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GOSSYPOLONE SUPPRESSES PROGESTERONE SYNTHESIS IN BOVINE LUTEAL CELLS

Y. Gu,[1] P. K. Li,[1] Y. C. Lin,[1]* Y. Rikihisa[2] and R. W. Brueggemeier[3]

[1]Laboratory of Reproductive Endocrinology, Department of Veterinary Physiology and Pharmacology, [2]Department of Veterinary Pathobiology, College of Veterinary Medicine and [3]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH 43210-1092, U.S.A.

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Summary—Gossypolone, a proposed major metabolite of gossypol, was synthesized and investigated for its effect on progesterone synthesis in cultured bovine luteal cells. Gossypolone inhibited human chorionic gonadotropin(hCG)-stimulated progesterone secretion, reduced substrate-enhanced conversions of 25-hydroxycholesterol to pregnenolone and of pregnenolone to progesterone in a dose-dependent fashion. These findings indicate that gossypolone inhibits not only 3β-hydroxysteroid dehydrogenase (3β-HSD) activity, as gossypol does, but also side-chain cleavage enzyme complex (cytochrome *P*450scc) activity. However, the two compounds appear to have a similar potency in inhibiting progesterone secretion. Both gossypolone and gossypol (8.5 μM) induced morphological changes in cellular organelles.

INTRODUCTION

Gossypol is a potent male antifertility agent. It alters hormone levels, especially steroids, in both males and females. Despite the encouraging results in antifertility studies, gossypol is not without toxic and side-effect problems. In order to maintain the desirable antifertility properties of gossypol while reducing detrimental side-effects, considerable efforts have been devoted to the synthesis and evaluation of experimental gossypol analogues and its derivatives.

Numerous gossypol analogues and derivatives have been studied [1–4]. Gossypolone is a proposed major metabolite of gossypol [5] and has been shown to have spermicidal effect, although it is less potent than gossypol [6]. Gossypol has been reported to alter steroidogenesis in both male [7–10] and female [11–14] reproductive systems. The promising reports of gossypol treatment in endometriosis and menorrhagia patients in China [15], and our previous finding that gossypol inhibits progesterone synthesis in cultured bovine luteal cells [16, 17] led us to examine and compare certain effects of gossypolone with gossypol on progesterone synthesis in bovine luteal cells *in vitro*.

*To whom correspondence should be addressed.

EXPERIMENTAL

Collagenase (type I, lot 77380) was purchased from Worthington Biochemical Co. (Freehold, NJ). Fetal bovine serum (FBS, lot B79306) was obtained from Armour Pharmaceutical Co. (Kankakee, IL). [1,2,6,7,21-^{3}H](*N*)-Progesterone (lot 2370–059) and δ-5[4,7-^{3}H]pregnenolone were purchased from New England Nuclear (Boston, MA) and Amersham (Arlington Heights, IL), respectively. Progesterone antiserum was obtained from Endocrine Sciences (Tarzana, CA) and pregnenolone antiserum (lot T463C) was purchased from Scantibodies Lab. (Santee, CA). Gossypol acetic acid was purchased from Sigma Chemical Co. (St Louis, MO) and checked for purity by thin-layer chromatography (TLC). TLC plates were purchased from Analtech Inc. (Newark, NE). Dulbecco's Modified Eagle's Medium, Ham's nutrient mixture F-12 (DME/F-12) and remaining chemicals were purchased from Sigma Chemical Co. or Aldrich Chemical Co. (Milwaukee, WI).

Preparation and culture of bovine luteal cells

Bovine ovaries containing corpora lutea were collected from a local slaughterhouse and transported back to the laboratory in iced phosphate buffered saline (PBS) within 1 h of slaughter.

