Steroidal Drug Cyproterone Acetate Is Activated to DNA-binding Metabolites by Sulfonation

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ABSTRACT

The antiandrogenic and gestagen steroid cyproterone acetate (CPA) has been widely used in human therapy. There is currently a debate about the safety of CPA, since it proved to be genotoxic in rat liver and human hepatocytes [I. Neumann et al., Carcinogenesis (Lond.), 13: 373–378, 1992; J. Topinka et al., Carcinogenesis (Lond.), 14: 423–427, 1993; L. R. Schwarz et al., Biological Reactive Intermediates: V. Basic Mechanistic Research in Toxicology and Human Risk Assessment, pp. 243–251, 1996; A. Martelli et al., Carcinogenesis (Lond.), 16: 1265–1269, 1995].

The results of these in vitro studies were confirmed by in vivo experiments. CPA-DNA adducts were formed in the livers of female rat liver, we have examined whether sulfoconjugation plays an essential role in the activation of CPA to DNA-binding metabolites which are detectable with 32P-postlabeling. Incubation of hepatocyte cultures with 30 μM CPA for 6 h caused the formation of several DNA adducts; the total adduct level amounted to about 12,400 adducts/109 nucleotides.

When the cells were incubated in sulfate-free medium to prevent the synthesis of the coumarate of sulfonation, 3'-phosphohydroenine-5'-phosphosulfate (PAPS), formation of all CPA-DNA adducts was greatly reduced, amounting to only 5% of that determined in the presence of sulfate (810 μM). Activation of CPA is likely to be catalyzed by hydroxysteroid sulfotransferase(s), because the specific substrate dehydroepiandrosterone is almost completely inhibited DNA-binding of CPA. Our assumption that sulfonation plays a decisive role in the bioactivation of CPA is further supported by the results obtained with an in vitro system consisting of calf thymus DNA, various subcellular liver fractions, and the cofactor PAPS, NADPH, or NADH. Significant DNA binding only occurred when cytosol and both PAPS and the reduced pyridine nucleotides were present. The DNA adduct spot obtained was chromatographically identical to the adduct spot A detected in isolated liver cells, suggesting that the CPA-DNA adduct formed in vivo and in vitro is identical. Cytosol is known to contain not only sulfotransferases but also reductases. Thus, the requirement for NADPH or NADH suggests that in addition to sulfotransferase(s), reductases are involved in the activation of CPA.

We propose that bioactivation of CPA involves reduction of the keto group at C-3 followed by sulfonation of the hydroxysteroid. The resulting sulfoconjugate is most likely unstable and supposed to generate a reactive carbonium ion.

INTRODUCTION

The antiandrogen and gestagen CPA2 is used in the treatment of acne, hirsutism, prostate carcinoma, and to inhibit sexual drive in sexual deviants. In particular, Diane and Diane-35, which contain CPA in combination with ethinyl estradiol, have obtained widespread acceptability with other known liver tumor promoters in the rat, such as the induction of growth of the liver and preneoplastic hepatocytes (1, 6–9), a decrease in apoptosis (10), and induction of cytochrome P450 (9), and by the lack of genotoxicity of CPA in standard in vitro mutagenicity tests (11, 12). However, our laboratory has recently presented evidence that CPA has not only tumor-promoting but also genotoxic and tumor-initiating activity. This evidence is based on the following findings: (a) CPA induces DNA repair and the formation of DNA adducts in liver cells of rat and man (1–3, 13, 14); (b) persistent CPA-DNA adducts are formed in the rat (2, 15); and (c) the synthetic steroid initiates the formation of preneoplastic hepatocytes in female rats when tested in the rat liver foci bioassay (16).

The genotoxicity of CPA is characterized by two special features in the rat: it is largely restricted to the liver (2, 3) and it exhibits a marked sex difference, female rats being much more sensitive to the genotoxic action than male rats (2). In view of the fact that CPA did not bind to calf thymus DNA in the absence of subcellular fractions in vitro (2), these findings collectively suggest that CPA is activated to DNA-reactive intermediates by drug-metabolizing enzymes of the liver which are sex specific. However, the pathway of the activation of CPA is unknown. Preliminary experiments indicated that cytochrome P450 does not play a major role in the generation of DNA-binding metabolites from the steroid (3). Another metabolic route which could lead to activation of CPA may involve reduction of the steroid at C-3 (17) and subsequent sulfonation of the 3-hydroxy derivative. The sulfoconjugate will most likely be unstable, giving rise to a reactive carbonium ion. In the present investigation, we therefore studied the possible involvement of sulfoconjugation in the activation of CPA using isolated hepatocytes and subcellular fractions.

