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CHOLESTEROL SIDE-CHAIN CLEAVAGE AND 11β-HYDROXYLATION ARE INHIBITED BY GOSSYPOL IN ADRENAL CORTEX MITOCHONDRIA

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Summary—Cholesterol side-chain cleavage and 11β-hydroxylation were assessed in isolated adrenal cortex mitochondria by formation of pregnenolone and corticosterone, respectively, in the presence and absence of gossypol. Pregnenolone biosynthesis was inhibited when increasing concentrations of gossypol were added. The control value of 4 nmol min^{-1} mg^{-1} dropped to 2 nmol min^{-1} mg^{-1} with 30 μM of the drug in the incubation medium. A more pronounced inhibitory effect was observed upon 11β-hydroxylation of steroids; I_{50} was 11 μM. Seventy-five percent of corticosterone production was impaired when 30 μM of gossypol were present. Bovine serum albumin prevented and reversed the inhibitory action of the drug. Kinetic studies showed a linear mixed type inhibition, suggesting a direct action of the drug upon the enzymatic complex. This study demonstrates a direct inhibitory effect of gossypol upon the steroidogenic enzymes located in the inner mitochondrial membrane of the adrenal cortex.

INTRODUCTION

Gossypol (GSP), a polyphenolic compound isolated from the stem and root of the cotton plant, exerts a wide variety of effects on cells and tissues [1]. Much of the current interest in GSP is due to its ability to suppress male fertility [2–4]. The mechanism of action of GSP is still unknown, but the results from *in vivo* and *in vitro* experiments have shown that it inhibits different enzymes associated with biological membranes [5–7]. Studies on the distribution of [^{14}C]GSP in testicular subcellular fractions of rat testis have shown that, among all organelles, mitochondria show the highest incorporation rate [8]. GSP treatment leads to dramatic changes in both mitochondrial function and structure, such as uncoupling of oxidative phosphorylation, swelling and vacuolation [9]. The effect of GSP upon testicular steroidogenesis is controversial [10], although the inhibition of testosterone biosynthesis *in vitro* in purified mouse Leydig cells has been reported [11–13]. To further characterize the effects of GSP in an active steroidogenic tissue, we studied its action upon cholesterol side-chain cleavage and 11β-hydroxylation in isolated adrenal cortex mitochondria.

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EXPERIMENTAL

Preparation of mitochondria

Adrenocortical mitochondria were prepared as previously described [14] from adrenal glands of mongrel dogs, except that the homogenate prepared in 0.25 M sucrose, 1 mM EDTA pH 7.4 (SET medium), was centrifuged twice at 600 *g* for 10 min. Before the last centrifugation, the mitochondrial pellet was suspended in 30 ml SET medium supplemented with 0.5% of defatted bovine serum albumin. The suspension was incubated for 10 min under slow constant stirring at 4°C. This preparation was then centrifuged at 10,000 *g* for 10 min. The final pellet was resuspended in a small volume of SET medium to reach a final concentration of 30 mg protein per ml. Mitochondrial protein concentration was measured by the method of Lowry *et al.* [15] using bovine serum albumin (BSA) (fraction V) as standard.

Standard incubation medium

The medium (SIM), used in all our experiments, was as follows: 0.25 M sucrose, 20 mM KCl, 10 mM potassium phosphate buffer pH 7.4, 5 mM $MgCl_2$ and 15 mM triethanol-

amine buffer pH 7.4. SIM was oxygenated for 1 min before using.

Enzyme assays

Cholesterol side-chain cleavage activity was measured through pregnenolone formation by intact adrenal cortex mitochondria as previously described [16]. The assay mixture consisted of a suspension of intact mitochondria (0.8 mg protein/ml) in SIM supplemented with 50 μM trilostane and 40 μl of a suspension of cholesterol–BSA [17]. This mixture was incubated in a shaking water bath for 2 min at 30°C. Where indicated, GSP was added. The reaction was started by the addition of 10 mM malate and stopped, after 2 min, by transferring duplicate aliquots of 25 μl of the reaction mixture into 1 ml hexane. All the samples were kept at 4°C until required for analysis.