The corpora lutea with a size about 14 × 14 × 12 mm³ were selected for luteal cell isolation. Selected corpora lutea were carefully dissected from ovaries on ice. The luteal cells were isolated as described by Poff *et al.* [18]. Briefly, the corpora lutea were sliced, minced and dissociated at 35°C with collagenase (2000 U/g tissue) and 0.5% bovine serum albumin (BSA). At 10 min intervals the tissue and medium were triturated with a 10 ml glass pipet to facilitate cell dissociation. The cells were then washed 4 times with fresh DME/F-12 containing no BSA by centrifugation for 10 min in a series of descending speeds, from 150 to 100 *g* at 4°C. The resulted bovine luteal cells were counted under a microscope with a hemocytometer. The viability of bovine luteal cells was determined by the trypan blue exclusion method [19]. By this method, about 17.36×10^6 viable cells/g of luteal tissue were yielded, with an approx. 20–30/80–70 ratio of large (> 20 μm) to small (8–20 μm) luteal cell populations [20]. The viability of yield cells was about 70–80%.

The culture dishes (60 × 15 mm) were treated with 5 ml of DME/F-12 containing 10% FBS for 2 h at 37°C. This step of procedure provided the necessary factors for cell attachment and spreading. Dishes were then washed twice with 5 ml of fresh DME/F-12 to remove FBS prior to luteal cell plating.

Approx. 1×10^6 viable bovine luteal cells were plated into each dish in a total volume of 5 ml DME/F-12 supplemented with insulin, 5 μg/ml; transferrin, 5 μg/ml; epidermal growth factor (EGF), 10 ng/ml; sodium selenite, 10 ng/ml; penicillin G sodium, 100 U/ml; streptomycin sulfate, 0.1 mg/ml and amphotericin B, 0.25 μg/ml (DME/F-12/S). At the end of 16 h culture at 37°C, under an atmosphere of 95% air/5% CO_2, the medium was removed and the dishes were washed twice with 5 ml of fresh DME/F-12. At this point, the steroidogenic activity of cultured bovine luteal cells was confirmed by the 3β-hydroxysteroid dehydrogenase (3β-HSD) stain method, as described by Wiebe [21]. An additional 3 h culture was carried out in a total volume of 5 ml DME/F-12/S with variable treatments. Briefly, the bovine luteal cells were cultured with human chorionic gonadotropin (hCG) (1 IU/ml) or 25 hydroxycholesterol (25-OH cholesterol, 100 μM) or pregnenolone (25 μM) in the presence or absence of gossypolone or gossypol. Gossypolone, gossypol and pregnenolone were first dissolved in dimethyl sulfoxide (DMSO), then diluted in the DME/F-12/S. The highest concentration of DMSO in the medium was 0.25%, at which level the progesterone secretion of bovine luteal cells was not affected (data not shown). At the end of each treatment, the medium was collected and stored at −20°C for steroid determination.

Synthesis of gossypolone acetic acid

Gossypolone used for the present study was synthesized at the Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University. Gossypol acetic acid (1 g, 1.75 mmol), acetone (50 ml) and acetic acid (100 ml) were mixed and heated on a steam bath. Ferric chloride (4.85 g, 30 mmol) in 60 ml of water was slowly added (in 10 min) to the gossypol solution. The resulting solution was heated for an additional 5 min. The reaction mixture was cooled to room temperature and orange crystals precipitated. The precipitate was filtered, dried under vacuum and recrystallized with acetic acid/water to yield 0.55 g of orange crystals (yield = 52%) (Fig. 1). The purity of resultant gossypolone acetic acid was > 99.0%, which was determined by HPLC and melting point analysis. Melting points were obtained on a Thomas–Hoover capillary melting apparatus and were uncorrected, m.p. 256–258°C [22]. Infrared (i.r.) spectral data was recorded on an RFX-40 FTIR spectrometer of Laser Precision Analytical, i.r. (KBr) 3490, 2940, 2920, 1655, 1630 and 1370. NMR spectra were obtained with an IBM AF/250 spectrometer in the pulse mode, ^{1}H NMR ($CDCL_3$)δ 1.43–1.48 (m, 12H, CH_3 of isopropyl), 2.06 (s, 6H, CH_3), 4.15 (m, 2H, CH of isopropyl), 6.57 (s, 2H, OH of 6 and 6′ position), 10.6 (s, 2H, CH of aldehyde), 13.0 (s, 2H, OH of 7 and 7′ position).