MATERIALS AND METHODS

Biochemicals. Spleen phosphodiesterase and proteinase K were purchased from Boehringer Mannheim (Mannheim, Germany); CPA, DHEA, PAPS, micrococcal nuclease, potato apyrase (grade VI), RNase T1 (R 1003) from Sigma (Deisenhofen, Germany); calf thymus DNA and RNase A from Serva (Heidelberg, Germany); T4 polynucleotide kinase from Amersham (Frankfurt, Germany); polyethyleneimine-cellulose TLC sheets, 0.1 mm, from Macherey-Nagel ( Düren, Germany); and (γ-32P)ATP, tetraethylammonium salt (specific activity, 3000 Ci/mmol) from NEN-DuPont ( Dreieich, Germany).

Animals. Female Wistar rats (8–10 weeks old, inbred strain; Neuherberg) were fed a standard diet (Altromin; Lage) and had free access to tap water.

Isolation and Culturing of Hepatocytes. Rat hepatocytes were isolated by collagenase perfusion of the liver (24), except that the perfusion media did not contain sulfate. Viability of the cells was routinely determined by staining with trypan blue. More than 80% of the hepatocytes excluded the dye.

Hepatocytes (7 × 106 viable cells in 7 ml) were seeded into collagen-coated 100-mm dishes. A modified DMEM was used which excluded L-cysteine, L-methionine, and sulfate (MgSO4 × 7H2O was replaced by MgCl2 × 6H2O) and was supplemented with 10 μM HEPES, 10 mM TES, 0.1 μM dexamethasone, 5 milliunits insulin, and, during the short attachment period, with 10 ng/ml epidermal growth factor. Cells were allowed to attach for 1 h at 37°C in a humidified atmosphere of 5% CO2 in air. After changing the medium, incubation was continued in the presence of 30 μM CPA and various concentrations of MgSO4 × 7H2O or DHEA. CPA was dissolved in DMSO. The final concentration of DMSO in the medium was 0.25% (v/v). After incubation for 3 h or 6 h, the cells were washed twice with 10 ml ice-cold PBS, scraped from the plates, and transferred into tubes used for DNA isolation.

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The abbreviations used are: CPA, cyproterone acetate (6-chloro-17a-acetoxyl-1,2a-methylenepregna-4,6-diene-3,20-dione); DHEA, dehydroepiandrosterone; PAPS, 3'-phosphohydroenine-5'-phosphosulfate; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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Bioactivation of CPA in Vitro. CPA (50 μM) was incubated in the the assay buffer with calf thymus DNA (0.2 mg/ml) and facultatively with liver cytosol (4 mg protein/ml), S9-mix (liver homogenate obtained after centrifugation at 9000 × g; 4 mg protein/ml), liver microsomes (4 mg protein/ml), PAPS (0.2 mM), NADH (0.25 mM), and NADPH (0.1 mM) at 37°C for 1 h. The assay buffer consisted of KH₂PO₄ (1.5 mM), Tris (10 mM), KCl (150 mM), MgCl₂ × 6H₂O (2 mM), EDTA (0.2 mM), and 5'-AMP-Na₂ (0.5 mM) and was adjusted to pH 7.0 at 37°C. Glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (2 units/ml) were added as components of a NADP+-regenerating system. To test whether both NADPH and NADH can function as reduction equivalents in the metabolism of CPA, the regenerating system was omitted and replaced by 2 mM NADPH or 2 mM NADH. Cytosol, S9-mix, and microsomes were prepared according to Remmer et al. (19) with the following modifications. The homogenization solution (250 mM sucrose) was supplemented with DTT (0.1 mM) and KH₂PO₄ (10 mM; pH 7.2 at 4°C). Microsomes were washed with KCl (150 mM), DTT (0.1 mM), KH₂PO₄ (10 mM), and EDTA (10 mM). Cytosol was dialyzed three times against 2 liters of homogenization solution for about 6 h at 4°C. The repetitions of the experiments were performed with independent preparations of the subcellular fractions.

