laboratories suggests an involvement of DNA topoisomerase I and II activities, the enzymes that modify the topological states of DNA [4], in the transcriptional control mechanisms of certain genes [5–10], replication [11,12] and chromatin assembly [13,14].

The aim of the present study was to determine the possible role of DNA topoisomerase I in FSH-induced differentiation of granulosa cells cultured in a defined medium.

EXPERIMENTAL

Granulosa cell preparation and culture

Ovaries were obtained from 24–25-day-old female Sprague–Dawley rats after diethylstilboestrol (DES) treatment (subcutaneous sytastic implants containing 5 mg of DES) for 3 days. Granulosa cells were prepared according to Campbell [15] with

some modifications. Briefly, the ovaries were punctured with a 30-G needle and incubated in Dulbecco's modified Eagle's medium (DMEM) (4.5 g of glucose/litre)/Ham's F12 (1:1, v/v)/EGTA (6 mM)/Hepes (10 mM) for 15 min at 37 °C. Ovaries were then washed twice and incubated in DMEM/F12 (1:1, v/v)/sucrose (0.5 M)/Hepes (10 mM) for 5 min at 37 °C. After the incubation, the medium was diluted with 2 vol. of DMEM/F12/Hepes (10 mM) and the ovaries were allowed to sediment. Granulosa cells were obtained by gently pressing the ovaries between two pieces of nylon mesh (Nytex 50), and were resuspended in DMEM/F12 (1:1, v/v)/Na₂CO₃ (2.2 g/l). Cells were seeded in plastic dishes (Falcon) at a density of 3 × 10⁵ cells/cm², in DMEM/Ham's F12 (1:1)/Na₂CO₃ (2.2 g/l) in the presence of different factors and were maintained in a humidified incubator (37 °C, 5% CO₂).

Preparation of whole-cell extracts

Cells (2 × 10⁶) were washed three times with buffer (10 mM-Tris/HCl, pH 7.5, 10 mM-NaCl and 1 mM-MgCl₂), harvested in the same buffer and centrifuged at 500 g for 10 min. The cellular pellet was resuspended in 0.5 ml of buffer D (20 mM-Hepes, pH 7.0, 1 mM-EDTA and 1 mM-dithiothreitol), kept at 0 °C for 15 min, homogenized (Pyrex all-glass homogenizer, no. 7727, 10 strokes), and KCl was added to 300 mM (final concentration). After 15 min, the homogenate was subjected to three cycles of freezing in liquid N₂/thawing and centrifuged at 15000 g for 10 min, then 2 vol. of saturated ammonium sulphate was slowly added to the supernatant, which was kept overnight at 4 °C. After centrifugation at 15000 g for 15 min, the salt-precipitated proteins were resuspended in 30 µl of buffer D. This extract was diluted to 6 µg of protein/µl with buffer D and stored at –70 °C. Protein concentrations were estimated by the method of Lowry *et al.* [16].

DNA topoisomerase I assay

The assay was carried out in a total volume of 10 µl. The standard reaction mixture containing 0.5 µl of supercoiled pUC12 DNA [17] was incubated with 1–8 µl of whole cell extract in the presence of 5 mM-EDTA in buffer D at 30 °C. Reactions were stopped by the addition of 2 µl of 80 mM-EDTA and 1 µl of

Abbreviations used: FSH, follicle-stimulating hormone; LH, luteinizing hormone; DES, diethylstilboestrol; DMEM, Dulbecco's modified Eagle's medium; Bt.cAMP, dibutyryl cyclic AMP.

† To whom correspondence should be addressed.

proteinase K (10 mg/ml). The mixture was then incubated at 37°C for 30 min and loaded on to 1% agarose gels for electrophoresis in Tris/borate buffer, followed by ethidium bromide staining and photography under u.v. light.

Radioimmunoassays

Progesterone and oestradiol in the culture media were determined by specific radioimmunoassays as described by Chiauzzi *et al.* [18].

Determination of DNA and protein synthesis

To monitor DNA synthesis, granulosa cells (1×10^5 in 0.1 ml) were preincubated for 24 h in the presence of factors as indicated below. [^3H]Thymidine (0.5 $\mu\text{Ci}/\text{well}$; 1.2 Ci/mmol) was added to the cultures and incubation was prolonged for 24 h. Cells were then harvested in hollow glass fibres with a multiwell cell harvester (Nunc). Radioactivity was measured in a scintillation counter.

