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IN VITRO INHIBITION BY GOSSYPOL OF OXIDOREDUCTASES FROM HUMAN TISSUES

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Abstract—The effect of gossypol, a polyphenolic compound with antifertility action on human males, has been investigated on the following oxidoreductases purified from human tissues: lactate dehydrogenase (EC 1.1.1.27) isozymes 1 or B_4 from heart, 5 or A_4 from liver and X or C_4 from spermatozoa; malate dehydrogenase (EC 1.1.1.37) mitochondrial and "soluble" isozymes from heart and NADP-glutamate dehydrogenase (EC 1.4.1.4) from liver. Gossypol proved to be a powerful inhibitor of the six enzymes studied. For all of them, inhibition was of the competitive type with respect to the coenzyme and non-competitive in relation to substrate. The lowest k_i values were shown for lactate dehydrogenase isozyme 1 or B_4 and for the two isozymes of malate dehydrogenase. Results did not show selectivity of gossypol for the sperm-specific isozyme X or C_4 of lactate dehydrogenase.

In 1978, a group of Chinese investigators [1] reported studies with gossypol [1,1′,6,6′,7,7′-hexahydroxy-3,3′-dimethyl-(2,2′-binaphthalene)-8,8′-dicarboxaldehyde], a compound isolated from cotton seeds. This substance has an antifertility effect on males of several species, including human. Oral administration of the drug to 4000 healthy men (20 mg daily) during periods of longer than 6 months produced oligo- and azoospermia without significant undesirable effects [1]. Hypokalemia has been reported as a low-incidence side-effect of gossypol administration [2].

The potential of the compound as a male contraceptive has aroused the interest of many investigators trying to elucidate its mechanism of action. Some observations [3, 4] have shown its inhibitory activity on enzyme systems related to energy production. Lee *et al.* [5, 6] have reported selective inhibition of isozyme X of lactate dehydrogenase (LDH X). This finding would offer a satisfactory explanation of the effect of gossypol. Isozyme X or C_4 of lactate dehydrogenase is a highly specific enzyme present in spermatozoa and gametogenic cells [7–9]. The enzyme, involved in metabolic processes providing energy for spermatozoal motility and survival [10–12], would certainly be an appropriate target for a specific male antifertility agent.

On the other side, there is some evidence [13] suggesting that inhibition of LDH X may not be a major mode of action of gossypol.

To substantiate this problem, we have studied the effect of gossypol upon several oxidoreductases purified from human tissues. Although LDH X is inhibited significantly by gossypol, data presented in this paper indicate that the antifertility action of the drug cannot be assigned to selective inactivation of the sperm-specific isozyme.

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MATERIALS AND METHODS

Enzymes. Human tissues were obtained, within 6 hr after death, during autopsy of normal individuals who died in accidents.

Mitochondrial and "cytosolic" or "soluble" malate dehydrogenases (MDHs) (EC 1.1.1.37) and isozyme 1 or B_4 of lactate dehydrogenase (EC 1.1.1.27) were purified from heart by means of DEAE cellulose column chromatography. All operations were carried out at 4°. The homogenate was prepared with 5 g of heart tissue in 10 ml of 0.01 M sodium phosphate buffer, pH 6.8. The suspension was centrifuged at 20,000 *g* for 20 min, and the supernatant fraction was poured into an 18 × 1.9 cm DEAE cellulose column previously equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. A stepwise pH and NaCl elution gradient was established by adding successively: (I) 100 ml of 0.01 M sodium phosphate buffer, pH 6.8; (II) 60 ml of 0.01 M phosphate buffer, pH 6.8, 2 mM NaCl; (III) 60 ml of 0.01 M phosphate buffer, pH 6.8, 5 mM NaCl; (IV) 60 ml of 0.01 M phosphate buffer, pH 6.8, 25 mM NaCl; (V) 60 ml of 0.01 M phosphate buffer, pH 6.8, 50 mM NaCl; (VI) 60 ml of 0.01 M phosphate buffer, pH 6.8, 100 mM NaCl; (VII) 60 ml of 0.01 M phosphate buffer, pH 6.6, 150 mM NaCl; and (VIII) 60 ml of 0.01 M phosphate buffer, pH 6.4, 150 mM NaCl. The eluate was collected in 4.5-ml fractions. Mitochondrial malate dehydrogenase was recovered in eluates from fraction I (tubes 6–14); the cytosolic isozyme, in fractions II and III (tubes 25–45); and LDH 1 or B_4, in fraction VII (tubes 94–102).

