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GOSSYPOL IS A POTENT INHIBITOR OF THE NAD$^+$-DEPENDENT DEHYDROGENASE RESPONSIBLE FOR THE INACTIVATION OF PROSTAGLANDINS

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ABSTRACT The effects of gossypol as inhibitor of prostaglandin 15-hydroxydehydrogenase (PGDH) were studied using several enzyme sources and a radiochemical technique. Sulphasalazine, an established PGDH inhibitor, was used as reference compound. Gossypol inhibited human placental and bovine lung PGDH dose-dependently with IC_{50} values of 22 and 20 μM, respectively. Inhibition was non-competitive with respect to substrate but competitive for NAD$^+$, and favoured at pH 6.6 compared to pH 9.6. *In vivo* this property may affect the gonadal prostaglandin system with implications for reproductive function, but caution is indicated before extrapolating this effect to the known antifertility properties of gossypol in males because the drug inhibits other NAD$^+$-dependent dehydrogenases, notably

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lactate dehydrogenase isozyme X specific to spermatozoa.

KEY WORDS gossypol; prostaglandin 15-hydroxydehydrogenase; salicylazosulfapyridine; NAD; binding sites; [9 β-^{3}H]prostaglandin $F_{2\alpha}$; radiochromatography

Gossypol is a male contraceptive agent which has found wide human application in China. Whilst gossypol may have direct spermicidal actions[1] and cause morphological abnormalities of spermatozoa[2], its action at the biochemical level remains unclear. Gossypol is an effective inhibitor of the lactate dehydrogenase family of enzymes, and especially of lactate dehydrogenase isozyme x[3] found exclusively in gametogenic cells of the mammalian testis[4]. This effect appears to depend upon competitive interaction with the NAD^+-cofactor binding site[5], and gossypol also inhibits other NAD^+-dependent dehydrogenases[6]. The present study was prompted by the fact that prostaglandins, which are found in large amounts in seminal fluid of humans and other species, may have important physiological functions in human reproduction[7] and are also inactivated by NAD^+-dependent dehydrogenases. In this report we show that gossypol is an effective inhibitor of prostaglandin inactivation by type-I NAD^+-dependent prostaglandin dehydrogenase and in crude cytosolic supernatants which contain this enzyme.

METHODS

Four systems capable of the inactivation of prostaglandins were used: purified human placental prostaglandin 15-hydroxydehydrogenase (PGDH), a kind gift from Dr J Jeffery, Aberdeen University; semi-purified bovine lung PGDH (purchased from BDH Ltd, Poole, UK); the 100000 × *g* cytosolic supernatants from homogenised rat colon and from homogenised guinea pig kidney. The supernatants were prepared in 4 volumes phosphate buffer as described previously[8].

Prostaglandin inactivation by these preparations was studied using a radiochemical method[8]: incubations contained 0.05 μCi [9β-^{3}H]prostaglandin $F_{2\alpha}$ (sp act 14 Ci/mmol, Amersham International), unlabelled prostaglandin $F_{2\alpha}$ (10 μg/ml, Sigma London Ltd), NAD^+ at varying concentrations up to 5 m*M*, and phosphate buffer to a final volume of 0.2 ml, and were incubated at 37°C. The reactions were stopped by addition of 0.2 ml ethanol, the samples acidified with 0.2 ml 1 N formic acid and the prostaglandins extracted twice into 1 ml ethyl acetate. After evaporation to dryness under a stream of air, the samples were resuspended in 15 μl aliquots of acetone and the extent of enzymatic inactivation measured by radiochromatography using a solvent of ethyl acetate/acetone/glacial acetic acid (90:10:1) and 70 min development time. The plastic plates were sectioned and the portions subjected to scintillation counting. By comparing counts in substrate and metabolite zones, the extent of inactivation could be calculated. Data values are given as mean ± SD.

