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DEHYDROEPIANDROSTERONE PRETREATMENT PROTECTS RATS AGAINST THE PRO-OXIDANT AND NECROGENIC EFFECTS OF CARBON TETRACHLORIDE

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Abstract—A single intraperitoneal injection of dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one, DHEA) 17 hr before carbon tetrachloride (CCl_4) poisoning protects rats against liver injury induced by the haloalkane. In liver homogenates, both the increase in malondialdehyde production and the formation of fluorescent lipid peroxidation products are significantly reduced. Also, liver microsomes obtained from DHEA-pretreated rats incubated *in vitro* with CCl_4 are less susceptible to lipid peroxidation than microsomes from normal animals. The release of liver enzymes into the blood is much reduced in DHEA-pretreated rats, confirming a cause–effect relationship between lipid peroxidation and hepatocyte death. Treatment with DHEA inhibits neither glucose-6-phosphate dehydrogenase activity in the cytosol, nor the microsomal mixed function oxidase system (cytochrome P450 content, aminopyrine demethylase and ethoxycoumarin de-ethylase activities). In animals treated with DHEA, the liver content of total glutathione and vitamin E is not modified. These results support the hypothesis that DHEA protects against CCl_4-induced liver injury through its own antioxidant activity, rather than by interfering with the metabolism of the toxin or with the tissue level of primary antioxidants.

Dehydroepiandrosterone (3β-hydroxy-5-androsten-17-one) (DHEA§) is a highly lipophilic 17-keto-steroid, primarily produced by the adrenal gland and the testes. The physiological activity of DHEA is not yet well defined, apart from its being an intermediate in the synthesis of testosterone and estrogen [1].

A series of pharmacological properties of DHEA have been demonstrated, including: a possible antiobesity effect [2]; inhibition of fat synthesis [3]; preventive action on the development of atherosclerosis [4]; physiological regulation of the immune response [5]; protective action against certain viral infections in animal models [6]; and a modest inhibition of human immunodeficiency virus type 1 infection *in vitro* [7].

In humans, it has been observed that a good correlation exists between pre-diagnostic serum levels of DHEA and its metabolites, and the risk of developing premenopausal breast cancer [8–10]. Lower blood levels of DHEA and DHEA sulphate are associated with the development of breast cancer or death from cardiovascular disease [11–13].

Certain steroid hormones, in particular methylprednisolone, whose chemical structures are similar to DHEA, have been shown to inhibit lipid peroxidation under some experimental conditions [14]. Recently, a novel series of 21-aminosteroids (lazaroids) have been demonstrated to be potent inhibitors of iron-dependent lipid peroxidation, comparable in potency to α-tocopherol [15, 16].

An *in vitro* antioxidant effect of DHEA has been suggested by J. M. Braughler, as reported by J. E. Schauer *et al.* [17].

The work here reported was designed to verify the possible antioxidant effect of DHEA *in vivo*. In all the studies reported in the literature, DHEA was administered to animals in the diet for extended periods of time, in some cases for the animals' entire lifetime. With the purpose of excluding any hormone activity, in this experimental model rats received a single dose of DHEA 17 hr before intoxication with CCl_4, a well-known prooxidant hepatotoxin.

MATERIALS AND METHODS

Materials. DHEA, DL-α-tocopherol, aminopyrine, 7-ethoxycoumarin, 4-androstene-3,17-dione and 17α-hydroxypregnenolone were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, NADH and glutathione were from Boehringer (Mannheim, Germany). All other chemicals were from Merck (Darmstadt, Germany).

Animals. Male Wistar rats, 180–200 g body weight, from Morini (S.Polo d'Enza, R.E., Italy) were used in this study. Animals were maintained on a standard pellet diet (Piccioni, Gessate Milanese, Italy) and water *ad lib.*

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§ Abbreviations: DHEA, dehydroepiandrosterone; MDA, malondialdehyde; GSH, total glutathione; AST, L-aspartate aminotransferase; ALT, L-alanine aminotransferase; SDH, sorbitol dehydrogenase.

