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PREVENTION OF CARBON TETRACHLORIDE-INDUCED LIPID PEROXIDATION IN LIVER MICROSOMES FROM DEHYDROEPIANDROSTERONE-PRETREATED RATS

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Dehydroepiandrosterone (DHEA), a lipid soluble steroid, administered to rats (100 mg/kg b.wt) by a single intraperitoneal injection, increases to twice its normal level in the liver microsomes. Microsomes so enriched become resistant to lipid peroxidation induced by incubation with carbon tetrachloride in the presence of a NADPH-regenerating system: also the lipid peroxidation-dependent inactivation of glucose-6-phosphatase and gamma-glutamyl transpetidase due to the haloalkane are prevented. Noteworthy, the liver microsomal drug-metabolizing enzymes and in particular the catalytic activity of cytochrome P_{450}IIE1, responsible for the CCl_4-activation, are not impaired by the supplementation with the steroid. Consistently, in DHEA-pretreated microsomes the protein covalent binding of the trichloromethyl radical ($CCl_3°$), is similar to that of not supplemented microsomes treated with CCl_4. It thus seems likely that DHEA protects liver microsomes from oxidative damage induced by carbon tetrachloride through its own antioxidant properties rather than inhibiting the metabolism of the toxin.

KEY WORDS: Dehydroepiandrosterone, carbon tetrachloride, lipid peroxidation, covalent binding, antioxidant.

INTRODUCTION

Dehydroepiandrosterone (DHEA) is an human steroid hormone isolated over 50 years ago, whose biological role has been intensively investigated. Epidemiological evidence has recently been obtained that DHEA exerts a role in the prevention of cardiovascular disease,[1,2] DHEA levels are one factor in the prediction of both the development of breast cancer[3] and the progression of HIV infection.[4] Experimental studies suggest an antiproliferative effect,[5] a decrease of fat synthesis[6] and an inhibition of the growth of spontaneous[7] or DMBA - induced[8] rat tumours.

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Aragno *et al.*[9] have recently demonstrated that a single intraperitoneal dose of DHEA, 17 hours before carbon tetrachloride (CCl_4) poisoning, protects rats against CCl_4-induced lipid peroxidation and the consequent necrosis of the liver. The observed protection against CCl_4 injury suggests a new role of DHEA as an antioxidant. We may thus hypothize that the peroxidative decomposition of structural lipids in the endoplasmatic reticulum of liver cells caused by CCl_4 is inhibited by DHEA through a radical-scavenging antioxidant process, rather than a prevention of free radical generation.

In order to explain the antioxidant mechanism of DHEA we have investigated the CCl_4 metabolism and its consequences in microsomal suspensions isolated from normal and DHEA-pretreated rats. Our aim was to point out the mechanisms of the protective action of DHEA on CCl_4 induced toxicity investigating both the behaviour of some targets of CCl_4-induced lipid peroxidation and the covalent binding to microsomal protein of the trichloromethyl-radical generated during the activation of carbon tetrachloride.

MATERIALS AND METHODS

Reagents

Dehydroepiandrosterone; aminopyrine; 7-ethoxycoumarin; 2, amino-2, methyl-propan-1,3diol; L-γ-glutamyl p-nitroanilide; cytochrome C; N-nitrosodimethyl-amine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Glucose-6-phosphate; glucose-6-phosphate dehydrogenase; $NADP^+$; NADH were from Boerhinger Mannheim (Germany). All other chemicals were from Merck (Darmstadt, Germany).

Animals and Experimental Protocol

Male Wistar rats, 180–200 g b.wt, from Nossan (Correzzana, Italy), were used. Animals were maintained on a standard pellet diet (Piccioni, Gessate Milanese, Italy) and water *ad libitum*. Normal rats were sacrificed under ether anesthesia and the liver was immediately excised, weighed in ice and utilized to prepare the microsomal fraction. Microsomes were isolated from 20% liver homogenates in saccarose-Hepes (0.25 M – 10 mM), pH 7.4. The homogenates were centrifuged once at 27,500 g at 4°C for 10 minutes and the supernatant recentrifuged at 105,000 g at 4°C for 50 minutes. Microsomes were stored at −80°C.