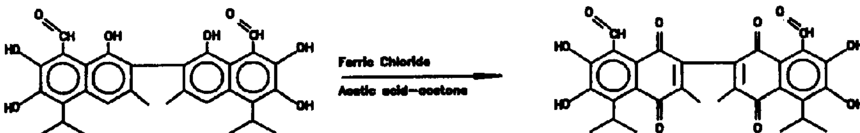

Fig. 1. Structures of gossypol and gossypolone. 6,6′,7,7′-Tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-(2,2′-binaphthalene)-1,1′,4,4′-tetraone-8,8′-dicarboxaldehyde (gossypolone).

Determination of steroids

The collected culture medium was extracted in a 1:7 (v/v) ratio of medium/ethyl ether. The ether layer was separated from the medium by freezing the extracted samples in dry ice/methanol mixture. The ether phase was evaporated and reconstituted with PBS containing 0.1% gelatin. The progesterone and pregnenolone contents were determined by RIA as described by Stouffer *et al.* [23]. The intra- and interassay coefficients of variation in our RIA were 4.3 and 14% for progesterone, and 4.9 and 16% for pregnenolone, respectively. The cross-reactivity of progesterone antiserum with pregnenolone was 15%, using 50% inhibition of binding method, as described by Abraham [24]. The cross-reactivity of pregnenolone antiserum with progesterone was 1%. The secretions of progesterone and pregnenolone were expressed as ng/h/10^6 cells.

Ultrastructural study of cultured bovine luteal cells

The cultured bovine luteal cells were fixed with 3.5% glutaraldehyde, 2% formaldehyde and 0.03% trinitrophenol in 0.1 M sodium cacodylate buffer (pH 7.4) overnight and post-fixed with 1% osmium tetroxide in 1.5% potassium ferrocyanide. After block staining with 1% uranyl acetate in maleate buffer (pH 5.2), the cells were dehydrated in a graded series ethyl alcohols (60–100%). At the end of dehydration, the cells were gently scraped out from dishes by a rubber policeman and centrifuged at 150 *g* for 5 min. The cell pellet were dehydrated with propylene oxide and embedded in Poly/Bed 812 (Polysciences Inc., Warrington, PA). Thin sections (60–90 nm) were cut under a microtome and stained with uranyl acetate/lead citrate, and examined with a Phillips 300 electron microscope.

Statistical analysis

Differences between treatment means were measured by analysis of variance and Duncan or Bonferroni multiple comparison procedure. Differences were considered significant at $P < 0.05$ in all comparisons.

RESULTS

Both gossypolone and gossypol inhibited hCG-induced progesterone secretion in cultured bovine luteal cells in a dose-dependent fashion

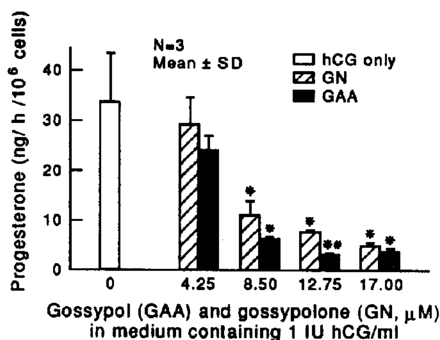

Fig. 2. hCG-induced progesterone section was inhibited by both gossypol (GAA) and gossypolone (GN) with a dose-dependent pattern. At 8.5 μM or greater concentrations, the inhibition became statistically significant for both GAA and GN. *Significantly different from hCG only group ($P < 0.05$); # significantly different between GN- and GAA-treated groups ($P < 0.05$).

(Fig. 2). Gossypolone and gossypol significantly ($P < 0.05$) inhibited hCG-induced progesterone secretion at concentrations of 8.5, 12.75 and 17 μM. Gossypolone appeared less potent than gossypol. However, the differences in progesterone secretion were not statistically significant between two compound-treated groups, except the 12.75 μM group.

Addition of exogenous 25-OH cholesterol (100 μM) greatly increased the progesterone secretion in cultured bovine luteal cells. Gossypolone, at 8.5 and 17 μM, significantly ($P < 0.05$) inhibited the substrate-enhanced progesterone secretion (Fig. 3).