Total pregnenolone was recovered from the quenched samples by adding 10 ml diethylether and shaking them for 1 min. The solvent phase was evaporated to dryness, and the solid residue was resuspended in 2 ml phosphate buffered saline, pH 7.2, 1% gelatin. Duplicate aliquots were assayed for pregnenolone concentration by RIA as previously described [16].

The 11β-hydroxylase activity was assessed by means of the transformation of DOC into corticosterone by the mitochondrial preparation. Intact mitochondria (1 mg protein/ml) were incubated in SIM supplemented with 100 μM DOC in a shaking water bath for 2 min at 30°C. Where indicated, different amounts of GSP were added. The hydroxylation reaction was started by the addition of 10 mM malate. After the incubation periods indicated under Results, the reaction was stopped with 1 ml of $HgCl_2$ 0.5% (w/v). Corticosterone was extracted with 15 ml ice-cold dichloromethane plus 0.2 ml 0.1 N NaOH, and then quantitated by a fluorescence assay [14].

Spectroscopic measurements

The rate of substrate binding to the active site of the enzyme was measured in a double beam spectrophotometer, operated in the dual wavelength mode, using 420–390 nm wavelengths at 25°C. Adrenal cortex mitochondria were diluted in SIM to yield 0.8 mg protein/ml, adding 2.5 μM rotenone to inhibit the respiratory chain. From this preparation two aliquots were taken and run in parallel. To sample A, 15 μM GSP in ethanol was added, and sample B received only the same volume of ethanol. Absorbance changes were recorded immediately after the addition of varying concentrations of DOC (0.5–10 μM) to the cuvette containing 2 ml of mitochondrial preparation. The rate of DOC binding to the mitochondrial cytochrome *P*-450 was calculated and plotted as described in Results.

Materials

Gossypol acetic acid (99% pure) was donated by Dr Gustavo García de la Mora, Facultad de Química, Universidad Nacional Autónoma de México. Trilostane was provided by the Sydney Ross Co. (México), tritiated pregnenolone (SA 24.2 Ci/mmol) was purchased from New England Nuclear (Boston, Mass), and anti-pregnenolone-3-mono-hemisuccinate-human serum albumin serum was obtained from RSL Inc. (Carson, Calif.).

Deoxycorticosterone and corticosterone were obtained from Syntex Laboratories (México). All other reagents and substrates used were of the highest purity available and were used without further purification. Gossypol and steroids were dissolved in absolute ethanol, and trilostane was freshly prepared in acetone and maintained at −20°C. Equivalent amounts of these solvents were included in all control assays.

RESULTS

Viability of dog adrenal cortex mitochondria was assessed as previously reported [14, 16]. Cholesterol side-chain cleavage was estimated by pregnenolone production (nmol min^{-1} mg^{-1}) after 2 min of incubation of adrenal mitochondria as indicated in Methods. Figure 1 shows inhibition of pregnenolone biosynthesis produced when increasing concentrations of GSP were added to the mitochondrial reaction mixture. The calculated control value of 4 nmol min^{-1} mg^{-1} dropped to 2 nmol min^{-1} mg^{-1} when 30 μM GSP was added.

To assess the action of GSP upon 11β-hydroxylase activity, corticosterone production was measured after incubating mitochondria with DOC, as substrate, in the absence and presence of GSP. Figure 2 shows corticosterone biosynthesis in adrenal cortex mitochondria incubated during different times. A fixed concentration of 30 μM GSP prevented the synthesis of corticosterone as early as 1 min after the reaction was started. When adrenal cortex mitochondria were incubated with increasing

