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Cyproterone acetate generates DNA adducts in rat liver and in primary rat hepatocyte cultures

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Cyproterone acetate (CPA), an active component of certain contraceptive and antiandrogenic drugs, has been shown recently to induce DNA repair synthesis in rat hepatocytes *in vitro*. In the present study we examined whether CPA can cause the formation of DNA adducts detectable by the ^{32}P-postlabeling technique in hepatic cells *in vitro* and *in vivo*. Incubation of primary cultures of hepatocytes from male Wistar rats with CPA resulted in the occurrence of radioactive spots in the radiochromatograms of ^{32}P-postlabeled DNA digests indicating the formation of two DNA adducts ('A' and 'B'). At 30 μM CPA, the highest concentration tested, ~50 'A' adducts and five 'B' adducts were found per 10^9 nucleotides. DNA of hepatocyte cultures from female rats was found to contain adduct A and a minor adduct termed 'D', but adduct B was not observed. Between 1 and 10 μM CPA, the relative level of adduct A was ~20-fold higher than the level observed in male hepatocytes. *In vivo* DNA adducts were detected almost exclusively in hepatic DNA. Hepatic DNA from male Wistar rats treated with single doses of CPA (1–100 mg/kg) by gavage, showed the major adducts A and B and two further spots of minor intensity ('C' and 'D') in the radiochromatograms. No adducts were detectable in extrahepatic tissues. The adduct pattern of liver DNA from females exposed to single oral doses between 0.1 and 30 mg CPA/kg body wt was similar to that observed in males; however, the relative levels for adducts A and D were ~100-fold higher. In females, linear relationships between dose and adduct levels were observed for all four adducts. The present findings show that CPA causes damage to hepatic DNA not only *in vitro*, but also *in vivo*. Thus it appears possible that DNA adduct formation is involved in the formation of hepatic tumors during long-term treatment of rats with the synthetic steroid.

Introduction

The synthetic steroid cyproterone acetate (CPA*) is widely used in humans as an active component of antiandrogenic, antiacnegenic and contraceptive drugs. When CPA was tested in animals for safety evaluation it was found to increase the liver tumor rate in rats of both genders following long-term feeding with high doses (1). Tumor incidence in females was higher than in males. Subsequent studies suggested that the tumorigenic activity was most likely attributable to a tumor promoting activity of the steroid, i.e. that an epigenetic mechanism was responsible.

*Abbreviations: CPA, cyproterone acetate (6-chloro-17-acetoxy-1,2-methylene-pregna-4,6-diene-3,20-dione, see Figure 1); PEI, polyethyleneimine; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline.

Like many other agents known to promote tumor formation in the liver, CPA was shown to induce hepatic cytochrome P450-dependent activities (2–4) and to stimulate DNA synthesis in the liver and liver growth (3,5). Moreover, it was found that CPA produces a higher proliferative activity in putative preneoplastic γ-glutamyltranspeptidase-positive hepatocytes *in vivo* and *in vitro* than in the unaltered surrounding tissue (6,7). The concept that CPA increases tumor formation in rat liver via epigenetic mechanisms was also supported by the lack of genotoxic activity in frequently used test systems. CPA does not induce gene mutations in *Salmonella typhimurium* (8) and does not increase the number of putative preneoplastic foci in rat liver when tested for tumor initiating activity using the Solt–Farber protocol (9).

Recently, however, we observed that CPA induces DNA repair synthesis in primary cultures of rat hepatocytes, indicating that CPA also has genotoxic activity (6). This finding led us to address the question of whether CPA or its metabolites may form DNA adducts as a possible cause of the DNA damage. Using the ^{32}P-postlabeling technique (10) we analyzed DNA from primary hepatocyte cultures exposed to CPA and from animals treated with single doses of CPA.

Materials and methods

Biochemicals

Spleen phosphodiesterase and proteinase K were purchased from Boehringer, Mannheim; micrococcal nuclease, potato apyrase, grade III and ribonuclease, grade V, from Sigma, Deisenhofen; ribonuclease A from Serva, Heidelberg; T4 polynucleotide kinase from Amersham, Frankfurt; polyethyleneimine (PEI)–cellulose TLC sheets, 0.1 mm, from Macherey-Nagel, Düren and adenosine 5′-triphosphate, tetra-(triethylammonium) salt, [γ^{32}P], sp. act. 3000 Ci/mmol, from NEN-Dupont, Dreieich. CPA, was kindly donated by Schering, Berlin.