Table 1. DHEA content in liver homogenates, microsomes and blood serum from normal and DHEA-pretreated rats, 2 hr after administration of CCl_4 or mineral oil

Groups	Liver homogenate (ng/g liver)	Microsomes (ng/g liver)	Blood serum (ng/100 mL)
−DHEA			
Control	55.28 ± 8.74	17.03 ± 3.02	55.25 ± 4.23
CCl_4	58.90 ± 2.58	19.11 ± 3.41	59.30 ± 13.12
+DHEA			
Control	334.21 ± 111.22*	42.40 ± 2.27*	521.00 ± 50.51*
CCl_4	294.49 ± 56.56*	52.90 ± 5.15*	980.50 ± 124.48*†

Data are expressed as means ± SE of five to six animals per group.
Statistical significance: * vs control, † vs DHEA control ($P < 0.05$).

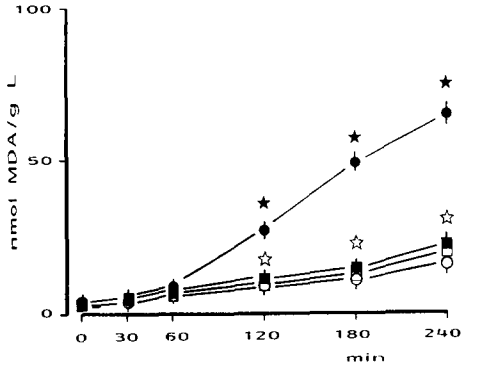

Fig. 1. Susceptibility to lipid peroxidation, evaluated in terms of MDA production, in liver homogenates of normal and DHEA-pretreated rats, 2 hr after CCl_4 poisoning. Control (○), CCl_4 (●), DHEA (□), DHEA + CCl_4 (■). Each point represents the mean of five to seven animals ± SE. Statistical significance: (★) vs control; (☆) vs CCl_4 ($P < 0.05$).

Experimental protocol. DHEA was dissolved in 1 vol. of 95% ethanol, followed by the addition of 9 vol. of 16% Tween 80 in 0.9% NaCl, and administered to the animals i.p. (100 mg/kg). Control rats were similarly injected with the vehicle only. Seventeen hours later the animals were poisoned with CCl_4, 1250 μL/kg, diluted in mineral oil, by gastric intubation. Controls received mineral oil only.

The rats were killed, 2 or 24 hr after poisoning, by cervical dislocation or by bleeding through the abdominal aorta, under light diethyl ether anaesthesia. The liver was immediately excised, weighed and utilized for the following biological analyses. Subcellular fractions were isolated from liver homogenates in 1.15% KCl. The homogenates were centrifuged once at 15,000 *g* at 4° for 18 min, and the supernatant recentrifuged at 105,000 *g* at 4° for 40 min. Microsomes and cytosolic fractions were stored at −80°.

Biochemical analyses. DHEA concentration was measured in the serum, in the 7% (w/v) liver homogenates prepared in 0.015 M Na-phosphate buffer pH 7.4 and in the derived microsomal subfractions. DHEA was determined after solvolysis and liquid chromatography. Samples were extracted with ethyl ether. After evaporation of ethyl ether, the residue was redissolved with 0.3 mL of isooctane–ethyl acetate (94:4) and chromatographed on celite–ethylene glycol (2:1, w/v) microcolumns, using isooctane–benzene (96:4) as the mobile phase for the DHEA fraction [18]. Radioimmunoassay was performed on the DHEA chromatographic fraction (Coat-A-Count DHEA, DPC, Los Angeles, CA, U.S.A.).

Lipid peroxidation was evaluated by measuring the steady-state concentration of malondialdehyde (MDA) and fluorescent chromolipids in liver homogenates (10%, w/v) 2 hr after CCl_4 poisoning. MDA production was estimated as described by Torrielli and Ugazio [19]. For the analysis of fluorescent chromolipids, total lipids were extracted by the method of Folch *et al.* [20]. The fluorescence intensity of the samples was measured at an excitation wavelength of 360 nm and an emission wavelength of 430 nm, with an LS-5 Luminescence Spectrometer [21].

