OXYGEN RADICAL FORMATION INDUCED BY GOSSYPOL
IN RAT LIVER MICROSOMES AND HUMAN SPERM

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Gossypol, a polyphenolic compound found in cotton plants, has many potential uses, including use as a male antifertility drug and spermicide. Gossypol affects a variety of cell processes and many of these effects may be explained by a common underlying mechanism. Here we report that gossypol promotes the formation of oxygen radicals when incubated with rat liver microsomes and human sperm suggesting that oxygen radical production may be the underlying basis of its biological activity.

Gossypol, a polyphenolic compound found in cotton plants, has a variety

of possible pharmacologic uses. It has been clinically tested as a male

antifertility drug (1) and anticancer agent (2,3) and its value in the

treatment of certain viral infections, including herpes (4), and

trypanosomal diseases, such as Chagas' disease (5) is also currently under

investigation. However, gossypol is not devoid of acute and chronic toxic

effects in mammals when taken in sufficient doses (6), and its mode of

action is not clearly understood. Previous investigations have shown that a

variety of cell processes are altered in the presence of even low con-

centrations of gossypol. These processes include mitochondrial respiration

(7,8), glycolytic and Krebs cycle enzyme activity (9), microsomal enzyme

induction (10) and steroidogenesis (11). Certain sperm-specific enzymes

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ABBREVIATIONS

SOD = superoxide dismutase; O_2^- = superoxide anion radical; OH$^•$ = hydroxyl radical; DMPO = 5,5'-dimethyl-1-pyrroline-N-oxide; DMPO-OOH = 5,5'-dimethyl-2-hydroxyperoxypyrrolidino-1-oxyl; DMPO-OH = 5,5'-dimethyl-2-hydroxyl pyrrolidino-1-oxyl; DTPA = diethylenetriamine penta-acetic acid; DMSO = dimethyl sulfoxide.

appear to be particularly sensitive to gossypol's effects (12). It is

likely that gossypol exerts its myriad effects via several different bioche-

mical pathways but a common underlying mechanism could also explain many of

these effects. One such mechanism could be the production of free radicals.

Gossypol has antioxidant properties similar to other aromatic phenols.

It has been used in industry to retard the autooxidation of rubber and oils

(13) and also inhibits lipid peroxidation in mammalian liver microsomes

(14). This behavior suggests that gossypol may participate in free radical

chain reactions. Moreover, the fertilizing capability of sea urchin sperm

in vitro is destroyed when gossypol is added to the medium; however, when

superoxide dismutase (SOD) or catalase is also present fertility is normal

(15). There are also similarities between gossypol's gross toxic effects in

animals and signs of vitamin E deficiency (16) or a histopathology which is

characteristic of exposure to chemical oxidants (17). We have therefore

evaluated gossypol's ability to promote the generation of free radicals in

the presence of rat liver microsomes and human sperm.

MATERIALS AND METHODS

Reagents: Partially succinylated cytochrome c (Type IV, Sigma) was prepared
as in (18) and stored at 0°C as a lyophilized powder. Gossypol acetic acid
and $NADP^+$ were obtained from Calbiochem-Behring, La Jolla, CA. SOD, cata-
lase, glucose-6-phosphate dehydrogenase, glucose-6-phosphate and DTPA were
from Sigma, St. Louis, MO. 5,5'-dimethyl-1-pyrroline-N-oxide (DMPO) was
from Aldrich, Milwaukee, WI.
Methods: Microsomes were prepared from the saline perfused livers of male
Sprague-Dawley rats (200-250 g) according to Ernster et al. (19). They were
washed, suspended and stored under liquid N_2 in 0.15 M KCl. Microsomal pro-
tein was assayed according to (20) using bovine serum albumin as standard.
Human sperm were collected from the ejaculate of a healthy donor of proven
fertility. They were washed and resuspended in Krebs-Ringer solution, pH
7.4, containing 2 mg/ml glucose. Motility of washed sperm was 80%. The
final concentration used for incubations was 2.4 x 10^6 sperm/ml.
Superoxide (O_2^-) production was measured using partially succinylated
cytochrome c and the wavelength pair 550-557 nm (18). Microsomal incuba-
tions were performed at 25°C in 1 ml cuvettes containing an NADPH generating
system (1mM $NADP^+$, 1 mM glucose-6-phosphate, 1 IU/ml glucose-6-phosphate
dehydrogenase) and 0.2 mg/ml succinylated cytochrome c in 50 mM tris-HCl
buffer, pH 7.4, containing 100 mM KCl. SOD (60 μg/ml) was added to test the
specificity of the reaction and the amount of cytochrome c reduced calcu-
lated using an extinction coefficient of 21.0 mM^{-1} cm^{-1} (18). Incubations
with washed sperm were performed similarly but in the Krebs-Ringer solution
described above and in the absence of NADPH.
The production of active oxygen species (O_2^- and $OH\cdot$) was also deter-
mined by electron spin resonance (ESR) spectroscopy. Spectra were recorded
using a Varian E-3 spectrometer with a 9.5 g Hz microwave source and the