To monitor protein synthesis, cell cultures were incubated with [^{35}S]methionine (10 $\mu\text{Ci}/\text{ml}$) for 4 h. At the end of the incubation, SDS was added to the cultures (2% final concentration) and the plates were incubated for a further 10 min. Total trichloroacetic acid-precipitable radioactive proteins were determined according to Vignon *et al.* [19].

Statistical analysis

Results are expressed as means $\pm$ S.E.M. of triplicate cultures. Statistical comparison of the results was made using a one-way ANOVA and Scheffe's tests for multiple comparisons [20]. Experiments were carried out at least three times with similar results.

RESULTS

To determine whether the DNA topoisomerase I activity was required for the FSH-induced differentiation of granulosa cells, we tested the effect of increasing doses of the specific DNA topoisomerase I inhibitor camptothecin (5 μM) [21] on basal and gonadotropin-stimulated steroid production. As can be seen in Fig. 1, production of both progesterone and oestradiol in FSH-treated cultures was markedly inhibited by camptothecin. It is noteworthy that different IC_{50} values (concns. causing 50% of maximum inhibition) were observed for the inhibition of the progesterone and oestradiol responses (320 and 60 nM respectively). The lowest dose tested (0.05 μM) produced a 50% decrease in FSH-stimulated oestradiol production, whereas the progesterone response was not altered.

Camptothecin, at high concentrations, has been shown to inhibit both transcription and replication [22]. Since stimulation of steroid production by FSH is known to require *de novo* protein synthesis [1], it was important to test whether the inhibition seen of cyclic-AMP-dependent steroid production was due to a complete shut-off of protein synthesis. Therefore the effects of camptothecin on protein synthesis were determined by measuring [^{35}S]methionine incorporation into cellular proteins (Fig. 2). Camptothecin did not significantly inhibit protein synthesis during the first 24 h, whereas a 60% decrease was observed after 48 h with the highest dose tested (5 μM). Even at this concentration, the general pattern of proteins synthesized by granulosa cells was not altered, as indicated by autoradiography after PAGE (results not shown). On the other hand, in agreement with previous reports [9,21], camptothecin treatment produced a marked inhibition of DNA synthesis, as measured by [^3H]thymidine incorporation (Table 1).

To test whether the FSH-induced differentiation of granulosa

Fig. 1. Effect of camptothecin on FSH-stimulated production of progesterone (a) and oestradiol (b).

Granulosa cells were cultured in a medium containing 0.3 μM -androstenedione in 24-well multiwells without (■) or with FSH (▨, 200 ng/ml) plus the indicated concentrations of camptothecin. Both control and camptothecin-treated cultures contained 0.1% dimethyl sulphoxide (final concentration). After 48 h, media were harvested for steroid determination by radioimmunoassay as described in the Experimental section. Results are means $\pm$ S.E.M. of triplicate cultures. Data bars not having common letters are significantly different ($P < 0.05$, by analysis of variance and Scheffe's multiple range test).

Fig. 2. Effect of camptothecin on protein synthesis

[^{35}S]Methionine incorporation (10 $\mu\text{Ci}/\text{ml}$) was measured during 4 h, starting 24 h (□) or 48 h (▨) after plating. Results are means $\pm$ S.E.M. of triplicate cultures. The asterisk indicates a significant difference ($P < 0.05$, Scheffe's multiple range test) when compared with the respective control cultures. No significant differences were observed in the final numbers of viable cells.

Table 1. Effects of camptothecin and Bt_2cAMP on DNA synthesis in cultured rat granulosa cells

Granulosa cells were cultured in 96-well multiwells and thymidine incorporation was measured from 24 to 48 h after plating. Results are means $\pm$ S.E.M. of triplicate cultures. Values with the same superscript are not significantly different ($P > 0.05$ by analysis of variance and Scheffe's multiple range test).

	[^3H]Thymidine incorporation (c.p.m./well)
Control	14937 $\pm$ 2611 ^a
Camptothecin (5 μM)	1035 $\pm$ 263 ^b
Bt_2cAMP (1 mM)	7653 $\pm$ 1270 ^c
Bt_2cAMP + camptothecin	469 $\pm$ 33 ^b

Fig. 3. Effect of FSH on DNA topoisomerase I activity

Supercoiled pUC12 DNA (1 μ g, lane C) was incubated with the indicated volumes of rat granulosa-cell-free extracts (6 μ g of protein/ μ l) as described in the Experimental section for the indicated times. The cells were previously cultured without (a, b) or with (c, d) FSH (200 ng/ml) for 24 h (a, c) or 48 h (b, d). After incubation the deproteinized DNA was subjected to electrophoresis on a 1% agarose gel. I, Supercoiled DNA; II, closed circular and nicked circular relaxed DNA.