Aliquots of liver homogenates or blood serum were analysed for α-tocopherol content as described by Burton *et al.* [22]. α-Tocopherol was extracted with 1 mL of *n*-heptane and, after brief centrifugation, the heptane phase was collected for HPLC analysis. A Supelcosil-plc-si column (25 cm × 4.6 mm, Supelco Inc., PA, U.S.A.) was used; the mobile phase was hexane:isopropanol (99:1, v/v) and the flow rate was 1.5 mL/min. The fluorescence detector was set to 298 nm excitation and 325 nm emission. The α-tocopherol content was estimated by comparison with a standard solution.

Microsomal total protein and cytochrome P450 content were determined spectrophotometrically by the methods of Lowry *et al.* [23] and Omura and Sato [24], respectively. Aminopyrine demethylase and 7-ethoxycoumarin de-ethylase activities were determined as described by Albano *et al.* [25]. Cytosol total glutathione (GSH) was assayed by Ellman's method [26].

Liver necrosis was evaluated in terms of serum levels of L-aspartate aminotransferase (AST, EC 2.6.1.1), L-alanine aminotransferase (ALT, EC 2.6.1.2) and sorbitol dehydrogenase (SDH, EC

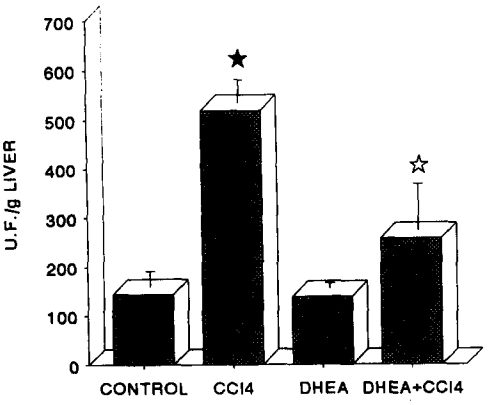

Fig. 2. Fluorescent chromolipid formation (EX/EM 360/430 nm) from liver homogenates of normal and DHEA-pretreated rats, 2 hr after poisoning with CCl_4. Data are expressed as means ± SE of four to six animals per group. Statistical significance: (★) vs control; (☆) vs CCl_4 ($P < 0.01$).

Table 4. Concentration of GSH in the cytosol and content of vitamin E in serum and liver homogenates of normal or DHEA-pretreated rats, 2 hr after administration of CCl_4 or mineral oil

Groups	GSH in cytosol (μmol/g liver)	Vitamin E Homogenate (nmol/g liver)	Vitamin E Serum (nmol/mL)
−DHEA			
Control	4.81 ± 0.53	41.5 ± 7.95	16.36 ± 1.38
CCl_4	4.11 ± 0.42	41.7 ± 1.43	12.96 ± 1.28
+DHEA			
Control	4.25 ± 0.26	38.5 ± 6.50	15.88 ± 1.52
CCl_4	4.48 ± 0.26	41.4 ± 1.78	12.58 ± 1.16

Data are expressed as means ± SE of three to four animals per group.

1.1.1.14) by UV combination kits Boehringer (Mannheim, Germany).

Hepatic microsomes from normal or 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 the oxidative stress. Microsomes (1 mg protein/mL) were incubated for 15 min at 37° in the presence of an NADPH regenerating system consisting of 5 mM glucose-6-phosphate, 0.25 mM $NADP^+$, 10 IU glucose-6-phosphate dehydrogenase with or without the addition of 8.6 mM CCl_4. Lipid peroxidation in terms of MDA production was measured as described by Slater and Sawyer [27].