Exogenous 25-OH cholesterol (100 μM) also greatly increased the pregnenolone level in the medium. Gossypolone inhibited the conversion of exogenous 25-OH cholesterol to pregnenolone. Again, at concentrations of 8.5 and 17 μM, gossypolone significantly ($P < 0.05$) inhibited the conversion (Fig. 4).

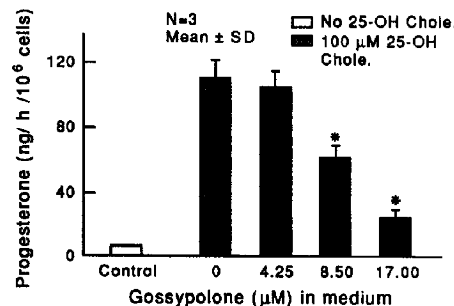

Fig. 3. Gossypolone reduced the conversion of exogenous 25-OH cholesterol to progesterone in a dose-dependent pattern. *Significantly different from 0 μM group ($P < 0.05$).

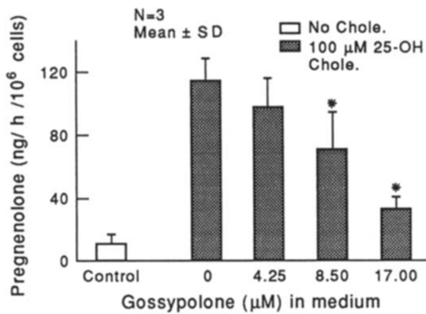

Fig. 4. Gossypolone reduced the conversion of exogenous 25-OH cholesterol to pregnenolone in a dose-dependent pattern. *Significantly different from 0 μM group ($P < 0.05$).

Addition of exogenous prognenolone (25 μM) drastically increased progesterone secretion. However, gossypolone, at 8.5 and 17 μM, significantly ($P < 0.05$) inhibited the substrate-enhanced conversion (Fig. 5).

Ultrastructural study revealed that 8.5 μM gossypolone or gossypol-induced observable changes in cellular structure of cultured bovine luteal cells (Figs 7 and 8). In comparison with untreated cells (Fig. 6), endoplasmic reticulum (ER) were dilated and normally elongated mitochondria became rounded. Several vacuoles were present in the cytoplasm. Loss of ground substance in both the cytoplasm and nuclei was also observed. All these changes occurred in both gossypolone- (Fig. 7) and gossypol- (Fig. 8) treated cells. However, the cell viability did not differ between groups at the end of incubation examined by using a trypan blue exclusion method [19].

DISCUSSION

We have previously reported that gossypol inhibited bovine embryo development during the preimplantation period [25]. However, gossypol metabolite(s) isolated from the livers of pigs fed with federally allowable gossypol (450 ppm) in the ration possessed a much higher potency in inhibiting the early development of mouse embryos *in vitro* [26]. The same metabolite(s) also exerted an inhibitor effect on the hCG-induced testosterone secretion in young male rats [10]. Those results suggest that the gossypol metabolite(s), which was isolated from animals receiving gossypol through a ration containing cottonseed meal, has a higher potency in influencing embryo development and androgen secretion, at least in the models used in our previous studies. It has been shown that hepatic cytochrome $P450$ activity decreased in neonatal rats

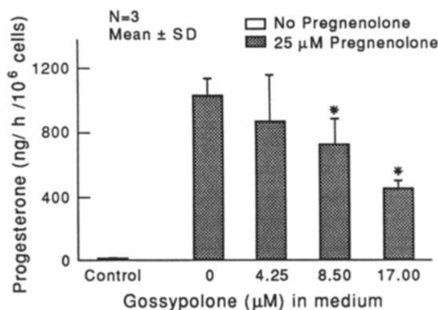

Fig. 5. Gossypolone decreased the conversion of exogenous pregnenolone to progesterone in a dose-dependent pattern. *Significantly different from 0 μM group ($P < 0.05$).