DHEA pretreated rats were supplemented i.p. with a single dose of the steroid (10–50–100–200 mg/kg). DHEA was dissolved in 1 vol. of 95% ethanol, followed by the addition of 9 vol. of 16% Tween 80 in 0.9% in NaCl. Seventeen hours later the rats were killed and the liver removed to prepare the microsomal fractions.

Biochemical Analyses

Hepatic microsomes from normal and DHEA-pretreated rats were resuspended in 0.15 M KCl:10 mM TRIS-HCl buffer pH 7.4 (3:2 v/v) to a concentration of 20 mg protein/ml, in order to evaluate oxidative stress. Microsomes (1 mg protein/ml) were incubated for 15 minutes at 37°C in the presence of a NADPH-regenerating system consisting of 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 I.U.

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glucose-6-phosphate dehydrogenase, with or without the addition of 8.6 mM CCl_4.

Lipid peroxidation in microsomal suspensions was measured in terms of MDA production as described by Slater and Sawyer.[10] Covalent binding of [^{14}C]CCl_4 to protein was tested by incubating aerobically normal or DHEA-pretreated microsomes (100 mg/kg b.wt.) with a NADPH-regenerating system at 37°C up to 30 min. The reaction was started by the addition of 8.6 mM [^{14}C]CCl_4, specific activity 61 mCi/mmol, (Amersham International, Amersham, U.K.) diluted in dimethylsulphoxide (DMSO). At the end of the incubation unbound radioactivity was removed by transferring the samples into conical tubes containing 8 ml 5% trichloracetic acid (TCA) in ice. The protein were separated and washed with the following centrifugates: once with 5 ml 5% TCA and four times with 5 ml of a methyl-ethyl ether (3/1 v/v) mixture. The remaining protein pellet was solubilized in 0.50 ml 1N NaOH and aliquots of 50 μl used for protein determination according to Lowry *et al.*[11] The remaining protein solution was added to 5 ml Ready Gel (Liquid Scintillation Cocktail, Beckman Instruments, Fullerton, CA, U.S.A.) and radioactivity counted with a LS 1801 beta scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.)

DHEA concentration was evaluated in microsomal fractions from liver of DHEA-pretreated and normal rats as reported by Boccuzzi *et al.*[12]

Microsomal total protein and cytochrome P_{450} content were determined spectrophotometrically by the method of Lowry *et al.*[11] and Omura and Sato,[13] respectively.

Aminopyrine demethylase and 7-ethoxycoumarin deethylase activities were determined as described by Albano *et al.*[14] N-nitroso dimethylamine demethylase was measured by the method of Yang *et al.*[15] and cytochrome C-reductase and glucose-6-phosphatase activities were determined as described by Slater and Sawyer.[16] Gamma-glutamyl transpeptidase activity was analyzed as described by Orlowsky and Meister.[17]

Statistical Analyses

Student's t test was used to determine the statistical significance of the difference between the experimental groups.

RESULTS

In Figure 1 is reported the effect of increasing doses of DHEA, given i.p. to rats, on liver microsomal lipid peroxidation induced by CCl_4. A good prevention (30–40%) of the MDA increased production due to the haloalkane is observed in microsomes isolated from rats pretreated with either 100 or 200 mg/kg body weight of DHEA. It is interesting to note that basal lipid peroxidation, in the CCl_4 untreated microsomes but enriched with the steroid, is even slightly lower than absolute controls (without DHEA-pretreatment).