Isozyme 5 (A_4) of lactate dehydrogenase was purified from 3 g of liver by DEAE cellulose column chromatography, in the same conditions described for MDHs and LDH 1. LDH 5 eluted with the first fraction (tubes 8–13).

Isozyme X (C_4) was purified from total semen. Ejaculates from normal donors obtained by masturbation were pooled, totalling a volume of 60 ml. It was submitted to three 30-sec bursts of sonication

at 100 W in a Faetron (Argentina) sonicator. Microscopic examination of the suspension showed complete disruption of spermatozoa after the treatment. All operations were carried out at 4°. The preparation was centrifuged for 20 min at 20,000 *g*; to the supernatant fraction was added solid ammonium sulfate up to 40% saturation and, after 2 hr, it was centrifuged for 20 min at 20,000 *g*. To the supernatant fraction was then added ammonium sulfate up to 70% saturation; it was left overnight and centrifuged. The sediment was resuspended in a small volume of 0.01 M sodium phosphate buffer, pH 6.8, and dialyzed against 1 litre of the same buffer, changed twice during 24 hr. The preparation was centrifuged again and the supernatant fraction was poured into a DEAE cellulose column (15 × 1.2 cm) previously equilibrated with 0.01 M sodium phosphate buffer, pH 6.8. The elution was performed by adding: (I) 50 ml of 0.01 M phosphate buffer, pH 6.8; (II) 30 ml of the same buffer containing 5 mM NaCl; (III) 30 ml of the buffer with 25 mM NaCl; (IV) 30 ml of buffer with 35 mM NaCl; and (V) 30 ml of buffer with 50 mM NaCl. The eluate was collected in 3-ml fractions. LDH X or C_4 eluted with fractions IV and V (tubes 40–50). Eluates from these tubes were pooled, and ammonium sulfate was added up to 70% saturation. The sediment, collected by centrifugation 12 hr later, was resuspended in 0.1 M sodium phosphate buffer, pH 7.4. Specific activity of the final preparation was 12.5-fold greater than that of the original sonicate.

Specific activities of the final preparations were: 2.3 units/mg protein for LDH 1; 3.4 units/mg for LDH 5; 0.5 units/mg for LDH X; 3.15 units/mg for mitochondrial MDH; and 3.4 units/mg for "soluble" MDH.

Electrophoretic control of the isolated isozymes was performed on acrylamide and starch gels, stained for protein and enzyme activity. Although the preparations were not absolutely pure (they showed traces of protein contaminants), the isozymes did not show contamination with other molecular forms when stained for enzyme activity.

Glutamate dehydrogenase (EC 1.4.1.4) was purified from liver as indicated by Corman and Inamdar [14]. Specific activity of the final preparation was 10.5 units/mg protein. The enzyme was pure according to electrophoretic criteria.

Assays. Lactate dehydrogenase activity was determined with the method described by Blanco *et al.* [9]. Malate dehydrogenase was assayed by using the technique of Kitto [15], and glutamate dehydrogenase with the procedure of Fahien and Cohen [16]. Assays were carried out at 37°. One unit of enzyme is the amount producing oxidation of 1 μmole of NADH or NADPH per min in the conditions of assay.

K_i *value determination.* Gossypol was diluted immediately before use in 0.04% Na_2CO_3, at room temperature, in tubes protected from light (black paper wrapping). In the following operations, all tubes containing gossypol were equally protected.

