ON THE PARTICIPATION OF CYTOCHROME P-450 IN THE FORMATION OF 16, 17-DIHYDROXYLATED C_{19} STEROIDS FROM 16-DEHYDRO-C_{19} STEROIDS

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1. Introduction

Liver microsomal preparations from rats were recently shown to convert various neutral 16-dehydro-C_{19} steroids into 16, 17-dihydroxylated metabolites [1]. This reaction probably proceeds via the intermediary formation of a 16, 17-epoxide. The present study was carried out in order to determine whether cytochrome P-450 is involved in the microsomal oxygenation of the Δ^{16} double bond.

2. Materials and methods

4, 16-Androstadien-3-one was supplied by Dr. B.W.L. Brooksbank (West Park Hospital, Surrey, England) and 16α, 17α-epoxy-5α-androstan-3α-ol was donated by Dr. C.L. Hewitt (Organon Laboratories, Newhouse, Scotland). 4, 16-[7α-^{3}H]-Androstadien-3-one (specific activity 12.5 Ci/mole) was given by Dr. D.B. Gower (Guy's Hospital Medical School, London, S.E. 1, England).

Liver microsomes were prepared from male rats of the Sprague-Dawley strain as described previously [1]. 50–100 μg unlabelled 4, 16-androstadien-3-one and 50,000 cpm of 4, 16-[7α-^{3}H]-androstadien-3-one were dissolved in 50 μl of acetone and added to varying amounts of microsomal suspension together with 0.03 μmoles of $MnCl_2$, 3 μmoles NADP, 12.5 μmoles of isocitrate and 10 μl of isocitric dehydrogenase solution [2] in a total volume of 4 ml of Bucher medium [3] and incubated for 10 min at 37° (standard conditions). The incubations were terminated by the addition of 20 vol of chloroform–methanol, 2:1 (v/v). The precipitate was filtered off and 0.2 vol of a 0.9% (w/v) aqueous sodium chloride solution were added. The chloroform phase was reduced under vacuum and the residue was chromatographed on a 10 g column of Sephadex LH-20 prepared in and eluted with chloroform–heptane–ethanol, 5:5:1 (by vol). Two fractions were collected: fraction A (0–50 ml of effluent) and fraction B (100 ml of methanol). One tenth of each fraction was measured for radioactivity in a Packard liquid scintillation counter (Model 4322) using Insta-gel®. Aliquots of fractions A and B were also taken for preparation of trimethylsilyl (silyl) ethers which were then analysed by gas chromatography–mass spectrometry. Spectra were recorded on magnetic tape using the incremental mode of operation and were then treated in an IBM 1800 computer [4].

In a separate experiment four rats were given intraperitoneal injections of phenobarbital (80 mg/kg) for three days. Four control rats were injected with saline. The rats were killed and liver microsomal fractions were prepared as described above. Microsomal fractions from two rats in the same experimental group were pooled prior to incubation. 100 μg of 4, 16-androstadien-3-one was incubated for 10 min with 0, 0.2, 0.5 and 1.0 ml of microsomal fraction. The incubations were stopped and the extracts were further processed as described above.

Liver microsomes for spectral measurements were isolated according to Ernster et al. [5]. The microsomal pellets were suspended in 0.15 M Tris-Cl buffer (pH 8.0) and recentrifuged at 105,000 *g* for 40 min. The pellets were resuspended in a 0.25 M sucrose so-