In order to study in detail the mechanism responsible for the protection exerted by DHEA on CCl_4-induced lipid peroxidation, further experiments were performed on the microsomal fractions isolated from rats pretreated with a single dose of DHEA (100 mg/kg b.wt). DHEA concentration in liver microsomes of pretreated rats was strongly increased with respect to that present in microsomes of control animals (42 ng/g liver vs 17 ng/g liver). Under this condition, DHEA did not

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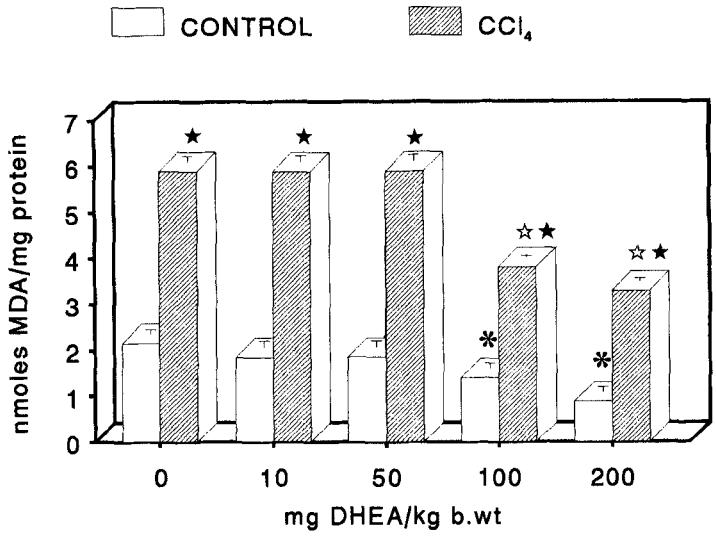

FIGURE 1 *Protective effect of DHEA-pretreatment on lipid peroxidation.* A range of DHEA doses (10–50–100–200 mg/kg) was given to animals by single i.p. injections. DHEA was dissolved in 1 vol. of ETOH plus 9 vol. of 16% Tween 80 in 0.9% NaCl. Seventeen hours later normal and pretreated rats were killed. Microsomes (1 mg protein/ml) were incubated with the NADPH-regenerating system, consisting of 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 I.U. glucose-6-phosphate dehydrogenase, with or without the addition of CCl_4 (8.6 mM), for 15 minutes at 37°C (3 ml final volume). Values are the means ± SE of three experiments in duplicate. Statistical significance: * vs CCl_4-untreated group without DHEA, ★ vs internal CCl_4-untreated group; ☆ vs CCl_4 without DHEA.

interfere with liver cytochrome P_{450} content or NADPH-cytochrome C-reductase activity. However, the supplementation with the steroid does not protect microsomal preparations from CCl_4-induced impairment of both hemo- and flavoproteins (Table 1). In a similar way, the activities of aminopyrine demethylase, of 7-ethoxycoumarin deethylase and of the marker of cytochrome P_{450}IIE1 catalytic

TABLE 1

No effects of DHEA-pretreatment on CCl_4 activation. Cytochrome P_{450} content and NADPH-cytochrome C reductase activity in liver microsomes obtained from normal or DHEA-pretreated (100 mg/kg) rats. Microsomes (1 mg prot/ml) were incubated with the NADPH regenerating system consisting of 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 I.U. glucose-6-phosphate dehydrogenase, with or without addition of CCl_4 (8.6 mM) diluted in DMSO, for 15 minutes at 37°C (3 ml final volume).

Groups	Cytochrome P_{450} (nmol/mg protein)	NADPH-cytochrome C reductase (μmol/min/mg protein)
−DHEA		
Control	0.466 ± 0.030	125.17 ± 6.03
CCl_4	0.225 ± 0.015*	79.25 ± 3.93*
+DHEA		
Control	0.460 ± 0.020	118.27 ± 6.02
CCl_4	0.209 ± 0.010*	74.65 ± 8.26*

Data are means ± SE of three experiments in duplicate. Statistical significance: * vs control ($P < 0.001$).

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action, i.e. dimethyl-nitrosamine demethylase were not modified by DHEA treatment, which on the other hand did not provide any protection against the marked enzymatic changes exerted by the haloalkane (Table 2).