Fig. 4. Effect of Bt₂cAMP, insulin and camptothecin on DNA topoisomerase I activity

Supercoiled pUC12 DNA (1 μ g, lane C) was incubated with the indicated volumes of rat granulosa-cell-free extracts (6 μ g of protein/ μ l) as described in the Experimental section for the indicated times. The cells were previously treated without (a) or with 1 mM-Bt₂cAMP (b), 1 μ g of insulin/ml (c) or 5 μ M-camptothecin (d) for 24 h. After incubation the deproteinized DNA was subjected to electrophoresis on a 1% agarose gel. I, Supercoiled DNA; II, closed circular and nicked circular relaxed DNA.

cells was associated with changes in the levels of DNA topoisomerase I activity, cells were cultured in the absence or the presence of this gonadotropin. Cell extracts were prepared 24 and 48 h after hormone addition. DNA topoisomerase activity was measured by the relaxation rate of pUC12 supercoiled DNA. As shown in Fig. 3, a 2-fold increase in DNA topoisomerase I activity was observed in FSH-treated cultures after 24 h (compare 5 μ l of extract in control cells with 2.5 μ l of extract in FSH-treated cells). This effect was markedly decreased after 48 h of hormone treatment.

A cyclic AMP derivative, dibutyryl cyclic AMP (Bt₂cAMP), produced an even larger increase (4-fold) in DNA topoisomerase I activity (Fig. 4), whereas insulin treatment had no effect. On the other hand, camptothecin completely abolished this activity

when added to the culture medium. A similar blockade of DNA topoisomerase I activity was observed when 5 μ M-camptothecin was added directly to the cell extracts from non-treated cultures during the enzyme assay (results not shown).

DISCUSSION

A specific inhibitor of DNA topoisomerase I, camptothecin, was able to block FSH-induced steroidogenesis in cultured rat granulosa cells. Although both progesterone biosynthesis and oestradiol formation from added androstenedione were affected, the latter response showed a comparatively higher sensitivity to inhibition by camptothecin. The rate-limiting step in progesterone biosynthesis is the conversion of cholesterol into pregnenolone by the cytochrome P-450 cholesterol side-chain-cleavage (P-450_{sc}) enzyme that resides in the mitochondrial inner membrane. In addition, stimulation of progesterone production by FSH under serum-free conditions is associated with increased cholesterol synthesis *de novo* [23]. Another cytochrome P-450 enzyme, the aromatase P-450 (P-450_{arom}), is required for oestradiol formation from androgens. FSH has been shown to increase both P-450_{sc} and P-450_{arom} mRNA levels. Even though both responses are supposed to be mediated by cyclic AMP, different sensitivities to gonadotropin stimulation have been reported. Furthermore, certain factors have opposite effects on progesterone and oestradiol responses [1]. Therefore it seems likely that different regulatory mechanisms are involved in the control of the expression of the steroidogenic enzymes from the two pathways, and this may explain their different sensitivities to the DNA topoisomerase I inhibitor.

The inhibition of cyclic AMP-dependent steroid production seen with camptothecin under the present conditions was specific and could not be accounted for by an overall blockage of protein synthesis. On the other hand, this inhibitor did block DNA synthesis, as measured by thymidine incorporation. DNA synthesis, however, does not seem to be required for differentiation of granulosa cells. Furthermore, increased FSH-induced steroidogenesis in rat granulosa cell cultures has been shown after inhibition of cell proliferation [23].

The results presented herein also show that both FSH and a cyclic AMP derivative can induce activation of DNA topoisomerase I which precedes the rise in steroid production. Insulin, acting through a different mechanism, has been shown to increase granulosa cell differentiation to a limited extent [1]. However, under the conditions of the present study, this hormone was unable to significantly modify DNA topoisomerase I activity.

Several lines of evidence suggest an association between elevated DNA topoisomerase I activity and increased gene expression [5–9,24]. Crespi *et al.* [25] have shown that treatment of adult rats with LH, another gonadotropin known to act through cyclic AMP, increased DNA topoisomerase I activity in Leydig cells. Stimulation of intracellular DNA topoisomerase activity has been reported in rat aortic smooth muscle cells by vasopressin and thrombin [26] and in human monoblast cells incubated with leukotriene D₄ [27]. In addition, Dainiak *et al.* [28] have proposed that DNA topoisomerases are critical in the differentiation of human marrow erythroid progenitor cells and stem cells in culture.