Isolation of DNA from Hepatocytes and in Vitro Incubations. Hepatocytes were scraped into 1 ml of extraction buffer consisting of Tris (10 mM), EDTA (0.1 mM), and SDS (0.5%; pH 8.0). After the addition of 10 μl RNase A (10 mg/ml) and 10 μl RNase T₁ (4000 units/ml), the suspension was incubated for 2 h at 37°C. Then the proteins were digested by proteinase K (0.4 mg/ml) at 37°C for 2 h. DNA was extracted with phenol and subsequently with chloroform/isoamylalcohol (24:1, v/v). DNA was precipitated by the addition of 100 μl NaCl (5 mM) and 1 ml of absolute ethanol. The DNA pellet was washed twice with 70% ethanol. Finally, DNA was dissolved in a SSC solution consisting of NaCl (10 mM) and sodium citrate (10 mM; pH 8.0).

In vitro incubations were stopped by the addition of phenol. Extraction of DNA was performed as mentioned above. The precipitates were kept at −20°C until resuspension in 500 μl TESSC buffer (20 mM Tris, pH 8.0). After incubation with 15 μl RNase A (10 mg/ml) and 15 μl RNase T₁ (4000 units/ml) for 30 min, the samples were treated with 0.25 mg/ml proteinase K at 37°C for another hour. Extraction, precipitation, and washing of DNA were performed as described above.

Measurement of DNA Adducts. DNA adducts were determined according to the procedure of Gupta (15), as described recently (15). To compare the adduct spots detected on the autoradiographs performed with DNA of in vitro incubations and cultured hepatocytes, both samples were chromatographed with a modified solvent system as well. In the modified system, 0.6 M ammonia was used in direction D2 instead of 3.5 M lithium formiate and 8.5 M urea (pH 8.0) to achieve a higher resolution in the cochromatography experiments.

All values were corrected for the background level determined on a control chromatogram at the corresponding position. The DNA digest used for these present study than previously (2, 15) and additional minor DNA adducts, i.e., adducts E-H, became visible. Incubating the cells for 6 h with the synthetic steroid attained total DNA adduct levels of about 12,400/10⁶ nucleotides. Similar to previous studies, DNA adduct A was by far predominant and adduct D was the second most frequent; the levels of these two adducts amounted to about 11,000 and 400/10⁶ nucleotides, respectively (Table 1).

To study the possible involvement of sulfoconjugation in the activation of CPA to DNA-binding metabolites, hepatocytes were incubated in sulfate-free medium. Sulfate is required for the biosynthesis of the cofactor of sulfonation, PAPS. The medium used in these experiments was free of inorganic sulfate and the amino acids methionine and cysteine, which may undergo oxidative metabolism to inorganic sulfate (22–24). As shown in Figs. 1b and 2, formation of CPA-DNA adducts in hepatocytes was strongly dependent on the presence of sulfate in the medium. In the absence of sulfate, total DNA binding of CPA amounted to only 5% of the binding determined...
at the normal sulfate concentration of 810 μM. At a concentration of 25 μM sulfate in the medium, almost maximum CPA-DNA adduct formation was attained (Fig. 2, note that the X axis is interrupted). A more detailed analysis of the effect of sulfate on the levels of the individual CPA-DNA adducts shows that formation of all DNA adducts detected in the hepatocyte cultures strongly depends on the presence of sulfate in the medium (Table 1).

Of the various sulfotransferase isoenzymes catalyzing sulfonation of endogenous and foreign compounds, hydroxysteroid sulfotransferase(s) may possibly catalyze sulfation of CPA. We therefore performed cell incubations in the presence of DHEA, DHEA-sulfate, which is most likely formed in hepatocytes, are known to selectively inhibit hydroxysteroid sulfotransferase(s) (25). As shown in Fig. 3, DHEA strongly inhibited the formation of CPA-DNA adducts in hepatocytes. Half-maximal inhibition is already attained at 20 μM, and at a concentration of 100 μM DHEA, almost no CPA-DNA adducts were formed (Fig. 3).