While, inefficient versus the CCl_4-dependent derangement of cytochrome P_{450} related enzyme activities, the enrichment of hepatic DHEA content afforded complete protection against glucose-6-phosphatase and γ-glutamyl-transpetidase (Table 3) inactivation by *in vitro* treatment with the toxic agent.

The covalent binding of CCl_4 metabolites to microsomal protein in the presence or absence of DHEA, at different time (5–15–30 minutes) of incubation, is reported in Table 4. The presence of DHEA did not significantly interfere with the trichloromethyl-radical binding to microsomal protein.

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TABLE 2

No effect of DHEA-pretreatment on impairment induced by CCl_4 on microsomal P_{450}-dependent enzymes. Aminopyrine-demethylase, 7-ethoxycoumarin deethylase and dimethyl-nitrosamine demethylase activities in liver microsomes obtained from normal or DHEA-pretreated (100 mg/kg) rats. Microsomes (1 mg protein/ml) were incubated with the NADPH regenerating system consisting of 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 I.U. glucose-6-phosphate dehydrogenase, with or without CCl_4 (8.6 mM) diluted in DMSO, for 15 minutes at 37°C (3 ml final volume).

Groups	Aminopyrine demethylase (nmol/min/mg prot)	Ethoxycoumarin deethylase (nmol/min/mg prot)	DMN demethylase (nmol/min/mg prot)
−DHEA			
Control	2.43 ± 0.16	0.15 ± 0.02	2.11 ± 0.14
CCl_4	0.53 ± 0.01*	0.03 ± 0.01*	0.94 ± 0.15*
DHEA			
Control	2.53 ± 0.18	0.16 ± 0.02	2.02 ± 0.09
CCl_4	0.48 ± 0.05*	0.03 ± 0.01*	0.79 ± 0.08*

Data are means ± SE of three experiments in duplicate. Statistical significance: *vs control ($P < 0.0001$).

TABLE 3

Protective effect of DHEA-pretreatment on markers of CCl_4-injury. Glucose-6-phosphatase and gamma-glutamyltranspeptidase activities in liver microsomes obtained from normal or DHEA pretreated (100 mg/kg) rats. Microsomes (1 mg protein/ml) were incubated with the NADPH regenerating system consisting of 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 I.U. glucose-6-phosphate dehydrogenase with or without CCl_4(8.6 mM) diluted in DMSO, for 15 minutes at 37°C (3 ml final volume).

Groups	Glucose-6-phosphatase (μmol phosphorus/15 min/mg prot)	γ GT (UI/mg prot)
−DHEA		
Control	2.51 ± 0.26	3.74 ± 0.20
CCl_4	1.36 ± 0.25*	2.81 ± 0.14*
+DHEA		
Control	2.93 ± 0.35	3.78 ± 0.23
CCl_4	2.59 ± 0.51	3.77 ± 0.17

Data are means ± SE of three experiments in duplicate. Statistical significance: *vs control.

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TABLE 4

No effect of DHEA-pretreatment on covalent binding to protein of CCl_4. Covalent binding of [^{14}C]CCl_4 (8.6 mM, specific activity 61 mCi/mmol) to protein in liver microsomes obtained from normal and DHEA-pretreated (100 mg/kg) rats. Microsomes (1 mg protein/ml) were incubated with the NADPH regenerating system consisting of 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 I.U. glucose-6-phosphate dehydrogenase, at 37°C up to 30 min (3 ml final volume).

Time (minutes)	[^{14}C]CCl_4 (nmol/mg protein)	[^{14}C]CCl_4 (nmol/mg protein)
5	1.24 ± 0.23	1.23 ± 0.19
15	1.65 ± 0.25	1.78 ± 0.25
30	2.19 ± 0.38	2.18 ± 0.35

Data are means ± SE of three experiments in duplicate.