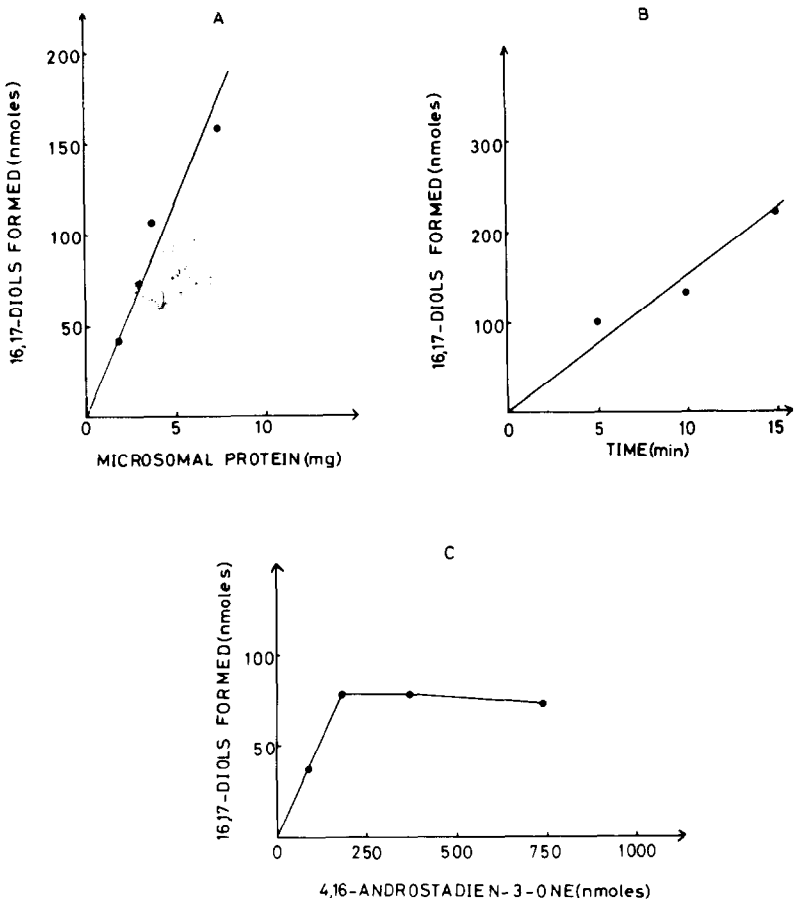

Fig. 1. Effect of enzyme concentration, time and substrate concentration on hepatic microsomal formation of 16β, 17α-dihydroxylated C_{19} steroids from 4, 16-[7α-^{3}H]androstadien-3-one. A) Effect of enzyme concentration; B) effect of time; C) effect of substrate concentration. Standard conditions were used (cf. Materials and methods).

lution. Protein was measured according to Lowry et al. [6] and cytochrome P-450 according to Schenkman et al. [7].

The spectral changes produced by the addition of the various compounds to the microsomal suspensions were recorded with an Aminco-Chance dual-wavelength split-beam scanning spectrophotometer. Each cuvette contained microsomal suspension, 50 mM Tris-Cl (pH 7.5) and 15 mM KCl in a final volume of 3 ml. The substance under study was added to the sample cuvette and an equivalent amount of the solvent was added to the reference cuvette.

In some experiments we studied the influence of carbon monoxide on the formation of 16, 17-dihydroxylated metabolites from 4, 16-androstadien-3-one. During these experiments the incubation medium was bubbled with a gas mixture containing 4% O_2, 40% CO and 56% N_2. Incubations performed with microsomes bubbled with 4% O_2 and 96% N_2 served as controls.

3. Results

Gas chromatography–mass spectrometry analysis of fractions A and B after chromatography of extracts obtained from incubation of 4, 16-androstadien-3-one with liver microsomes from male rats showed that fraction A contained the incubated substrate whereas fraction B contained a mixture of the following ring

D-saturated 16, 17-dihydroxylated steroids (for identification procedure, see [1]): 3α, 17β-dihydroxy-5α-androstan-16-one (minor metabolite), 16β, 17α-dihydroxy-5α-androstan-3-one, 16β, 17α-dihydroxy-4-androstan-3-one (major metabolite), 5α-androstane-3α, 16β, 17α-triol and 5α-androstane-3β, 16β, 17α-triol.

Fig. 1 shows the effect of enzyme concentration, substrate concentration and time on the oxygenation of 4, 16-[7α-^{3}H]androstadien-3-one to 16, 17-dihydroxylated compounds by microsomal fraction in the presence of an NADPH-regenerating system.

Microsomes from rats pretreated with phenobarbital converted 13.7 nmoles of 4, 16-androstadien-3-one/mg protein × 10 min to 16, 17-dihydroxylated metabolites. The corresponding figure for control rats was 8.1 nmoles/mg protein × 10 min. Gas chromatography–mass spectrometry analysis of fraction B from the Sephadex LH-20 chromatography of the incubation extract showed the same qualitative and quantitative composition of 16, 17-dihydroxylated metabolites after incubation with control (cf. above) and phenobarbital microsomes.

Treatment of microsomes with carbon monoxide during the incubation gave 75% inhibition of the conversion of 4, 16-androstadien-3-one to 16, 17-dihydroxylated metabolites.

4, 16-Androstadien-3-one elicited the type I spectral change upon addition to microsomal suspensions. The spectral dissociation constants (K_s-values) [7] ranged from 20 to 54 μM and the maximal optical change from 0.004 to 0.009 O.D./mg protein in microsomes from untreated rats. In microsomes from phenobarbital treated rats the latter parameter ranged from 0.028–0.031 O.D./mg protein.

Since it is plausible that epoxides are intermediates during the formation of 16β, 17α-dihydroxylated C_{19}-steroids from 16-dehydro-C_{19} steroids we performed spectral studies on the microsomal interaction on 16α, 17α-epoxy-5α-androstan-3α-ol (16α, 17α-epoxy-4-androsten-3-one was not available) and 5α-androst-16-en-3α-ol. Both these compounds elicited the type I spectral change (fig. 2) the magnitudes (Δ.O.D. max/mg protein) of which were considerably higher in phenobarbital microsomes than in control microsomes. The K_s-values of 16α, 17α-epoxy-5α-androstan-3α-ol and 5α-androst-16-en-3α-ol were similar (∿ 45 μM).