In Vitro Studies. To further substantiate the role of sulfoconjugation in the activation of CPA and to study the involvement of reduction in the formation of reactive metabolites in addition to sulfonation, activation of CPA has been studied in vitro using subcellular fractions of the livers of female rats and cofactors. The in vitro systems contained calf thymus DNA and the following components in various combinations: cytosol or S9-mix as a source of sulfotransferases and reductases, and the cofactors PAPS, NADH, and NADPH (+NADPH-regenerating system). Under the in vitro conditions, only one DNA adduct was detectable (Fig. 4). This adduct was chromatographically identical to adduct A, the predominant DNA adduct determined in hepatocytes (Fig. 5). Similarly, the two adducts showed identical retention times using high-performance liquid chromatography. As shown in Fig. 6, significant DNA binding only occurred when the incubations contained both PAPS and reduced pyridine nucleotides in addition to cytosol or S9-mix. Additional experiments showed that NADPH can be replaced by NADH. In the presence of 2 mM NADPH or 2 mM NADH (and absence of the NADPH-regenerating system), DNA binding amounted to 568 ± 20 and 688 ± 63 (n = 2), respectively. Bioactivation of CPA was prevented when the cytosol was denatured by heating to 95°C for 15 min (data not shown). Similarly, formation of CPA-DNA adducts was insignificant when the cytosol or S-9-mix was replaced by microsomes. In the presence of microsomes, reduced pyridine nucleotides, the NADPH-regenerating system, and PAPS, DNA binding of CPA amounted to only about 0.23% ± 0.07% (n = 3) of that determined in the presence of liver cytosol and the cofactors. The requirement for reduced pyridine nucleotides in the generation of DNA-binding metabolites suggests the involvement of reductase(s), which is present in the cytosol of the liver cell.

**DISCUSSION**

Sulfonation plays a key role in a number of essential biological pathways, including biotransformation of steroidal hormones, xenobiotic detoxification, and carcinogenic activation (compare Ref. 22). Transfer of SO₃⁻ from the sulfonate donor PAPS to endogenous and foreign compounds is catalyzed by various sulfotransferases. During the past four years, several c-DNAs of sulfotransferase have been cloned, such as phenol sulfotransferases, hydroxysteroid sulfotransferases, and estrogen sulfotransferase (26–29). Several sulfotransferases have overlapping substrate specificities. For a long time, sulfonation has been known to play an important role in the activation of several xenobiotics. The first electrophilic metabolite of a carcin-

![Graph](image-url)
ogen to be discovered was the sulfoconjugate 2-acetylaminofluorene-\(N\)-sulfate (30, 31). Since then several carcinogens have been shown to be metabolically activated by sulfonation, e.g., safrol, 4-aminoazobenzene, and numerous benzylic alcohols of polycyclic aromatic hydrocarbons (22, 32–34). The sulfonates of these carcinogens are unstable, and elimination of the sulfate results in an electrophilic nitrenium ion or carbonium ion which can bind to DNA.

In the present investigation, we show that sulfonation is involved in
the bioactivation of the synthetic steroid CPA. This is evident from the marked dependence of CPA-DNA adduct formation in hepatocytes on inorganic sulfate and the requirement for cytosol (sulfotransferases) and PAPS in vitro. Half-maximal formation of CPA-DNA adducts is observed even at the low sulfate concentrations of 15 µM. Sulfonation of CPA is most likely mediated by hydroxysteroid sulfotransferase(s) (29, 35), as indicated by the inhibition of CPA-DNA adduct formation by DHEA, which is a substrate of hydroxysteroid sulfotransferases (36).

Involvement of hydroxysteroid sulfotransferases in the activation of CPA would be in line with our previous findings, which showed that the genotoxicity of CPA was largely restricted to the liver and was much higher in female rats than in male rats (2, 3). Similarly, certain hydroxysteroid sulfotransferases have been shown to be preferentially expressed in the liver and to exhibit much higher activity in the livers of adult female rats as compared to male rats (36, 37). In contrast to hydroxysteroid sulfotransferases, expression of aryl sulfotransferase IV, which bioactivates N-hydroxy-2-acetylaminofluorene, and of estrogen sulfotransferase is much higher in male rat liver (38, 39). The activity of rat hepatic hydroxysteroid sulfotransferase toward androsterone has been shown to exhibit characteristic alterations during postnatal development (37). It increased after birth in both sexes until the weaning stage, thereafter decreasing in males. Compatible with this developmental pattern, we found high CPA-DNA adduct levels in the liver of male rats when 21-day-old animals were treated with CPA (40).