Isolation and treatment of rat hepatocytes

Hepatocytes were isolated by collagenase perfusion of the liver as described recently (11). Viability of the cells was routinely determined by staining with trypan blue. Hepatocyte preparations were only used when >80% of the cells excluded the dye. Viable cells (6×10^6) were seeded per 100 mm collagen-coated dish containing 8 ml of a modified Waymouth MB 752/1 medium prepared according to Klose *et al.* (12) with the modification that the concentrations of Na_2SeO_3

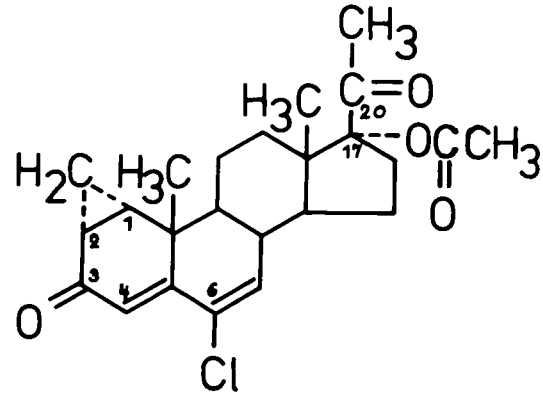

Fig. 1. Structure of cyproterone acetate.

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and $SnCl_2$ were 15 and 0.25 nM respectively. Tocopherol, retinol and trasylol were added at concentrations of 0.53 μM, 0.50 μM and 0.2 U/l. Na_3VO_4 was omitted. Cells were allowed to attach for 1 h in the presence of 5% fetal calf serum. After changing the medium, the hepatocytes were cultured in the absence of serum but in the presence of 0.1 μM dexamethasone and 5 mU insulin.

Appropriate amounts of CPA were dissolved in dimethylsulfoxide (DMSO). Added volumes of DMSO were 16 μl (0.2% of total incubation volume) in all cases including control incubations. After incubation for 6 h, the cells were washed twice with 10 ml ice-cold phosphate-buffered saline (PBS), harvested using a rubber policeman and transferred into tubes used for DNA isolation.

Treatment of animals and isolation of tissues

Seven to 8 week old Wistar rats (inbred strain Neuherberg) were treated orally with CPA dissolved in 300 μl DMSO. Controls were treated with the vehicle only. Twenty-four hours later the animals were killed by cervical dislocation, the organs quickly removed, frozen in liquid nitrogen and stored at −80°C. White blood cells were isolated from heparinized blood by centrifugation at 900 *g* for 15 min (13).

DNA isolation

For the isolation of DNA, hepatocytes from one dish or white blood cells prepared from one animal were suspended in 1 ml of extraction buffer (10 mM Tris−HCl, 100 mM EDTA, 0.5% SDS, pH 8.0). Tissues were homogenized in 20 volumes of the extraction buffer. DNA from tissue homogenates, hepatocytes and white blood cells was isolated by a published procedure (14) using RNases A and T1 and proteinase K treatment followed by phenol/chloroform/isoamyl alcohol extraction. DNA was precipitated at room temperature by addition of absolute ethanol and washed twice with 70% ethanol. After redissolving the DNA in 0.01× SSC buffer, its amount was estimated fluorometrically using bisbenzimide (15). Calf thymus DNA was used as a standard.