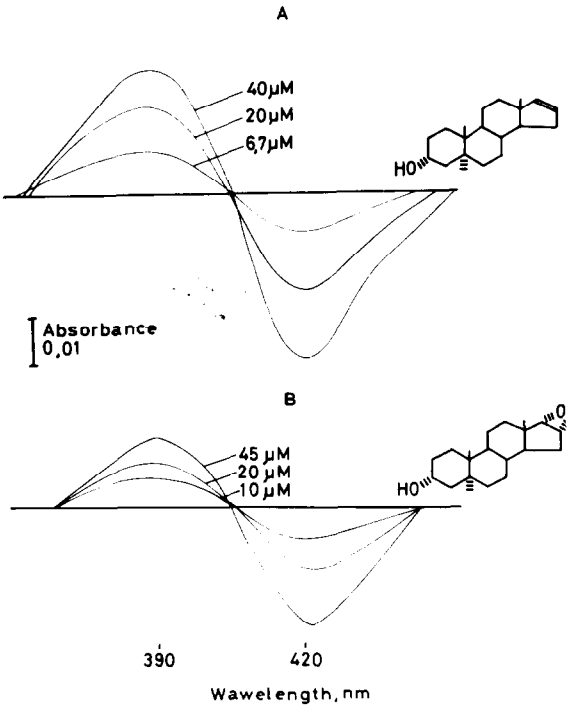

Fig. 2. Difference spectra elicited by 5α-androst-16-en-3α-ol (A) and 16α, 17α-epoxy-5α-androstan-3α-ol (B) to a suspension of rat liver microsomes from rats treated with phenobarbital. The concentration of protein was 1.8 mg/ml and the concentration of cytochrome P-450 2.5 nmole/mg protein. The compounds were added to the sample cuvette to give the final concentrations depicted in the figure.

4. Discussion

4, 16-Androstadien-3-one elicits the type I spectral change in rat liver microsomes. Phenobarbital treatmei of rats increased and carbon monoxide inhibited the microsomal *in vitro* formation of 16β, 17α-dihydroxylated metabolites from this substrate. The maximal mi nitude of spectral change (Δ.O.D. max/mg protein) induced by 4, 16-androstadien-3-one also increased upor phenobarbital treatment. These data indicate that the liver microsomal cytochrome P-450 is involved in the formation of 16, 17-dihydroxylated metabolites from 16-dehydro-C_{19} steroids.

It is plausible that the formation of 16β, 17α-dihydroxylated C_{19} steroids occur via an intermediate epoxide [1]. It is well known that phenobarbital and polycyclic hydrocarbon treatment of rats increases several cytochrome P-450 dependent oxidations (cf.

[8], and it has also been suggested that such treatment stimulates the formation of an epoxide from bromobenzene [9].

Furthermore, the rate of formation of a dihydrodiol from styrene oxide [10] is increased by phenobarbital and polycyclic hydrocarbon treatment. The conversion of an epoxide to a dihydrodiol has been proposed to be catalyzed by one or several hydrases [11], strictly located in the microsomal fraction [10]. The specific activity of this enzyme increases during maturation of rats, which is also true for several cytochrome P-450 dependent reactions [10].

Since the hydrase seems to follow a similar maturation and induction pattern and to have the same subcellular localization as liver microsomal cytochrome P-450 it is possible that the epoxide formation and the consecutive formation of dihydrodiols are catalyzed by two enzymes whose activities are governed by a common regulating system. Another possibility could be that cytochrome P-450 is directly involved in the formation of dihydrodiols from the epoxides.

In an attempt to test the latter hypothesis we performed spectral studies on the interaction of 16α, 17α-epoxy-5α-androstan-3α-ol and 5α-androst-16-en-3α-ol with rat liver microsomes. Both compounds elicited the type I spectral change, the magnitude of which was larger in microsomes from phenobarbital treated rats than in microsomes from control rats. When using microsomes from rats treated with phenobarbital, the K_s-values for the two compounds were similar ($\sim$ 45 μM) indicating that both steroids interact with cytochrome P-450 with similar affinities.

The data suggest that cytochrome P-450 could be involved in the metabolism of epoxides. Since both the Δ^{16} steroid and the corresponding 16α, 17α-epoxide bind to cytochrome P-450 they could conceivably compete with each other's interaction with the cytochrome. If this is true the displacement of an epoxide from cytochrome P-450 by its parent compound might influence the rate of biotransformation of the epoxide. It cannot be excluded that cytochrome P-450 itself catalyzes the enzymatic formation of dihydrodiols from epoxides and thus is identical with the so called hydrase. The dihydrodiol might then possibly be formed during the dissociation of the enzyme–substrate complex.

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