The buffer used for the assay, the coenzyme (NADH or NADPH), and gossypol were mixed by shaking in a vortex for 20 sec. Then, the enzyme preparation was added, and the mixture was incubated for 10 min at room temperature (20–23°). The reaction was started by substrate addition. Coenzyme, substrate and gossypol concentrations are indicated in the Results. K_m and V values were obtained by the graphical method of double reciprocals of initial velocity and substrate or coenzyme concentration, by assaying with or without gossypol added to the reagent mixture. K_i values were calculated from those of K_m and V.

Chemicals. NADH, disodium salt, grade III, approximately 98%; NADPH, tetrasodium salt, type I, 95–97%; pyruvate, sodium salt, type II, 99%; oxaloacetic acid, grade I, 90–95%; and α-ketoglutarate, monosodium salt, crystalline, were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals used were analytical grade, of the best quality commercially available. Gossypol-acetic acid was supplied by the U.S. Department of Agriculture, Southern Region, New Orleans, LA, U.S.A., and was also purchased from Sigma. Gossypol from both sources was 100% pure, as determined by absorbance in cyclohexane solutions at 358 nm. Results obtained with gossypol from either of the above-mentioned sources were identical.

RESULTS

Effect of gossypol on enzymic activity at various coenzyme concentrations. Initial velocities were determined in the presence and absence of gossypol. Concentrations of the inhibitor are indicated in Table 1. Final coenzyme (NADH) concentrations used were 0.01, 0.015, 0.02, 0.03 and 0.04 mM for LDH 1 (B_4), LDH 5 (A_4) and LDH X (C_4). Pyruvate concentration was 0.2 mM in all lactate dehydrogenase assays. For mitochondrial and cytosolic malate dehydrogenases, NADH concentrations were 0.01, 0.015, 0.02, 0.03, 0.04 and 0.06 mM. Oxaloacetate at 0.1 mM was used in all cases. For glutamate dehydrogenase, the following concentrations of NADPH were utilized: 0.008, 0.01, 0.012, 0.015 and 0.02 mM; the final concentration of α-ketoglutarate was 8 mM. Curves obtained were hyperbolic for all cases.

For the six enzymes, double-reciprocal plots showed that the inhibition was of the competitive type. K_m, V and K_i values are presented in Table 1.

Effect of gossypol on enzymic activity at various substrate concentrations. For LDH 1 (B_4) and LDH X (C_4), final concentrations of pyruvate used were 0.02, 0.03, 0.05, 0.10 and 0.15 mM and for LDH 5 (A_4), 0.035, 0.05, 0.075, 0.10 and 0.20 mM. For both malate dehydrogenases, oxaloacetate at 0.01, 0.02, 0.03, 0.04 and 0.06 mM was used. For glutamate dehydrogenase, α-ketoglutarate at 0.05, 0.10, 0.20, 0.30 and 0.40 mM was utilized. Coenzyme concentration (NADH for LDHs and MDHs, and NADPH for glutamate dehydrogenase) was 0.114 mM in all cases. Hyperbolic curves were obtained with the six enzymes. Lineweaver–Burk representation of results for the six enzymes showed that inhibition was of the non-competitive type with respect to substrate. K_m, and V and K_i values are shown in Table 1.

The same experiments were performed by changing the sequence of the addition of the reagents.

Table 1. Effect of gossypol on oxidoreductases from human tissues

Enzyme	Variable Substrate	Gossypol (μM)	K_m (mM)	V	K_i (μM)	Type of inhibition
LDH 1 (B_4)	NADH	0	0.10	200		
	NADH	5.0	0.16	200	3.0	Competitive
	Pyruvate	0	0.27	285		
	Pyruvate	3.75	0.27	149	4.1	Non-competitive
LDH 5 (A_4)	NADH	0	0.04	154		
	NADH	15.0	0.07	154	22.0	Competitive
	Pyruvate	0	0.35	162		
	Pyruvate	15.0	0.35	118	40.0	Non-competitive
LDH X (C_4)	NADH	0	0.018	105		
	NADH	7.0	0.043	105	9.7	Competitive
	Pyruvate	0	0.12	65		
	Pyruvate	7.0	0.12	39.7	11.0	Non-competitive
MDH (mitoch.)	NADH	0	0.055	90.9		
	NADH	3.0	0.11	90.9	3.0	Competitive
	Oxaloacetate	0	0.08	294		
	Oxaloacetate	1.5	0.08	191	2.8	Non-competitive
MDH (cytosol.)	NADH	0	0.022	148		
	NADH	5.0	0.048	148	4.2	Competitive
	Oxaloacetate	0	0.06	185		
	Oxaloacetate	3.0	0.06	138	8.8	Non-competitive
GlutDH	NADHP	0	0.08	182		
	NADPH	5.0	0.11	182	13.0	Competitive
	α-Ketoglutarate	0	0.25	210		
	α-Ketoglutarate	2.5	0.25	118	3.3	Non-competitive