^{32}P-Postlabeling

The butanol enrichment procedure described by Gupta (10) was employed with minor modifications. DNA (2.5 μg) was digested to 3′-nucleoside monophosphates with a mixture of micrococcus nuclease (0.6 U) and spleen phosphodiesterase (5 μg). Covalently modified nucleotides were enriched by two steps of extraction with *n*-butanol containing 1 mM tetrabutylammonium bromide. The dried butanol extracts were 5′-endlabeled with 25−40 μCi/sample [γ-^{32}P]ATP, sp. act. 3000 Ci/mmol, in the presence of T4 polynucleotide kinase (2.5 U). After incubation with apyrase to degrade excess ATP, the samples were analyzed by two-directional PEI−cellulose chromatography using the following solvent systems: D1 to remove non-modified nucleotides: 1 M sodium phosphate, pH 6.8; D2 to separate the remaining adducted nucleotides: 3.5 M lithium formate, 7 M urea, pH 3.5; and D3: 0.5 M Tris, 8 M urea, pH 8.0. The chromatographic sheets were finally chromatographed with solvent D1 in direction 1 to reduce the background level of radioactivity. After washing and drying, the sheets were autoradiographed at −80°C for 4 to 96 h, depending on the presumed amount of adduct radioactivity. The radioactive spots on the chromatograms were cut out and the radioactivity was determined by Cerenkov counting. All values were corrected for the background level determined in the neighborhood of the spots. The detection limit was ~1 adduct/10^9 nucleotides.

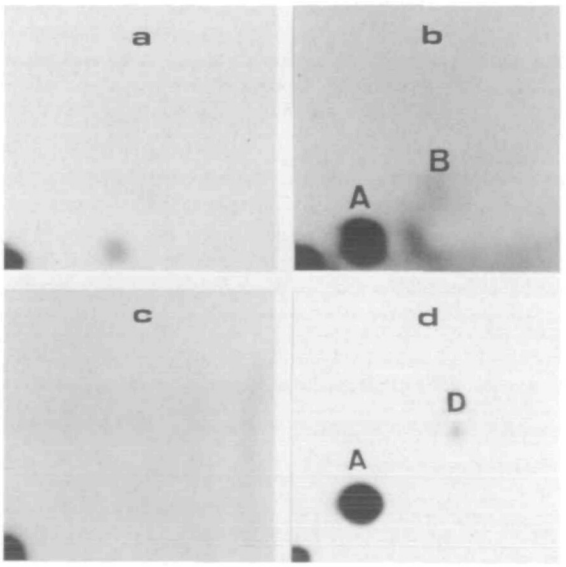

Fig. 2. DNA fingerprints from CPA-treated rat hepatocytes. Hepatocytes were incubated with CPA for 6 h. After isolation and digestion of the DNA, covalently modified nucleotides were extracted from the DNA digests into *n*-butanol, labeled with [γ-^{32}P]ATP, separated by PEI−celluose TLC and quantified. The autoradiograms shown are derived from: hepatocytes from males without CPA (**a**) or with 30 μM CPA (**b**); hepatocytes from females without CPA (**c**) or with 10 μM CPA (**d**). Autoradiograms (a) and (b) were exposed for 48 h, (c) for 24 h and (d) for 4 h. The position of adduct A in map d slightly differed from that in map b. However, subsequent cochromatography experiments confirmed the identity of the adduct in both samples.

Results

Formation of CPA-induced DNA adducts in primary rat hepatocyte cultures

In order to examine whether CPA can cause the formation of DNA adducts *in vitro*, primary hepatocyte cultures from male and female rats were exposed to various concentrations of the steroid for 6 h. Subsequently, the DNA was isolated, digested and the digest extracted with butanol. Radio-TLCs of ^{32}P-labeled butanol extracts, the 'DNA fingerprints', revealed several adduct spots not detectable in control incubations performed in the absence of CPA (Figure 2). One spot termed 'A' was predominant in the fingerprints obtained from DNA of animals of both genders (Figure 2b and d). Two additional spots of minor intensity were observed in males or females respectively: spot 'B' in DNA fingerprints from males and spot 'D' in DNA fingerprints from females (Figure 2b and d).