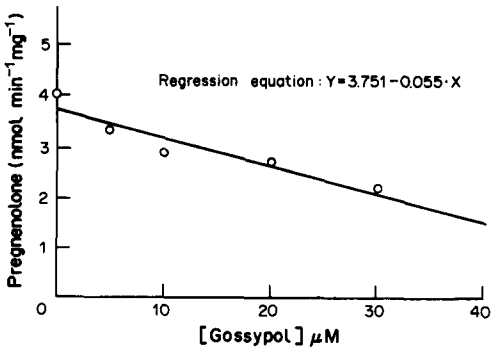

Fig. 1. Pregnenolone production by adrenal cortex mitochondria incubated in the presence of different concentrations of GSP. Intact mitochondria (0.8 mg protein/ml) were preincubated in a medium composed of: 0.25 M sucrose, 20 mM KCl, 10 mM phosphate buffer pH 7.4, 5 mM $MgCl_2$, 15 mM triethanolamine buffer pH 7.4, 50 μM trilostane and 40 μl cholesterol—BSA emulsion. The indicated concentrations of GSP were added at this stage. To start the reaction 10 mM malate was added at zero time. After 2 min, the reaction was stopped by transferring aliquots into 1 ml hexane. The regression line shows a negative slope with a correlation coefficient close to one ($R = 0.954$).

concentrations of GSP, inhibition of corticosterone synthesis showed a dose–response relationship, as shown in Fig. 3. The estimated I_{50} was found to be 11 μM GSP. To determine whether inhibition induced by GSP was reversible, the activity of 11β-hydroxylase was measured under different conditions: (a) 1% BSA was added to the medium at the beginning of the reaction and maintained throughout the experiment. The results in Table 1A show that, under these conditions, enzyme activity was reduced to only 5% of the control value; (b) mitochondria, preincubated with GSP, were washed with SET medium additioned with 1%

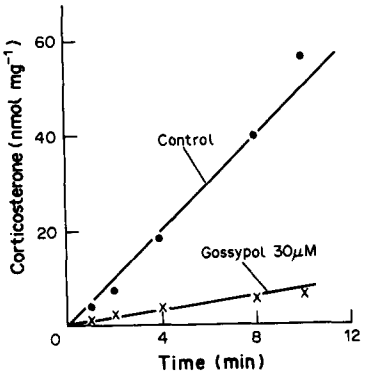

Fig. 2. Corticosterone biosynthesis inhibition in adrenal cortex mitochondria induced by GSP. Whole mitochondria (1 mg protein/ml) were preincubated in a medium composed of: 0.25 M sucrose, 20 mM KCl, 10 mM phosphate buffer pH 7.4, 5 mM $MgCl_2$, 15 mM triethanolamine buffer pH 7.4, and where indicated GSP was included. To start the reaction 10 mM malate was added at zero time. An aliquot of 1 ml was taken at the indicated incubation times and the reaction was stopped by adding 1 ml 0.5% $HgCl_2$.

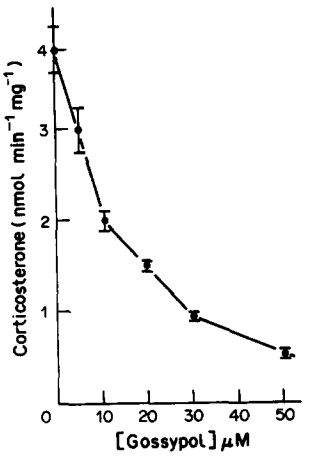

Fig. 3. Effect of varying concentrations of GSP on the rate of 11β-hydroxylation of DOC. Experimental conditions were similar to those described in Fig. 2. The calculated I_{50} was 11 μM GSP.

BSA and then assayed for activity. This treatment restored enzyme activity to 93% of that of the control sample treated in a similar manner (Table 1B).

In an attempt to further investigate the mechanism of action of the inhibition induced by GSP, kinetic assays were performed. The rate of corticosterone biosynthesis was measured in the absence and presence of a fixed concentration of 15 μM GSP. The results were analysed by linear regression analysis and expressed as a double-reciprocal plot as shown in Fig. 4.

To obtain further insight into the action of GSP on the enzyme catalysis, the rate of substrate binding to the active site was measured (see Methods), since this is the first step in the proposed model of the cycle for cytochrome *P*-450 catalyzed reactions [18]. The binding of substrate caused absorbance changes due to a shift from low-spin to high-spin in the hem-iron of *P*-450. These absorbance changes were recorded, and the rates of DOC binding to the

Table 1. Corticosterone biosynthesis

(A) Mitochondria were treated under the same condition as described in Fig. 2 except that 1% BSA was added and maintained throughout the experiment.