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DISCUSSION

It has been clearly established that carbon tetrachloride is activated through the microsomal drug-metabolizing enzymes[18] and that the haloalkylation, i.e. the covalent binding of reactive metabolites to macromolecules, and lipid peroxidation, i.e. the peroxidative degradation of polyunsaturated fatty acids, are the two events mainly responsible for the liver injury produced by CCl_4.[19]

Many physiological and synthetic antioxidants have been found to be effective inhibitors of lipid peroxidation induced by CCl_4, including vitamin E,[20] silybin,[21] propyl gallate[22] and promethazine.[23]

Dehydroepiandrosterone, the main steroid produced by the human adrenal gland, has recently been shown to exhibit, in the whole animal,[9] a strong protection against CCl_4-induced hepatic lipid peroxidation and necrosis.

Reported here are the attempts to characterize the antioxidant activity of DHEA using rat liver microsomal suspensions.

Figure 1 shows that DHEA-pretreatment (100 or 200 mg/kg of boby weight) significantly reduced the absolute extent of lipid peroxidation both in controls and in CCl_4-treated microsomes.

To assess the role of DHEA in protecting against damage induced by CCl_4, we investigated some microsomal mono-oxigenase P_{450}-dependent enzymes. The presence of DHEA did not appear to modify the microsomal P_{450} content nor the NADPH-cytochrome C reductase activity (Table 1). Likewise, the entire function oxidase system (assayed as aminopyrine demethylase and ethoxycoumarin deethylase) appeared not to be influenced by the presence of DHEA. In both systems, with or without DHEA, CCl_4 determined high and consistent inhibition of all these activities.[16,24]

With the aim of excluding any interference of DHEA with CCl_4 metabolism, the cytochrome P_{450}IIE1 was determined in our experimental conditions.

P_{450}IIE1 is an active catalyst for the oxidation of many xenobiotics, including CCl_4.[25] Dimethylnitrosamine (DMN) has been shown to be a specific effective substrate for P_{450}IIE1. DMN demethylase activity was thus taken as an index of the catalytic activity of P_{450}IIE1 on CCl_4 activation (Table 2). DHEA enrichment did not inhibit DMN demethylase activity compared to control microsomes. The presence of carbon tetrachloride in either system, with or without DHEA, provoked a similar decrease in DMN demethylase activity, so indicating that CCl_4 was nor-

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mally metabolized by the P_{450}-dependent monooxigenase system even in the presence of relatively high amounts of DHEA.

It is known that the addition of CCl_4 to microsomes induces a strong decrease of glucose-6-phosphatase and γ-glutamyl-transpeptidase activities.[16,26] In our experimental model the loss of activity of both enzymes, caused by CCl_4, was completely prevented in microsomes enriched with DHEA (Table 3).

Finally, the covalent binding of CCl_4 metabolites to proteins was not modified by the presence of DHEA supplementation, at least up 30 minutes of incubation (Table 4).

Previous works on the mechanisms of protection against CCl_4 by defined antioxidants, showed that both vitamin E and promethazine, inhibit the CCl_4-induced enhancement of lipid peroxidation but are completely inactive in protecting the protein covalent binding of CCl_4.[27,28]

Consistently, DHEA does not prevent the loss of cytochrome P_{450} and of related enzymes (aminopyrine demethylase, NADPH-cytochrome C reductase, DMN-demethylase, ethoxycoumarin- deethylase) which are known to be impaired through haloalkylation rather than lipid peroxidation.[29] On the other hand, DHEA affords full protection against the decrease of glucose-6-phosphatase and gamma-glutamyltranspetidase, targets of lipid peroxidation.[23]

In conclusion, relatively high concentrations of DHEA affords protection against CCl_4-dependent lipid peroxidation triggered in isolated rat liver microsomes. Since the supplementation with the hormone does not show any interference with the microsomal production of CCl_4-radical metabolites, DHEA appears to act as a chain-breaking and not like a preventive antioxidant.

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Accepted by Professor H. Sies

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