Statistical analyses. The significance of differences

Table 2. Serum concentration of SDH, ALT and AST in normal or DHEA-pretreated rats, 24 hr after intoxication with CCl_4

Groups	SDH (mU/mL)	ALT (mU/mL)	AST (mU/mL)
−DHEA			
Control	2.9 ± 0.3	26.5 ± 2.4	119.9 ± 9.8
CCl_4	2070.0 ± 35.8*	2028.1 ± 419.0*	6008.7 ± 780.3*
+DHEA			
Control	3.0 ± 0.3	24.4 ± 3.4	131.5 ± 8.6
CCl_4	864.8 ± 65.2*†	580.5 ± 113.3*†	1235.6 ± 158.5*†

Data are expressed as means ± SE of five to six animals per group.
Statistical significance: * vs control, † vs CCl_4 ($P < 0.05$).

Table 3. Cytochrome P450 content, 7-ethoxycoumarin de-ethylase and aminopyrine demethylase activities in microsomes of normal and DHEA-pretreated rats, 2 hr after poisoning with CCl_4

Groups	Cytochrome P450 (nmol/mg protein)	Ethoxycoumarin de-ethylase (nmol/min/mg protein)	Aminopyrine demethylase (nmol HCHO/min/mg protein)
−DHEA			
Control	0.394 ± 0.03	0.132 ± 0.02	2.06 ± 0.10
CCl_4	0.166 ± 0.01*	0.048 ± 0.01*	0.59 ± 0.05*
+DHEA			
Control	0.418 ± 0.01	0.132 ± 0.01	1.81 ± 0.23
CCl_4	0.146 ± 0.01*	0.055 ± 0.01*	0.44 ± 0.10*

Data are expressed as means ± SE of five to seven animals per group.
Statistical significance: * vs control ($P < 0.05$).

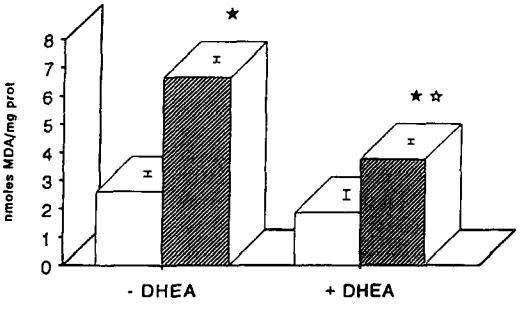

Fig. 3. MDA production by rat liver microsomes from control or DHEA-supplemented animals, tested *in vitro* with a NADPH regenerating system with or without 8.6 mM CCl_4 poisoning. (□) Microsomes incubated with NADPH regenerating system; (▨) microsomes incubated with NADPH regenerating system and with CCl_4. The results are the means of experiments in duplicate from three animals ± SE. Statistical significance: (★) vs control; (☆) vs CCl_4 ($P < 0.05$).

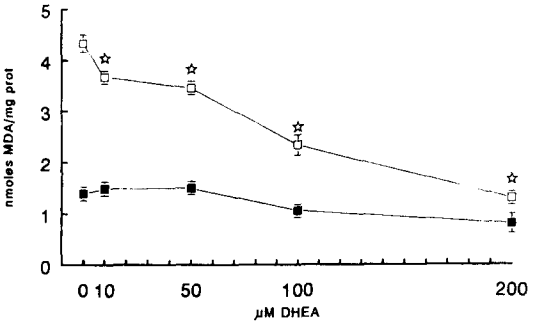

Fig. 4. MDA production in microsomes obtained from normal rats, incubated *in vitro* with a NADPH regenerating system without (■) or with 8.6 mM CCl_4 (□) in the presence of increasing concentrations of DHEA. Each point represents the mean of experiments in duplicate from three animals ± SE. Statistical significance: (☆) vs CCl_4 alone ($P < 0.05$).

between the means of the experimental groups was calculated by Student's *t*-test. The 0.05 level of probability was used as the criterion of significance.

RESULTS

Table 1 shows DHEA concentrations in total homogenates, in liver microsomes and in the blood serum of control animals and of rats injected with a single dose of the steroid, then treated with CCl_4 or mineral oil. The i.p. injection of DHEA produced a strong increase in steroid levels in all compartments, in both controls and poisoned animals. Already 2 hr after CCl_4 poisoning, the haloalkane-induced enhancement of liver MDA production (Fig. 1) and fluorescent chromolipid content (Fig. 2) was completely or strongly inhibited.