In both genders the adduct levels increased in a dose-dependent manner up to CPA concentrations of 10 μM. Substantial

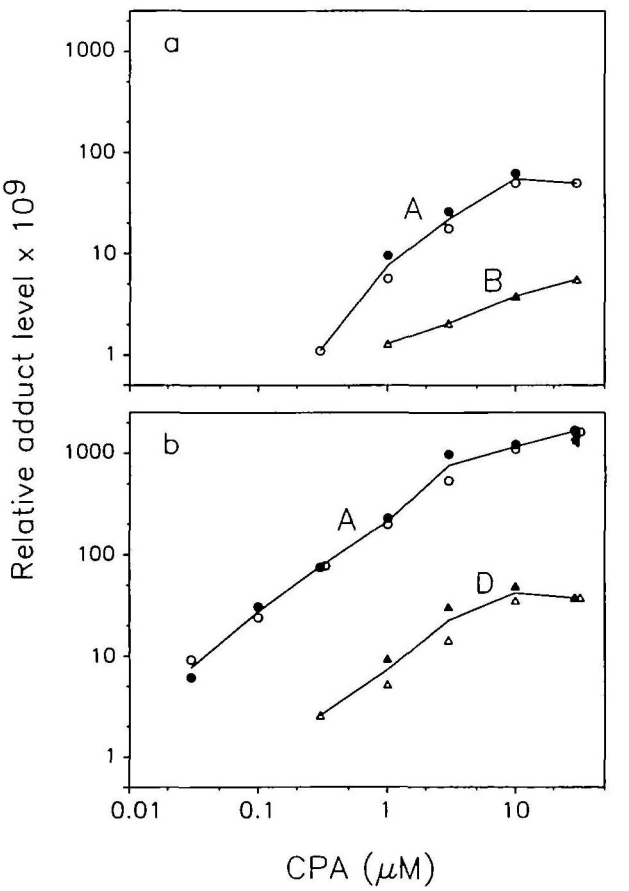

Fig. 3. CPA-dependent formation of DNA adducts in hepatocyte cultures from male (**a**) and female (**b**) rats. Hepatocytes were incubated for 6 h with the CPA concentrations indicated. Data from two independent cell preparations (open and closed symbols) are shown. A = adduct A, B = adduct B, D = adduct D.

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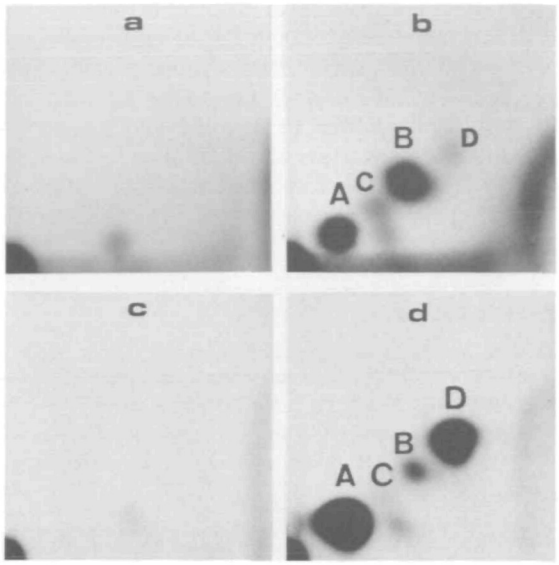

Fig. 4. Adduct fingerprints of hepatic DNA from male and female rats treated with single oral doses of CPA. The animals were killed 24 h after treatment. The figure shows autoradiograms of PEI–cellulose TLC maps of ^{32}P-postlabeled DNA digests. For experimental details see legend of Figure 2. Autoradiograms shown are derived from (a) DMSO-treated males (controls); (b) males treated with 30 mg CPA/kg body wt; (c) DMSO-treated females (controls); (d) females treated with 10 mg CPA/kg body wt. Autoradiograms (a) and (b) were exposed for 72 h, (c) and (d) for 24 h.