In contrast to hepatocytes of adult female and male rats, similar DNA adduct levels have been determined when human hepatocytes of both sexes were incubated with CPA (14). This finding would also be compatible with the involvement of hydroxysteroid sulfotransferase in the activation of the synthetic steroid, since expression of hepatic DHEA-sulfotransferase has been reported to be similar in women and men (41).

Finally, involvement of liver-specific hydroxysteroid sulfotransferase would also explain the lack of genotoxicity in standard in vitro mutagenicity tests in the presence of S9 and NADPH (12). External metabolic activation in these test systems did not include sulfonation due to the lack of the cofactor PAPS. Even if reactive sulfoconjugates were to be formed externally, mutations may not necessarily be induced in the indicator cells since sulfoconjugates may be short-lived or not be able to cross the membrane of the indicator cells (42).

The present in vitro studies suggest that, in addition to sulfonation, reduction of CPA represents an essential step in the bioactivation of the steroid, since binding to calf thymus DNA only occurred in the presence of cytosol/S9-mix, PAPS, and NADPH/NADH. Thus, we propose that the keto group of CPA at C-3 is first reduced and subsequently sulfated, giving rise to a reactive sulfoconjugate (Fig. 7). This assumption is supported by preliminary data from our laboratory showing that CPA is efficiently metabolized to the 3-hydroxy steroid in the in vitro system used.4 Moreover, Kerdar et al. (17) recently showed that 3α-OH-CPA is a major metabolite of CPA in rat bile and suggested that this metabolite is involved in the bioactivation of the steroid. Up to now several keto reductases/3-hydroxysteroid dehydrogenases have been identified in rat and human liver cytosol and endoplasmic reticulum (43). The present results suggest the involvement of cytosolic reductase(s); it is not yet clear to what extent

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*Fig. 6. Formation of DNA adducts by CPA in vitro, dependence on the presence of cytosol or S9-mix and cofactors for reduction and sulfonation. CPA (30 µM) was incubated with cytosol or S9-mix (4 mg protein/ml) in the presence or absence of NADH (0.25 mM), NADPH (0.1 mM), the NADPH-regenerating system, and PAPS (0.2 mM). Adducts were purified, postlabeled with 32P, and separated as described in "Materials and Methods." Results represent the means of three independent experiments. Bars, SD. Cytosol, 100% = 2308 ± 429 adducts/10⁶ nucleotides; S9-mix, 100% = 864 ± 206 adducts/10⁶ nucleotides.

*Fig. 7. Proposed metabolic pathway of the activation of CPA by sequential reduction and sulfonation of the steroid at C-3. It is suggested that a reactive carbonium ion is formed by elimination of the sulfonate group.

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4 Unpublished results.
reductases of the endoplasmic reticulum may also take part in the bioactivation of CPA.

In the *in vitro* experiments, only one DNA adduct was detectable, which is likely to represent *adduct A*, the most predominant adduct in hepatocytes. The reason for the lack of the other adducts determined in the intact cell system is not clear. One may speculate that the combined action of reductase, sulfotransferase, and additional drug-metabolizing enzymes such as cytochrome P450-dependent monoxygenases may give rise to the *adducts B-H* which are detectable in hepatocytes. In the presence of PAPS, NADPH, and S9-mix (cytosol + endoplasmic reticulum), however, these adducts were not detectable.

It is interesting to note that the proposed pathway of activation of CPA has clear analogies to one of the pathways leading to the activation of the antiestrogen tamoxifen and the synthetic stilbene estrogen diethylstilbestrol. Recently, it has been reported that hydroxylation at the aliphic α-carbon of the ethyl side chain of these molecules is most likely followed by sulfonation to ultimate DNA-damaging metabolites (44, 45).

The present study indicates that sulfonation by hydroxysteroid sulfotransferase(s) plays a major role in the bioactivation of CPA. We put forward the hypothesis that CPA is reduced to the 3-hydroxy derivative prior to the formation of a reactive sulfoconjugate (Fig. 7). Following elimination of the sulfate anion, an electrophilic carbonium ion will be formed. This reaction will be facilitated, since the conjugated double bonds delocalize the positive charge, thereby stabilizing the carbonium ion. At present it is not clear whether sulfate elimination proceeds via a SN1 or a SN2 mechanism.

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