Addition	nmol min^{-1} mg^{-1}	% Activity
—	2.23	100
30 μM GSP	0.83	37
30 μM GSP + 1% BSA	2.11	95

(B) Mitochondria were washed, as indicated, after preincubation for 2 min under the same conditions as described in Fig. 2, and then assayed for activity.

Addition	Washing	nmol min^{-1} mg^{-1}	% Activity
—	SET medium	1.25	100
30 μM GSP	SET medium	0.30	23
30 μM GSP	SET medium +1% BSA	1.20	93

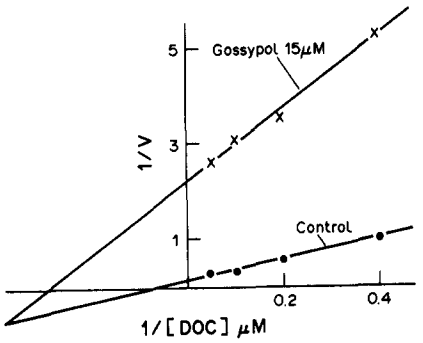

Fig. 4. Lineweaver–Burke plot for the inhibition of corticosterone biosynthesis in adrenal cortex mitochondria by GSP. The experimental procedure was the same as described in Fig. 2. Velocity is expressed as nmol of product formed per mg of mitochondrial protein per min. The lines were calculated by linear regression analysis (Control $R = 0.999$; GSP $R = 0.994$).

enzyme were fitted to the respective equations by the least-squares method and plotted as a double-reciprocal plot (Fig. 5). Kinetic assay data showed that 11β-hydroxylase exhibits a linear mixed-type inhibition by GSP, indicating that there is a decrease in both V_{max} and K_m [19]. However, V_{max} diminishes to a greater extent than K_m, as implied by the fact that curves intersect below the 1/[S] axis, which results in the formation of the product at a lower rate than in the control experiment.

DISCUSSION

It is well established that adrenal cortex mitochondria possess the ability to cleave the side-chain of cholesterol to yield pregnenolone [20] and to hydroxylate steroid substrates at the C-11 position [21, 22]. The experiments described here demonstrate that GSP induces inhibition of the activity of these hydroxylases. In fact, addition of 30 μM GSP to adrenal cortex mitochondria induced a decrease of 50% (I_{50}) in the basal transformation of cholesterol to pregnenolone (Fig. 1). This finding strongly suggests the presence of another site exposed to modifications by GSP in the steroidogenic pathway besides those prevously described [12]. Thus, this effect may be contributing to the diminution of testosterone output observed by Pearce *et al.* [12] in cultured mouse Leydig cells treated with GSP.

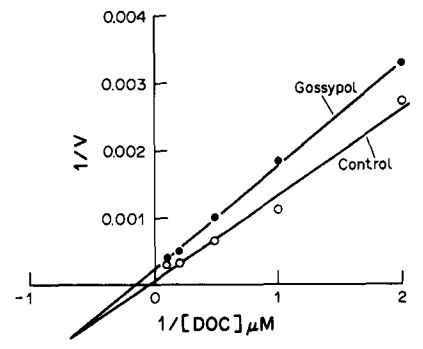

Fig. 5. Lineweaver–Burke plot of the binding of DOC to adrenal cortex mitochondria in absence and presence of a fixed concentration of 15 μM GSP. Intact mitochondria (1 mg protein/ml, 1.5 nmol P-450) were treated as indicated in Methods. Velocity is expressed as Δ absorbance (420–390 nm) per mg mitochondrial protein per min. Lines were calculated by linear regression analysis (Control $R = 0.991$; GSP $R = 0.999$). Each point represents an average of 4 experiments.

It has been suggested that one of the main factors involved in the regulation of the mechanism of cholesterol conversion to pregnenolone is the availability of cholesterol to the cytochrome P-450$_{scc}$ [23]. However, addition of GSP to the mitochondrial incubation medium did not modify the rate of mitochondrial [^{14}C]cholesterol-transport (data not shown), indicating that the observed inhibitory effect of GSP is probably not due to a blockade of this process.