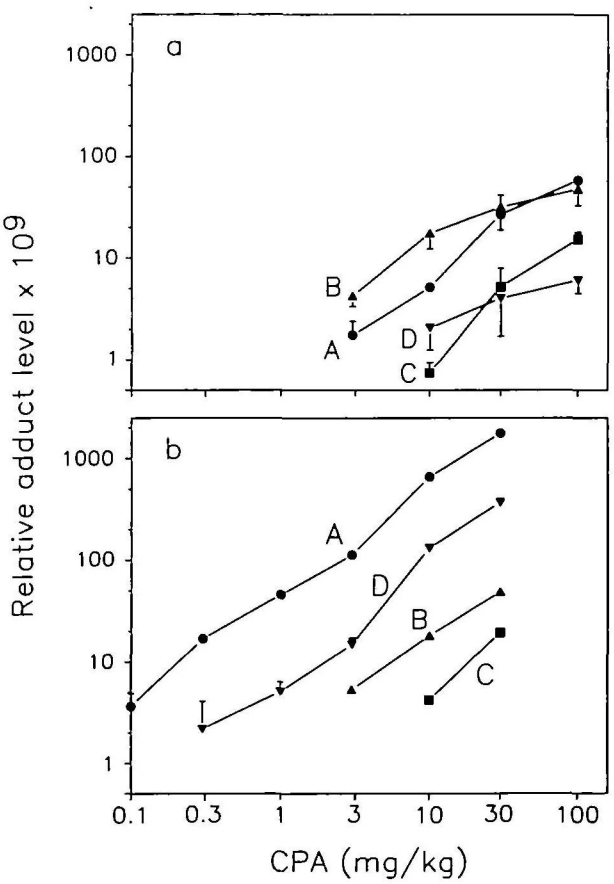

Fig. 5. Formation of CPA-induced DNA adducts in the liver of male (a) and female (b) rats. Groups of three animals received single oral doses of CPA in 300 μl DMSO. Twenty-four hours later DNA was isolated. Each symbol represents the mean value of three animals ± SD. A = adduct A, B = adduct B + adduct B_1, C = adduct C, D = adduct D. When no error bars are given, SD was less than the size of the symbol.

differences were noted between the genders in the quantity of adducts formed: at concentrations of CPA between 1 and 10 μM, the level of adduct A was ~20-fold higher in hepatocytes from females than in hepatocytes from males (Figure 3a and b). At 30 μM CPA, the highest concentration tested, there were ~2000 or 50 adducts/10^9 nucleotides, in DNA from females or males respectively.

Formation of CPA-induced DNA adducts in rat liver

The formation of CPA-induced DNA adducts *in vivo* was examined following treatment of sexually mature Wistar rats with single oral doses of CPA. Liver DNA was isolated 24 h after treatment. The DNA fingerprints from male animals showed the adducts A and B, which are also formed in hepatocyte cultures, as well as two further spots of minor intensity, 'C' and 'D' (Figure 4a and b). In some cases another minor spot was visible closely associated with B ('B_1'). The adduct level specified for adduct B comprises the total of the levels of adducts B + B_1. Adduct levels increased dose-dependently between 3 and 100 mg CPA/kg body wt (Figure 5a). Adduct B was predominant at 10 mg CPA/kg whereas adducts A and B were formed in similar amounts at higher doses. At a dose of 100 mg/kg body wt, adduct A was present at a level of ~60 adducts/10^9 nucleotides.

The hepatic DNA adduct pattern observed in CPA-treated females was similar to that in males. However, DNA adduct formation in females differed qualitatively and quantitatively from that in males. First, adduct D, a minor adduct in males, was a major adduct in females (Figure 4c and d). Second, all DNA adducts in female rat liver, except for adduct B, were formed at much higher amounts than in the liver of males. The level of adducts A and D in females increased with the dose administered over two orders of magnitude (Figure 5b). After a single dose of 30 mg CPA/kg body wt, 1700 adducts/10^9 nucleotides were found for adduct A, followed by adduct D with 350, adduct B with 47 and adduct C with 19 adducts/10^9 nucleotides respectively (Figure 5b). Four A adducts/10^9 nucleotides were still found at a dose of 100 μg CPA/kg body wt. Compared with males, the level of adducts in females was 65-fold higher at a dose of 30 mg CPA/kg and 110-fold higher at a dose of 10 mg CPA/kg. A similar quantitative relationship between females and males was observed when the total of all adduct levels was compared.

To examine whether CPA-induced DNA adducts are also formed in other tissues, male animals were given a single dose of either 10 or 100 mg CPA/kg and DNA from kidney, testes, intestinal mucosa and the lymphocyte fraction was analyzed. No DNA adducts were observed in these tissues at either dose. Females treated with a single dose of 20 mg CPA/kg showed low levels of DNA adduct A in some extrahepatic tissues: seven adducts/10^9 nucleotides in kidney, 61 (one animal) and three (two animals) in uterus and about two in intestinal mucosa, ovary and white blood cells respectively.