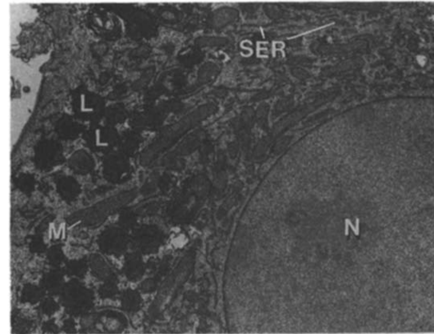

Fig. 6. A transmission electron micrograph of a bovine luteal cell incubated with 1 IU hCG/ml for 3 h. Note the ground substance in nucleus (N), elongated mitochondria (M), numerous smooth endoplasmic reticula (SER) and lipid droplets (L) in the cytoplasm. (Mag. × 9300).

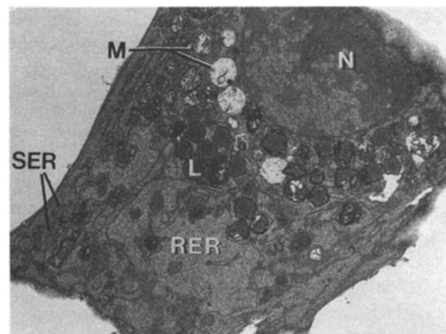

Fig. 7. An electron micrograph of a bovine luteal cell incubated with 1 IU hCG plus 8.5 μM gossypolone for 3 h. The cell was partially disrupted. Cytoplasmic and nuclear (N) ground substance were lost leaving fragmented smooth endoplasmic reticulum (SER) and halfway-disrupted mitochondria (M). Rough ER (RER) appeared dilated and a few lipid droplets (L) were present. (Mag. × 8500).

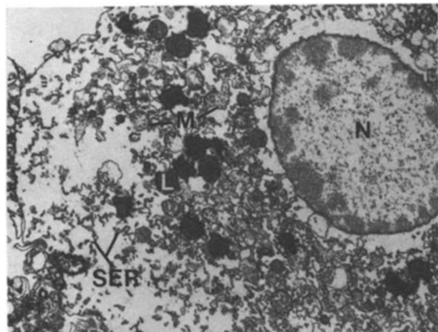

Fig. 8. An electron micrograph of a bovine luteal cell with 1 IU hCG/ml plus 8.5 μM gossypol for 3 h. Extremely dilated rough endoplasmic reticulum (RER) and round up mitochondria (M) were evident. Fewer lipid droplets (L) and smooth endoplasmic reticula (SER) were present in cytoplasm. The nucleus (N) contained more heterochromatin. (Mag. × 7400).

receiving gossypol-containing milk from their treated dams [27]. Recent evidence further demonstrated that gossypol-containing serum obtained from gossypol-treated nursing rat dams significantly suppressed cAMP formation in cultured bovine luteal cells [28]. The implication of those results raises the question of whether the biological activities of gossypol metabolite(s) affect reproductive functions in animals or humans who consume gossypol-containing animal products such as meat, liver and milk. This was another reason for conducting the present study to evaluate and compare the effectiveness of gossypolone and gossypol on progesterone synthesis in cultured bovine luteal cells.

Gossypolone is a proposed major metabolite of gossypol. Kim *et al.* [6] reported that gossypolone, as gossypol, possessed antimotility effects on human spermatozoa. They suggested that certain similar moieties in both compounds contributed to these antifertility effects. Recently, our laboratory has reported that gossypol inhibited 3β-HSD activity in bovine luteal cells *in vitro* [17]. The present study demonstrated that gossypolone inhibited not only the activity of 3β-HSD but also the activity of cytochrome P450scc, although the overall potency of gossypolone in inhibiting progesterone secretion was not greater than parent compound-gossypol. In the present case, in fact, gossypolone seemed to be less potent and at one point (12.75 μM), at least, the difference became statistically significant (Fig. 2). Gossypolone, at 8.5 μM or higher concentrations, significantly inhibited hCG-induced progesterone secretion (Fig. 2), the conversions of exogenous 25-OH cholesterol to progesterone (Fig. 3) and to pregnenolone (Fig. 4), and the substrate-enhanced conversion of pregnenolone to progesterone (Fig. 5).