When 11β-hydroxylation was studied, a more pronounced inhibitory effect of GSP was observed. Indeed, the I_{50} values of GSP inhibition were different between pregnenolone biosynthesis ($I_{50} = 30\ \mu$M) and corticosterone formation ($I_{50} = 11\ \mu$M). This last I_{50} value is similar to that reported previously for catechol-O-methyl-transferase activity in rat liver [24]. These unequal responses to GSP may be attributed to different reactivities between P-450$_{scc}$ and P-450$_{11\beta}$ towards the drug. In this regard Jefcoate *et al.* [25] found that in whole mitochondria both cytochromes showed dissimilar reactivities towards para-chloromercuribenzoate (PCMB), that is, when DOC was added before PCMB, the faster reaction of P-450$_{11\beta}$ is partially protected against the PCMB. Therefore, these authors attributed the low reactivity of P-450$_{scc}$ to the fact that, in whole mitochondria, this cytochrome is present in a high proportion as a cholesterol–P-450$_{scc}$ complex.

The kinetic analysis of the inhibition induced by GSP was performed taking into account that it is a reversible inhibitor, as shown by the experiments in which BSA was used (Table 1).

The linear mixed type inhibition of 11β-hydroxylase induced by GSP has also been described for lactate dehydrogenase isozymes [26], suggesting that a similar kinetic mechanism operates in the inhibition of both enzymatic complexes. Moreover, the same

pattern of inhibition was observed in corticosterone production (Fig. 4), and in enzyme–substrate complex formation (Fig. 5). Inhibition was lower in the latter, which can be explained considering the interaction of GSP at several sites throughout the catalytic cycle of the enzyme. The similarity in the kinetic patterns suggests that GSP exerts its inhibitory action directly on the enzyme. The possibility that GSP may also be modifying the conformation of the enzyme, through interactions with the hydrophobic environment of the mitocondrial membrane, should also be considered. Interactions of this type have been demonstrated in liposomes and artificial membranes exposed to GSP [27, 28].

The results of this study demonstrate that GSP inhibits side-chain cleavage of cholesterol and 11β-hydroxylase activity in isolated adrenal cortex mitochondria. These findings strongly suggest another site of action of GSP on steroidogenic tissues, and must be considered to explain the antifertility effect produced by the drug in males.