Discussion

The results show that the synthetic progestin CPA induces the formation of several DNA adducts in rat liver cells *in vitro* and *in vivo*. Several findings indicate that the spots observed in the autoradiograms of the ^{32}P-labeled digests represent CPA-specific DNA adducts. The radioactivity of the TLC spots quantitatively correlated with the concentration of CPA or the administered dose respectively. None of the spots induced by CPA treatment were observed in DNA fingerprints from untreated animals or from cultured hepatocytes. Moreover, the spots were not detectable

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in DNA fingerprints from incubations of CPA with isolated DNA indicating that metabolism by liver enzymes is required for adduct formation (data not shown).

It seems highly unlikely that the DNA adducts are related to the 'estrogen-induced endogenous DNA adducts' described by Liehr *et al.* (16). These adduct-like compounds were detected in Syrian hamster kidney only after long-term exposure to estrogens administered by kidney implants, whereas the DNA adducts in our experiments were observed in high amounts only 24 h after administration of a single CPA dose. Moreover, the adduct levels observed in the former studies were orders of magnitude lower than the adduct levels observed in the present study. The pattern of CPA-induced DNA adducts we obtained also differs from the estrogen-induced adduct pattern.

In order to explore the significance of the 1,2-cyclopropane group of CPA for genotoxic activity, two structural CPA analogs lacking this moiety, chloromadinone acetate and megestrol acetate, were examined for their ability to form adducts in hepatocytes from female rats. Both compounds yielded much lower adduct levels than did CPA (unpublished results). It is possible, therefore, that the ultimate reactive metabolite originates primarily from a metabolic transformation of the cyclopropane group and that the chlorine atom at the 6-position is of minor importance.

The DNA adduct patterns induced by CPA in cultured hepatocytes differed slightly from those observed *in vivo*. The major DNA adducts found *in vitro* were also formed *in vivo*, i.e. adducts A and B in males and adducts A and D in females. It seems possible that the other minor adducts, B and C, observed *in vivo* were not detected in cultured hepatocytes because of the short incubation time of only 6 h which might not be sufficient for the formation of detectable adduct levels.

The observation that CPA can cause modifications in hepatic DNA corroborates previous results from our laboratory showing that CPA induces DNA repair synthesis in cultured hepatocytes from female rats. Interestingly, we could not detect induction of repair in cultured hepatocytes from male rats (unpublished observation), a finding in line with the present observation of much higher adduct levels in females compared with males. It is tempting to speculate that the sex difference in the genotoxicity of CPA may be due to differences in the capacity of male and female rats to activate CPA. It appears likely that cytochrome P450-dependent mono-oxygenases are involved. Sex-specific expression of distinct P450 isoenzymes in the rat has been reported twice (17,18).

The present results indicating a genotoxic activity of CPA are in apparent contrast to previous findings of others that CPA neither induces gene mutations in *S.typhimurium* with or without metabolic activation (8), nor initiates putative preneoplastic foci in rat liver (9). The inability of CPA to induce mutations in *Salmonella* may be a consequence of the well-documented limitations of exogenous metabolic activation systems, such as the difficulties to detect very short-lived reactive species or to simulate the complex interaction of various enzymes which occurs in the intact liver cell. In this context it is interesting to note that attempts to generate CPA–DNA adducts in a system consisting of liver microsomes, NADPH, DNA and CPA were not successful (unpublished results). The failure of CPA to induce a detectable increase in the frequency of preneoplastic liver cell foci may be due both to the relatively low sensitivity of the Solt–Farber protocol used in these experiments (9) as compared with that of other test protocols (19), and to the fact that the Solt–Farber system employs male rats. In a recent study performed with female rats a foci initiating activity of CPA was clearly shown (Deml *et al.*, unpublished result).

In conclusion, the present results indicate that CPA very probably has not only a tumor promoting but also a tumor initiating potential in rat liver. In view of the higher susceptibility of female rats to the induction of liver tumors by CPA, and the much higher DNA adduct levels in females as compared with males, it is likely that the genotoxicity of CPA plays an essential role in the tumorigenicity of the steroid in rats.

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