Table 2 reports the serum levels of three marker enzymes of liver necrosis 24 hr after intoxication with CCl_4. While DHEA *per se* did not modify any of these parameters, CCl_4 caused a dramatic increase in the release in the blood serum of these enzymes. The severe CCl_4-induced hepatotoxicity was significantly reduced in rats pretreated with DHEA.

Table 3 reports microsomal cytochrome P450 content and 7-ethoxycoumarin de-ethylase and aminopyrine demethylase activities. Treatment with CCl_4 lowered all three parameters. DHEA *per se* did not affect microsome function nor, when given before CCl_4, did it protect against either enzymatic derangment.

The level of two major antioxidants (GSH and vitamin E) was also tested, 19 hr after DHEA injection (Table 4). The liver cytosol concentration of GSH and the serum and hepatic levels of vitamin E were not modified by any of the treatments.

Figure 3 shows MDA production by rat liver microsomes from control or DHEA-supplemented animals, tested *in vitro* with 8.6 mM CCl_4; DHEA pretreatment led to marked protection against lipid peroxidation process.

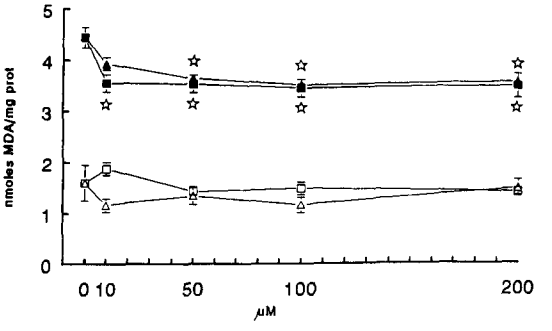

Fig. 5. MDA production by microsomes from normal animals tested *in vitro* with different concentrations of 17α-hydroxypregnenolone or 4-androstene-3,17-dione in the presence of a NADPH regenerating system with or without 8.6 mM CCl_4. (△) 17α-Hydroxypregnenolone; (□) 4-androstene-3,17-dione; (▲) 17α-hydroxypregnenolone + CCl_4; (■) 4-androstene-3,17-dione + CCl_4. Each point represents the mean of experiments in duplicate from three animals ± SE. Statistical significance: (☆) vs CCl_4 alone ($P < 0.05$).

In Fig. 4 is shown MDA production in microsomes obtained from normal animals, incubated *in vitro* with the NADPH regenerating system with or without CCl_4 8.6 mM in the presence of increasing concentrations of DHEA. This ketosteroid is able to reduce CCl_4-induced oxidative damage significantly from 10 μM concentration, showing a dose-dependent effect.

Figure 5 reports MDA production in the same experimental model in the presence of two other steroid compounds with a structure similar to DHEA, but with different functional groups. The results show that 17α-hydroxypregnenolone and 4-androstene-3,17-dione only slightly prevent lipid peroxidation, and in addition their effect is not dose dependent.

DISCUSSION

In spite of numerous investigations, the physiological role of DHEA has not yet been clearly established. DHEA, when administered to rodents as a dietary supplement in chronic or subacute experiments, shows a variety of chemoprotective activities and anti-carcinogenic action [8]. A possible antioxidant activity of DHEA has recently been postulated [17, 28]. Estabrook *et al.* [29] have suggested that, when added to the rodent diet, DHEA acts as a xenobiotic rather than a hormone. No other attempts have to our knowledge been made to clarify the possible antioxidant property of DHEA *in vivo*, using an experimental model designed in such a way as to exclude the hormonal activity of the compound.