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REFERENCES

1. Abou-Donia M. B.: Physiological effects and metabolism of gossypol. *Residue Rev.* **61** (1976) 125–160.
2. Quian S. Z. and Wang Z. G.: Gossypol: a potential antifertility agent for males. *A. Rev. Pharmac. Toxic.* **24** (1984) 329–360.
3. Poso H., Wichmann K., Janne J. and Lukkainen T.: Gossypol a powerful inhibitor of human spermatozoal metabolism. *Lancet* **1** (1980) 885–887.
4. Wichmann U., Kapyaho K., Sinervirta R. and Janne J.: Effect of gossypol on the motility and metabolism of human spermatozoa. *J. Reprod. Fert.* **69** (1983) 259–264.
5. Abou-Donia M. B. and Dieckert J. W.: Gossypol: uncoupling of respiratory chain and oxidative phosphorylation. *Life Sci.* **14** (1974) 1955–1963.
6. Reyes J. and Benos J. D.: Specificity of gossypol uncoupling: a comparative study of liver and spermatogenic cells. *Am. J. Physiol.* **254** (1988) C571–C576.
7. Ueno H., Sahni M. K., Segal J. Sh. and Koide S. S.: Interaction of gossypol with sperm macromolecules and enzymes. *Contraception* **37** (1988) 333–341.
8. Xue S. P.: Studies on the antifertility effect of gossypol, a new contraceptive for males. In *Recent Advances in Fertility Regulation* (Edited by C. C. Fen and D. Griffin). Atar S. A., Geneva (1981) p. 135, pp. 122–146.
9. Robinson J. M., Tanphaichitr N. and Bellve A. R.: Gossypol-induced damage to mitochondria of transformed Sertoli cells. *Am. J. Pathol.* **125** (1986) 484–492.
10. Prasad M. R. and Diczfalusy E.: Gossypol. *Int. J. Androl.* (Suppl. 5) (1981) 53–70.
11. Donaldson A., Sufi S. B. and Jeffcoate S. L.: Inhibition by gossypol of testosterone production by mouse Leydig cells *in vitro*. *Contraception* **31** (1985) 165–171.
12. Pearce S., Sufi S. B., O'Saughnessy P. J., Donaldson A. and Jeffcoate S. L.: Site of gossypol inhibition of steroidogenesis in purified mouse Leydig cells. *J. Steroid Biochem.* **25** (1986) 683–687.
13. Lin T., Murono P. E., Osterman J., Nankin R. H. and Coulson B. P.: Gossypol inhibits testicular steroidogenesis. *Fert. Steril.* **35** (1981) 563–566.
14. Cuéllar A., Escamilla E., Ramírez J. and Chávez E.: Adriamycin as an inhibitor of 11β-hydroxylase activity in adrenal cortex mitochondria. *Archs Biochem. Biophys.* **235** (1984) 538–543.
15. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193** (1951) 265–275.
16. Cuéllar A., Díaz-Sánchez V. and Altamirano A.: Impaired pregnenolone biosynthesis in adrenal cortex mitochondria by adriamycin. *J. Steroid Biochem.* **28** (1987) 437–440.
17. Martínez F., Eschegoyen S., Briones R. and Cuéllar A.: Cholesterol increase in mitochondria: a new method of cholesterol incorporation. *J. Lipid Res.* **29** (1988) 1005–1011.
18. Estabrook R. W., Mason J. I., Baron J., Lambeth D. and Waterman M.: Drugs, alcohol and sex hormones: a molecular perspective of the receptivity of cytochrome P-450. *Ann. N.Y. Acad. Sci.* **212** (1973) 27–56.
19. Segel H. I.: Mixed-Type Inhibition. In *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems*. Wiley Interscience, New York (1975) p. 182, pp. 182–189.
20. Stone D. and Hechter O.: Studies on ACTH action in perfused bovine adrenals: the state of action of ACTH in corticosteroidogenesis. *Archs Biochem. Biophys.* **51** (1954) 457–469.
21. Harding W. B., Wong H. S. and Nelson H. D.: Carbon monoxide combining substances in rat adrenal. *Biochim. Biophys. Acta* **92** (1964) 415–417.
22. Simpson E. R. and Estabrook R. W.: Mitochondrial malic enzyme: The source of reduced nicotinamide adenine dinucleotide phosphate for steroid hydroxylation in bovine adrenal cortex mitochondria. *Archs Biochem. Biophys.* **129** (1969) 384–395.
23. Kimura T.: Transduction of ACTH signal from plasma membrane to mitochondria in adrenocortical steroidogenesis. Effects of peptide, phospholipids and calcium. *J. Steroid Biochem.* **25** (1986) 711–716.
24. Tang F., Tsang A. Y. F., Lee Chung-po and Wong P. Y. D.: Inhibition of catechol-o-methyltransferase by gossypol: the effect of plasma proteins. *Contraception* **26** (1982) 515–519.
25. Jefcoate C. R., Simpson E. R., Boyd G. S., Brownie A. C. and Orme-Johnson W. H.: The detection of different states of the *P*-450 cytochromes in adrenal mitochondria; changes induced by ACTH. *Ann. N.Y. Acad. Sci.* **212** (1973) 243–261.
26. Stephens T. D., Whaley J. K., Klimkow M. N. and Hoskins D. D.: Kinetic characterization of the inhibition of purified cynomolgus monkey lactate dehydrogenase isozymes by gossypol. *J. Androl.* **7** (1986) 367–377.
27. Reyes J., Allen J., Tanphaichitr N., Bellve A. R. and Benos J. D.: Molecular mechanism of gossypol action on lipid membranes. *J. Biol. Chem.* **259** (1984) 9607–9615.
28. Peyster D. A., Hyslop P. A., Kuhn C. E. and Sauerheber R. D.: Membrane structural/functional perturbations induced by gossypol. *Biochem. Pharmac.* **35** (1986) 3293–3300.