The inhibition of FSH-stimulated steroidogenesis by camptothecin, together with the increase in DNA topoisomerase I activity observed in FSH- or Bt₂cAMP-treated cultures, is consistent with a key role for this enzyme in the regulation of gene expression in rat granulosa cells.

We thank Dr. Bret Jessee for the camptothecin. This work was supported by grants from the Consejo Nacional de Investigaciones

Científicas y Técnicas of Argentina, the Universidad de Buenos Aires, the Fundación Antorchas (G.C.G.) and The Rockefeller Foundation (J.L.B.).

REFERENCES

1. Hsueh, A. J. W., Bicsak, T. A., Jia, X. C., Dahl, K. D., Fauser, B. C. J. M., Galway, A. B., Czekala, N., Pavlou, S. N., Papkoff, H., Keene, J. & Boime, I. (1989) *Recent Prog. Horm. Res.* **45**, 209–276
2. Roesler, W. J., Vandenbark, G. R. & Hanson, R. W. (1988) *J. Biol. Chem.* **263**, 9063–9066
3. Bernath, V. A., Muro, A. F., Vitullo, A. D., Bley, M. A., Barañao, J. L. & Kornblihtt, A. R. (1990) *J. Biol. Chem.* **265**, 18219–18226
4. Wang, J. C. (1985) *Annu. Rev. Biochem.* **54**, 665–697
5. Bonven, B. J., Gocke, E. & Westergaard, O. (1985) *Cell* **41**, 541–551
6. Gilmour, D. S., Pflugfelder, G., Wang, J. C. & Lis, J. T. (1986) *Cell* **44**, 401–407
7. Gilmour, D. S. & Elgin, S. C. R. (1987) *Mol. Cell. Biol.* **7**, 141–148
8. Garg, L. C., DiAngelo, S. & Jacob, S. T. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3185–3188
9. Egyhazi, E. & Durban, E. (1987) *Mol. Cell. Biol.* **7**, 4308–4316
10. Schaak, J., Sched, P. & Shenk, T. (1990) *Nucleic Acids Res.* **18**, 1499–1508
11. Snapka, R. M. (1986) *Mol. Cell. Biol.* **6**, 4221–4227
12. Annunziato, A. T. (1989) *J. Cell Sci.* **93**, 593–603
13. Glikin, G. C., Ruberti, I. & Worcel, A. (1984) *Cell* **37**, 33–41
14. Caceres, J. F., Blangy, D. & Glikin, G. C. (1990) *Eur. J. Biochem.* **181**, 531–537
15. Campbell, K. L. (1979) *Biol. Reprod.* **21**, 773–786
16. Lowry, O. H., Rosebrough, N. J., Farr, R. J. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
17. Vieira, J. & Messing, J. (1982) *Gene* **19**, 259–268
18. Chiauzzi, V. A., Cigorraga, S., Escobar, M. E., Rivarola, M. A. & Charreau, E. H. (1982) *J. Clin. Endocrinol. Metab.* **54**, 1221–1228
19. Vignon, F., Lippman, M. E., Nawata, H., Derocq, D. & Rochefort, H. (1984) *Cancer Res.* **44**, 2084–2088
20. Li, C. C. (1964) *Introduction to Experimental Statistics*, McGraw-Hill, New York
21. Liu, L. F. (1989) *Annu. Rev. Biochem.* **58**, 351–375
22. Barañao, J. L. S. & Hammond, J. M. (1986) *Mol. Cell. Endocrinol.* **44**, 227–236
23. Epstein-Almog, R. & Orly, J. (1985) *Endocrinology (Baltimore)* **116**, 2103–2112
24. Stewart, A. F., Herrera, R. E. & Nordheim, A. (1990) *Cell* **60**, 141–149
25. Crespi, M. D., Radicella, J. P., Baldi, A., Charreau, E. H. & Calvo, J. C. (1987) *Mol. Cell. Endocrinol.* **53**, 221–226
26. Nambi, P., Mattern, M., Bartus, J. O., Aiyar, N. & Crooke, S. T. (1989) *Biochem. J.* **262**, 485–489
27. Mattern, M. R., Mong, S., Mong, S.-M., Bartus, J. O., Sarau, H. M., Clark, M. A., Foley, J. J. & Crooke, S. T. (1990) *Biochem. J.* **265**, 101–107
28. Dainiak, N., Kreczko, S., Hanspal, M. & Strauss, P. R. (1989) *J. Cell. Physiol.* **138**, 87–96

Received 29 November 1990/26 March 1991; accepted 23 April 1991