DHEA was injected i.p. into rats at a dose equivalent to the vitamin E dose which is effective in preventing acute CCl_4 poisoning [30]; the pretreatment time schedule was also identical, i.e. 17 hr before poisoning with CCl_4. It was therefore crucial to verify whether the administration method used was actually increasing the liver concentration of DHEA. Table 1 shows that the liver and serum concentrations of DHEA are about one order of magnitude higher in DHEA-pretreated rats. Interestingly, CCl_4 poisoning slightly lowers the liver concentration of DHEA, while, on the contrary, it significantly increases the blood level. These events could be due to a CCl_4-dependent moderated derangement of hepatocyte uptake of DHEA from plasma, not detectable with normal plasma levels of DHEA, but only when the animals are supplemented with the compound.

CCl_4 is metabolized, by the cytochrome P450-dependent microsomal enzyme system, to reacting metabolites which trigger off peroxidative events ending in the production of MDA and other aldehydes [31]. The CCl_4-induced stimulation of lipid peroxidation has been demonstrated to play a primary role in liver cell death [30, 32].

In the present experimental model, the prooxidant effect of a single dose of CCl_4 was assessed in liver homogenates and measured in terms of both MDA production (Fig. 1) and fluorescent chromolipid content (Fig. 2). The CCl_4-induced elevation of these two markers of oxidative damage is effectively counteracted by pretreating rats with DHEA. This is consistent with the fact that the severe hepatic necrosis caused by CCl_4 and evaluated in terms of plasma release of marker enzymes (Table 2) is markedly reduced in the animals supplemented with DHEA.

With the aim of investigating whether the compound interferes with CCl_4 metabolism, the effect of DHEA on the microsomal drug metabolizing system was studied. The results obtained (Table 3) exclude any change in the enzymatic pathway caused by the steroid. Furthermore, under certain conditions, DHEA has been demonstrated to inhibit glucose-6-phosphate dehydrogenase activity [33] and thus temporarily deplete the available NADPH cellular pool. This could depress the first step of CCl_4 metabolism. Under our experimental conditions, whether administered alone or prior to CCl_4 poisoning, DHEA did not affect glucose-6-phosphate dehydrogenase activity (unpublished data).

In conclusion, all these data support a direct antioxidant effect of DHEA, more probably in chain-breaking reactions than in the initiation phase of oxidative damage. DHEA seems to depress oxidative reactions directly, rather than through an increase in naturally occurring intracellular antioxidants.

Consistent with this hypothesis is the observation that the levels of GSH and vitamin E remain unchanged during the early phases of CCl_4 poisoning, with or without steroid supplementation (Table 4). As far as CCl_4 poisoning is concerned, these results are in agreeement with those of Danni *et al.* [34] in the isolated rat hepatocyte model, and of Comporti [35] in the whole animal.

To characterize better DHEA antioxidant activity against CCl_4-induced lipid peroxidation, an *in vitro* model was then used. In microsomes obtained from DHEA-supplemented rats, treated with CCl_4, MDA production was significantly reduced (Fig. 3), further confirming that DHEA is able to protect against oxidative processes.

In order to assess the role of DHEA in reducing lipid peroxidation reactions, we have also measured MDA production in non-treated microsomes using a NADPH regenerating system with or without CCl_4 in the presence of increasing concentrations of DHEA. *In vitro* this compound is able to prevent significantly MDA production at 10 μM concentration displaying a dose-dependent antioxidant activity; in fact, with increasing DHEA concentrations (100–200 μM) a more marked decrease in MDA production is evident (35–55%, respectively).

To determine whether the antioxidant activity is strictly related to the chemical structure of DHEA, two other steroid compounds were used. In particular we chose 17-α-hydroxypregnenolone, which has two hydroxyl functional groups in C3 and C17, and 4-androstene-3,17-dione, which does not have any hydroxyl substituent. The protective effect against CCl_4-induced lipid peroxidation exerted by these two compounds appears very slight: a 15–20% inhibition of MDA production is evident in the 10–50 μM range; no further effect on MDA can be obtained by increasing the concentration up to 200 μM. It seems thus likely that the antioxidant activity displayed by DHEA is specific, and not a common feature of hydroxy and keto-steroids.

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