Cytochrome P450scc is considered as a rate-limiting enzyme in progesterone synthesis from cholesterol in luteal cells [29–31]. 3β-HSD also plays an important role in the synthesis of progesterone. In luteal cells, cytochrome P450scc and 3β-HSD are located in mitochondria [32] and smooth ER [33], respectively. The location of morphological changes in either 8.5 μM gossypolone- or gossypol-treated bovine luteal cells is closely correlated to the altered functional activity, such as mitochondria, Golgi apparatus and ER (Figs 7 and 8). However, broad cellular structural changes in the luteal cells may contribute to the general cytotoxic effect of gossypolone as well as gossypol, since cytotoxic effect has been reported in several types of cells *in vitro* treated with gossypol at high concentrations and prolonged culture time [34, 35]. Furthermore, we recently found that synthetic gossypolone also inhibited adrenocorticotropic hormone (ACTH)-induced intracellular cAMP formation and corticosterone secretion *in vitro* in adrenocortico cells from young rats (21–25 days old) [36]. This implies a general cytotoxic effect of gossypolone.

We previously reported that gossypol metabolite(s) extracted from the livers of pigs fed gossypol exhibited a higher potency in certain biological activities [10, 26]; however, the chemically synthesized gossypolone does not exhibit a higher potency in inhibiting progesterone secretion in cultured bovine luteal cells than parent compound-gossypol, although it inhibits both 3β-HSD and cytochrome P450scc. Whereas, it seems less potent in the present study. This is in agreement with Kim *et al.* [6]. They reported a less potent spermicidal effect of gossypolone compared with gossypol. Also other investigators reported a variety of gossypol analogues with either reduced or lost antifertility effects as the functional groups of gossypol were substituted or deleted, such as aldehyde and hydroxy groups [1, 3, 4, 37]. The higher potency of pig liver extracts in certain biological activities probably contributes to the impurity or other substances contained in the liver extracts.

The antifertility effect of gossypol requires several weeks treatment with a dosage of 20 mg/kg body wt/day in most animal models.

The exact concentration of gossypol in gonads is not available, due to the lack of an appropriate method of *in vivo* measurement. However, when rats were administered with a single dose of either [^{14}C]gossypol or [^{3}H]gossypol, 0.021% of total administered radioactivity was detected in testes after 48 h (38) and 0.035% in ovary after 24 h (our unpublished data). Based on the available data and considering the accumulation of gossypol during the *in vivo* treatment, we believe that the concentrations of gossypol used in our *in vitro* study are fairly comparable with those in the *in vivo* study. However, the difference in the biological activity of gossypol between the *in vitro* and *in vivo* conditions is still questionable.

The present study clearly demonstrates that gossypolone, as well as gossypol, inhibits hCG-stimulated progesterone synthesis and secretion in cultured bovine luteal cells. Inhibited conversions of exogenous 25-OH cholesterol to pregnenolone and of pregnenolone to progesterone indicate that gossypolone not only inhibits the activity of 3β-HSD as gossypol reported previously [16, 17], but also inhibits the activity of cytochrome *P*450scc. The reason for gossypolone to possess such a specific inhibitory action in the biosynthesis of steroid which gossypol does not in cultured bovine luteal cells is not clear at the present time. The overall potency in inhibiting hCG-induced progesterone secretion does not significantly differ between these two compounds; however, gossypolone seems less effective than gossypol in the present study. The comparable morphological changes in bovine luteal cells treated with either gossypolone or gossypol indicate that both compounds exert a similar regulatory action on progesterone synthesis. However, more prominent morphological changes observed in gossypolone-treated bovine luteal cells may indicate a higher cytotoxic effect of gossypolone.

Based on the present results, we conclude that gossypolone, as well as gossypol, inhibits hCG-induced progesterone synthesis and secretion. However, a similar antisteroidogenic effect and a possible higher cytotoxic effect suggest that gossypolone may not be a suitable substitute for gossypol as an antifertility agent in both male and female species. However, further *in vivo* study is needed to confirm our observations made with the *in vitro* model.

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