RESULTS

Gossypol was a potent inhibitor of

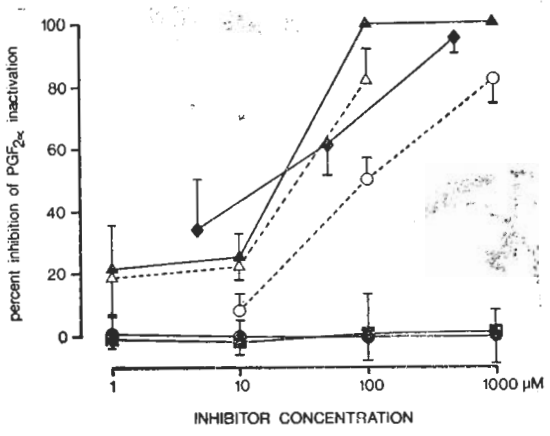

Fig 1. Inhibition of PGDH action by gossypol and sulphasalazine. Solid lines are for gossypol, dashed lines for sulphasalazine. △ ▲ –purified human placental PGDH (60 min); ♦ –bovine lung PGDH (60 min); ▩ –guinea pig kidney 100 000 × *g* supernatant (20 min); ○ ● –rat colon 100 000 × *g* supernatant (15 min). All incubations contained NAD^+ at 5 m*M*. Incubation times indicated in brackets above. n = 4.

both human placental and bovine lung PGDH preparations (Fig 1) with IC_{50} values (concentration to cause 50% inhibition) of 22 μM and 20 μM, respectively. These values are similar to that achieved by sulphasalazine on the human placental PGDH preparation (Fig 1). Sulphasalazine is a drug with well established capacity to inhibit PGDH-dependent prostaglandin inactivation in both subcellular and intact organ systems[8], and in these experiments had an IC_{50} value of 29 μM. However, gossypol at concentrations up to 1 mM had no effect on prostaglandin $F_{2\alpha}$ inactivation in the rat colon and guinea pig kidney cytosolic preparations (Fig 1), whereas as expected sulphasalazine was active, at least in the rat colon cytosolic preparation ($IC_{50} = 80$ μM).

The failure of gossypol to inhibit prostaglandin inactivation in the 2 cytosolic supernatants was surprising. However, these supernatants may contain NAD^+ and it is thought that gossypol interacts competitively with the NAD^+-binding site of other NAD^+-dependent enzymes[5]. Furthermore, NAD^+ was added to the cytosolic incubations at 5 mM final concentration. We therefore tested the effects of gossypol at 50 μM on prostaglandin $F_{2\alpha}$ inactivation by rat colon cytosolic supernatants to which different amounts of NAD^+ had been added. With 5 mM NAD^+, breakdown of prostaglandin $F_{2\alpha}$ was substantial (65.4±9.2% after 20 min incubation) but this was not affected by gossypol. At 1 mM and 0.5 mM NAD^+, breakdown of prostaglandin $F_{2\alpha}$ was reduced to 39.4±0.4% and 29.9±4.8%, respectively, but under these conditions gossypol at 50 μM was an effective inhibitor, reducing inactivation by 40.7±0.6% and 37.3±9.8% respectively. These results suggest that gossypol's capacity to inhibit PGDH may depend upon some interaction with the NAD^+ cofactor.

This was tested in the experiment shown in Fig 2. The double reciprocal plot was obtained after analysing data from an experiment in which the bovine lung PGDH preparation was incubated with 10 μM and 50 μM gossypol,

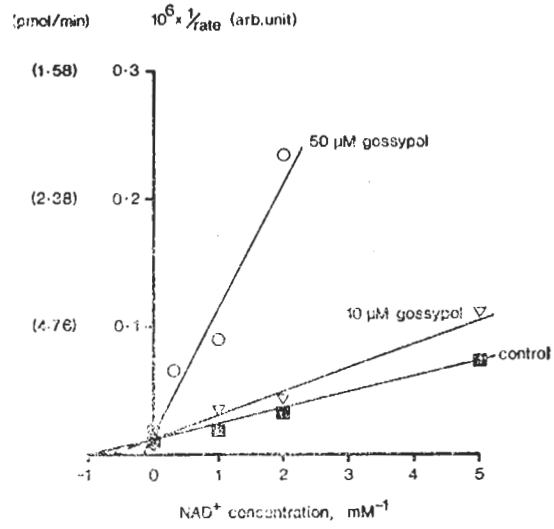

Fig 2. Double reciprocal plot of the inhibitory effect of gossypol on the rate of inactivation by PGDH of radiolabelled $PGF_{2\alpha}$ (10 μg/ml) incubated with semi-purified bovine lung PGDH for 60 min at 37℃ with various concentrations of NAD^+

at a variety of NAD^+ concentrations. The common intercept on the ordinate shows that gossypol acts as a competitive inhibitor for NAD^+; thus the K_m value for NAD^+ without inhibitor is 1.19 mM and this was increased to 1.52 mM at 10 μM gossypol and to 5.89 mM at 50 μM gossypol