following conditions: field set 3385 G, field scan $\pm$ 50 G, receiver gain 5
x 10^6 G, modulation amplitude 1.25 g, scan time 0.5 $\bar{h}$, time constant 10s and
microwave power 10 Hz. The spintrap DMPO was added to a final concentration
of 100 mM. Samples were placed in 100 μl glass disposable microsampling
pipets (Corning no. 7099-5).

RESULTS AND DISCUSSION

Gossypol promotes the formation of superoxide ($O_2^{\cdot-}$) in the presence of
rat liver microsomes and NADPH (Fig. 1). $O_2^{\cdot-}$ formation was linear with
microsomal protein concentration up to 0.2 mg/ml (Fig. 1A) when 0.5 mM
gossypol was added. At higher protein concentrations linearity was not
found (results not shown). In all further microsomal incubations the pro-
tein concentration used was 0.1 mg/ml. Fig. 1B shows O_2^{-} generation from
liver microsomes incubated with different concentrations of gossypol in the
millimolar range. Thus, although gossypol was able to stimulate O_2^{-} for-
mation in liver microsomes it is relatively ineffective in comparison to
menadione, paraquat and other redox-cycling compounds (21). No significant
cytochrome c reduction took place in the absence of microsomes.
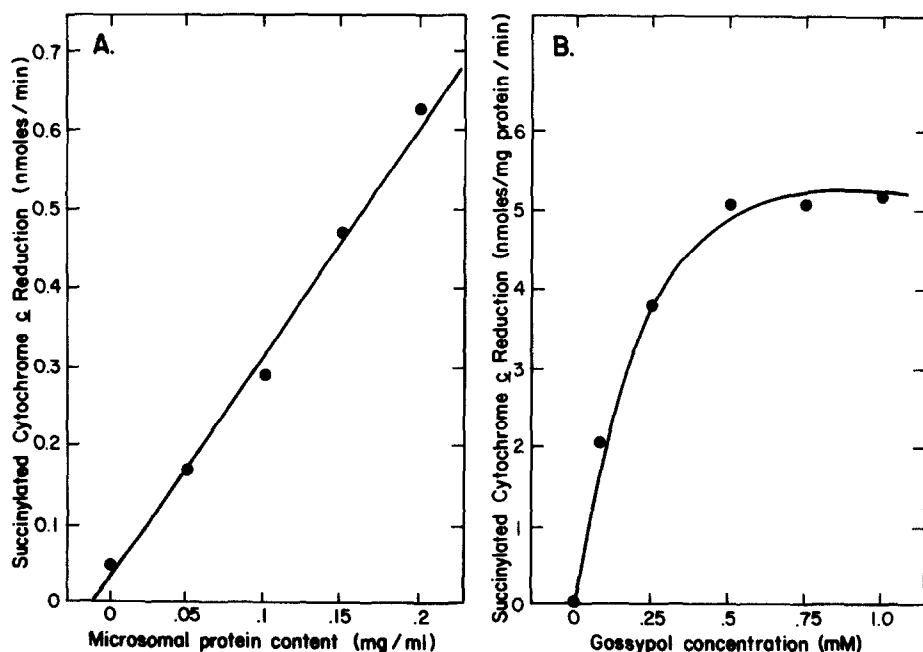

Figure 1.   Gossypol-induced superoxide formation in rat liver microsomes with
            NADPH.
            (A) Dependence on microsomal protein concentration.
            (B) Dependence on gossypol concentration.
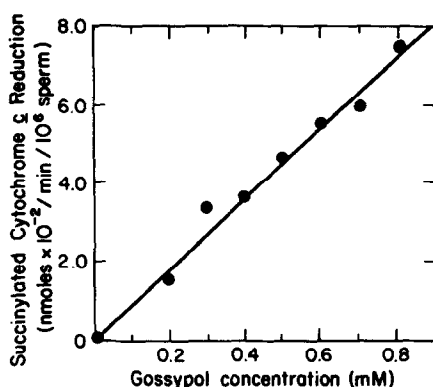   Gossypol was dissolved in DMSO immediately before use.

Figure 2. Gossypol-induced reduction of succinylated cytochrome c in human sperm.

Incubations contained 2.4×10^6 sperm/ml.