Substrate, gossypol and the enzyme were mixed first, incubated for 10 min, and then the reaction was started by adding the coenzyme; or gossypol and the enzyme were incubated and coenzyme plus substrate added after 10 min. In both cases, the inhibition was generally higher than that observed with the same substrate and coenzyme concentrations when the reagents were added in the order indicated in Materials and Methods. The curves obtained in those experiments were not hyperbolic but fluctuated erratically. It was impossible to calculate reliable K_i values from those results.

DISCUSSION

Results of studies with partially purified human enzymes demonstrate that gossypol is a powerful inhibitor of the NAD-linked lactate and malate dehydrogenase isozymes and of the NADP-glutamate dehydrogenase. The inhibition was competitive with respect to the coenzyme and non-competitive in relation with substrate. The K_i values show that, *in vitro*, the most sensitive enzymes are LDH 1 or B_4 and the two isozymes (mitochondrial and cytosolic) of malate dehydrogenase.

These observations do not agree with those reported by Lee and Malling [5] and Lee *et al.* [6], who proposed that the antifertility effect of gossypol would be explained by selective and irreversible inhibition of LDH X or C_4, the sperm-specific isozyme of lactate dehydrogenase.

Perhaps the discordance can be explained by differences in the performance of assays. We have observed that the order of addition of reagents is critical when studying inhibition by gossypol. Lee *et al.* [6] performed the assay in two different ways: (a) by mixing the buffer, coenzyme, gossypol and substrate first and starting the reaction by addition of the enzyme, and (b) by incubating the enzyme with buffer and gossypol and starting the reaction by adding substrate and coenzyme. In the first case, there is no contact between the enzyme and gossypol prior to initiation of the reaction. In the second, the enzyme is in contact with gossypol in the absence of coenzyme. In our conditions, the enzyme was exposed to the coenzyme and gossypol simultaneously for 10 min before the reaction was started by addition of substrate. When reagents were mixed in a different order, or when time for enzyme–coenzyme–inhibitor interactions was not allowed, results were quite erratic.

The observation of a higher inhibition when the enzymes were incubated with gossypol prior to addition of NADH or NADPH indicates that the coenzyme has some protective effect on the enzyme and suggests that the action of the drug may not be explained by coenzyme alteration.

As indicated, our data do not support the claimed selectivity of gossypol for LDH X. Instead, a rather general effect on NAD- and NADP-linked oxidoreductases was demonstrated, the most affected being some enzymes that are not sperm-specific. These observations, plus reports from different laboratories showing uncoupling of oxidative phosphorylation [2, 3, 17, 18], would support the assumption that gossypol acts by impairing metabolic processes related to energy production in cells. The question arises, then, as to whether this action may

explain the antifertility effect of the drug on males.

Present data do not answer this question. The ability of gossypol to interact with many proteins [19, 20] and to inhibit a variety of enzymes besides those involved in energy production [2] must be taken into account when trying to explain its mechanism of action.

Available evidence indicates that the antifertility effect of gossypol cannot be attributed to a single action on a very selective target. Its final effect might be the result of a multiple-site attack.

In any case, the very low K_i values demonstrated for lactate, malate and glutamate dehydrogenases suggest that inhibition of these enzymes may play a significant role in that action. On the other hand, since most of the affected enzymes are not unique to spermatozoa or spermatogenic cells, but are ubiquitous, further studies on possible toxic effects of gossypol are needed before use in humans is undertaken.

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