In the final series of experiments we investigated the effects of pH on the ability of gossypol to inhibit prostaglandin inactivation. Breakdown of prostaglandin $F_{2\alpha}$ in rat colon cytosolic supernatants containing 2.5 mM NAD^+ increased over the pH range 6.6 (50.0±3.8% inactivation) to pH 9.6 (91.8±4.2% in 70 min) but the inhibitory effects of 10 μM and 50 μM gossypol became less marked as alkalinity was increased, e g, 31.0% inhibition at pH 6.6 and 8.8% at pH 9.6 at 10 μM gossypol, and 49.8% at pH 6.6 reducing to 28.8% at pH 9.6 using 50 μM gossypol. Thus acidic conditions favour the inhibition of PGDH by gossypol.

DISCUSSION

The present experiments show that gossypol

is a potent inhibitor of PGDH derived from both human and animal sources, and has a similar potency to sulphasalazine which was used as a reference compound. However, the mode of action of gossypol differs in that it is competitive for the NAD^+ site, whereas sulphasalazine is non-competitive with regard to both substrate and cofactor[9]. However, both gossypol and sulphasalazine are more effective inhibitors under acidic than basic conditions, although the reasons for this in terms of molecular mechanisms have not yet been established.

The potency of gossypol on the 2 purified enzyme preparations (IC_{50} of 22 μM on human placental PGDH and IC_{50} of 20 μM on bovine lung PGDH) is quite similar in magnitude to the value of 12 μM obtained recently[5] in a study on bovine lactate dehydrogenase isozyme X. Moreover, kinetic analysis in this latter study also revealed that gossypol interacted competitively with the NAD^+ site. Thus in summary, it appears that gossypol exerts a wide range of interactions with NAD^+-dependent dehydrogenase enzymes as proposed by Montamat *et al.*[6] in their study of α-hydroxyacid dehydrogenase, malate dehydrogenase and other dehydrogenases from *Trypanosoma cruzi*. This means that it will be difficult to establish the validity of any claims that interaction of gossypol with any one of these enzymes might explain its antifertility actions.

Another feature that precludes ready acceptance of any single enzyme inhibition hypothesis for the mode of action of gossypol is that the drug has been found to exert numerous other effects. For example, in experiments in this laboratory we have found in both sheep seminal vesicle microsomal membrane and intact colonic fragment systems that gossypol acts both to enhance and to inhibit the formation of prostaglandins, depending upon the dose and experimental conditions. This may be due to the phenolic character of the molecule, as simple phenols have also been shown to exert similar effects on the prostaglandin system. In conclusion, while the present study demonstrates that gossypol is a potent inhibitor of NAD^+-dependent prostaglandin 15-hydroxydehydrogenase and that the compound may be a useful probe for the NAD^+-binding site of this and other dehydrogenase enzymes, a measure of caution is indicated before offering this as a possible mode of action for the therapeutic or side effects of the drug in practice.

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棉酚是个与失活前列腺素有关的 NAD^+ 依赖的脱氢酶强抑制剂

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摘要 用多种来源的酶和放射化学技术研究棉酚作为前列腺素 15-羟基脱氢酶(PGDH)抑制剂的作用。已知 PGDH 抑制剂 sulphasalazine 用作对照。棉酚抑制人胎盘和牛肺的 PGDH, IC_{50} 分别为 22 和 20 μM。其抑

制与底物是非竞争性的，与 NAD^+ 则为竞争性的，而在 pH 6.6 时抑制作用较 pH 9.6 时为强．体内，这种性质可能影响性腺前列腺素系统而干涉生殖功能．但将此效应推论到男人已知棉酚的抗生育作用上需十分谨慎，因为药物亦抑制其他 NAD^+ 依赖的脱氢酶，特别是精子特有的 LDH 同功酶 X．

关键词 棉酚；前列腺素 15-羟基脱氢酶；水杨酰偶氮磺胺吡啶；NAD；结合部位；[9 β-^{3}H] 前列腺素 $F_{2\alpha}$；放射色谱法

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