Gossypol also stimulated the production of radical species capable of reducing succinylated cytochrome c in sperm cell suspensions (Fig. 2), even in the absence of exogenous NADPH as an electron donor. SOD did not completely quench this activity, while catalase was more effective in doing so than SOD. These observations suggest that (a) O_2^- production may arise via different mechanisms in microsomes and sperm with gossypol, and (b) hydrogen peroxide may be an important mediator in these reactions.

Confirmation of O_2^- production by ESR spectroscopy was not achieved using microsomal incubations containing gossypol and NADPH at pH 7.4. This result is consistent with the relatively low production of O_2^- and the insta-bility of the DMPO-OOH adduct (22). At pH 8.3, however, a distinct ESR signal was observed (Fig. 3). Comparison of critical features from spectra derived from the incubation of microsomes and NADPH with and without gossy-pol (Figs. 3A-B) shows that gossypol markedly increased the quantity of radical species formed by microsomes and NADPH, although the identity of the radicals in each case appeared to be similar. Stimulation of DMPO-adduct formation by gossypol was shown to be concentration-dependent over the range 25-250 µM (not shown). It seems reasonable to conclude that this provides futher evidence of oxygen radical production by gossypol since these signals were short-lived (approximately 45 min) and NADPH and microsomes alone pro-duce both O_2^- and OH·(23). Comparison of these spectra with chemically
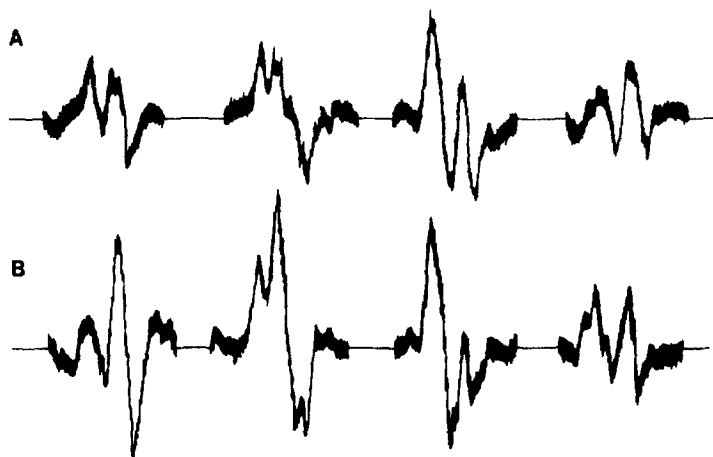
Figure 3. ESR spectra of gossypol-induced free radical formation in rat liver microsomes.

ESR spectra of microsomes and NADPH, in the absence (A) and presence (B) of 100 μM gossypol acetic acid. Gossypol was dissolved in 1N KOH, neutralized with 1N HCl to pH 8.5 and diluted to desired concentrations prior to addition, because DMSO can alter ESR spectra. Spectra shown are from 2-20 min of incubation.

generated DMPO-OOH and DMPO-OH adduct spectra in the literature (22,24) further indicated that most of the complex spectra observed could be attributed to O_2^- and OH·. Incubations of microsomes, NADPH, and gossypol with SOD showed that while SOD did alter the relative contributions of the two adducts, it did not eliminate the ESR signal. Catalase, however, effectively quenched it (not shown). This was interpreted as further evidence that hydrogen peroxide may be an important mediator in gossypol-induced radical formation.

The observation that radical formation appeared to be dependent on pH led us to investigate the possibility that radical generation by gossypol might be related to its tendency to autoxidize at higher pH. In fact, gossypol alone produced a DMPO-adduct signal which could be partially attributed to the DMPO-OH adduct on comparison with published spectra (22,24). The gossypol molecule is not stable for long periods at basic pH (13), but the finding that this degradation resulted in the generation of oxygen radicals was surprising. A more detailed investigation was not attempted, although radicals generated via this mechanism at pH 7.4 were barely detectable by ESR. Thus, although both enzymatic oxidation and autooxidation

could contribute to radical generation by gossypol, it seems likely that enzymatic oxidation would be the more important reaction at physiological pH.

Gossypol may generate oxygen radicals directly during its oxidation or may do so via extensive oxidation of its aldehyde groups to peroxy groups, which are unstable and thus dissociate. One further possibility is that redox-cycling quinone metabolites of gossypol (6) are responsible for the oxygen radical production in the presence of microsomes. These hypothetical mechanisms obviously require further investigation.

In conclusion, biochemical and ESR assays indicate that oxygen radical formation is stimulated in biological systems by gossypol. An important mediator in this event appears to be the production of hydrogen peroxide, which has been shown to exert unusually toxic effects in several of the target cells of gossypol, including sperm (25) and _Trypanasoma cruzi_ (26). Hence further investigations of gossypol's ability to generate oxygen radicals in more complex biological systems are warranted to determine the importance of this process in gossypol's mechanism of action.

ACKNOWLEDGEMENTS

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